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Genetic testing in pediatric disorders

Edited by
Merlin G. Butler and Jessica Duis

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Genetic testing in pediatric disorders

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CASE REPORT
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Alagille Syndrome: A Novel Mutation in *JAG1* Gene

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Alagille syndrome is an autosomal dominant multisystem disorder with variable phenotypic penetrance, caused by heterozygous mutations in *JAG1* or *NOTCH2*, encoding for the components of the Notch signaling pathway. In this paper, we described a novel mutation not yet reported in literature. This 3-years old male child was referred to our Clinical Genetics Unit because of delayed psychomotor development, systolic murmur, dysmorphic facial features, and hypertransaminasemia. The novel *JAG1* heterozygous *c.2026delT* variant in exon 16 was found. *JAG1* mutations are classified as protein truncating and non-protein truncating, without any genotype-phenotype correlation. The detected mutation determines a stop codon (p.Cys676AlafsTer67) in the gene sequence, encoding a truncated protein. Our report broadens the spectrum of *JAG1* gene mutations.

Keywords: Alagille syndrome, *JAG1*, stop codon mutation, Next Generation Sequencing, hypertransaminasemia

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BACKGROUND

Alagille syndrome (ALGS; OMIM 118450) is an autosomal dominant multisystem disorder with variable phenotypic penetrance first described in 1969 by Daniel Alagille (1). The incidence rate is 1:70,000–100,000 live births (2, 3). Molecular diagnosis has increased the number of cases detected and the true incidence is probably close to 1 in 30,000 (1, 4). Although clinical features may differ significantly, the diagnosis of ALGS is mainly based on clinical findings (5, 6). Initial diagnosis is based on the presence of intrahepatic bile duct paucity and at least of 3 other clinical features: chronic cholestasis, cardiac disease (pulmonary stenosis), ocular abnormalities (posterior embryotoxon), skeletal abnormalities (butterfly-like vertebrae), and peculiar facial features (broad forehead, deep-set eyes, bulbous nose, and small pointed mandible) (7–9). Patients have a high prevalence of renal and vascular disease as well (1).

ALGS is caused by mutations in genes that impair Notch signaling, a highly conserved pathway that is fundamental to the transcription of genes for cell fate and differentiation of multiple organ systems (10–14). Almost all cases of ALGS are caused by mutations in the *JAG1* gene (20p12.2), which consists of 26 exons, while a small rate of patients has a heterozygous mutation in the Notch receptor, *NOTCH2* gene (1p13) (15–18). Although mutations causing ALGS have now been identified, diagnostic challenges remain because there are not genotype–phenotype correlations (19). Albeit several genotype-phenotype correlation studies have been performed, they have not shown a link between mutation type and clinical manifestation or severity, leading to the hypothesis that a second gene could work to modify the effects of a *JAG1* or *NOTCH2* mutation (1).

The diagnosis is based on clinical features. Liver biopsy typically shows paucity of the intrahepatic bile ducts, but it is no longer considered mandatory to make a diagnosis of ALGS, and the presence of cholestasis is acceptable to fulfill this criterion. Confirmatory diagnosis is based on gene sequencing. However, about 4% of patients with clinical diagnosis of ALGS may not show any genetic variant, suggesting that further genetic mechanisms are still to be elucidated (1).

In this paper, we describe a patient carrying a novel mutation not yet reported in literature. Signed informed consent has been acquired from the patient's parents for the publication of this case report and any potentially identifying information was removed.

CASE REPORT

The patient is a Caucasian 3-years old male child, late-preterm born (36 weeks) from vaginal delivery, after a pregnancy complicated by placental detachment. Birth weight was 2,490 g (26° centile). He was the first child of an unrelated couple. Family history was negative for cardiac or hepatic disorders. The main stages of psychomotor development were delayed (sitting position at 8 months with hypotonia; walking at 18 months; speaking at 3 years). At 20 months of age a systolic murmur was found at the cardiac auscultation and heart ultrasound was performed, showing a mild stenosis of the pulmonary branches. Screening for metabolic diseases was negative, except for the finding of hypertransaminasemia. Because of dysmorphic facial features, delayed neurological development and elevated liver enzymes, a genetic condition was suspected and the patient was referred to the Clinical Genetics Unit of the Giovanni XXIII Children's Hospital in Bari.

At referral, height, weight and head circumference were normal (>50° centile). He featured prominent frontal bossing, saddle nose with a bulbous tip, 2/VI systolic cardiac murmur, severe psychomotor retardation suggesting an autistic phenotype. His stools were hypocholic with remains of undigested food. Fundus oculi and brain resonance were normal. Karyotype and FRAXA analysis resulted negative. After patient's parents signed the informed consent, gene sequencing of *JAG1* (NM_000214) was performed by Next Generation Sequencing. Target enrichment was done by TruSeq custom amplicon (Illumina, San Diego, CA, United States) according to the manufacturer's instructions. Template library was prepared and was sequenced using MiseqIllumina platform

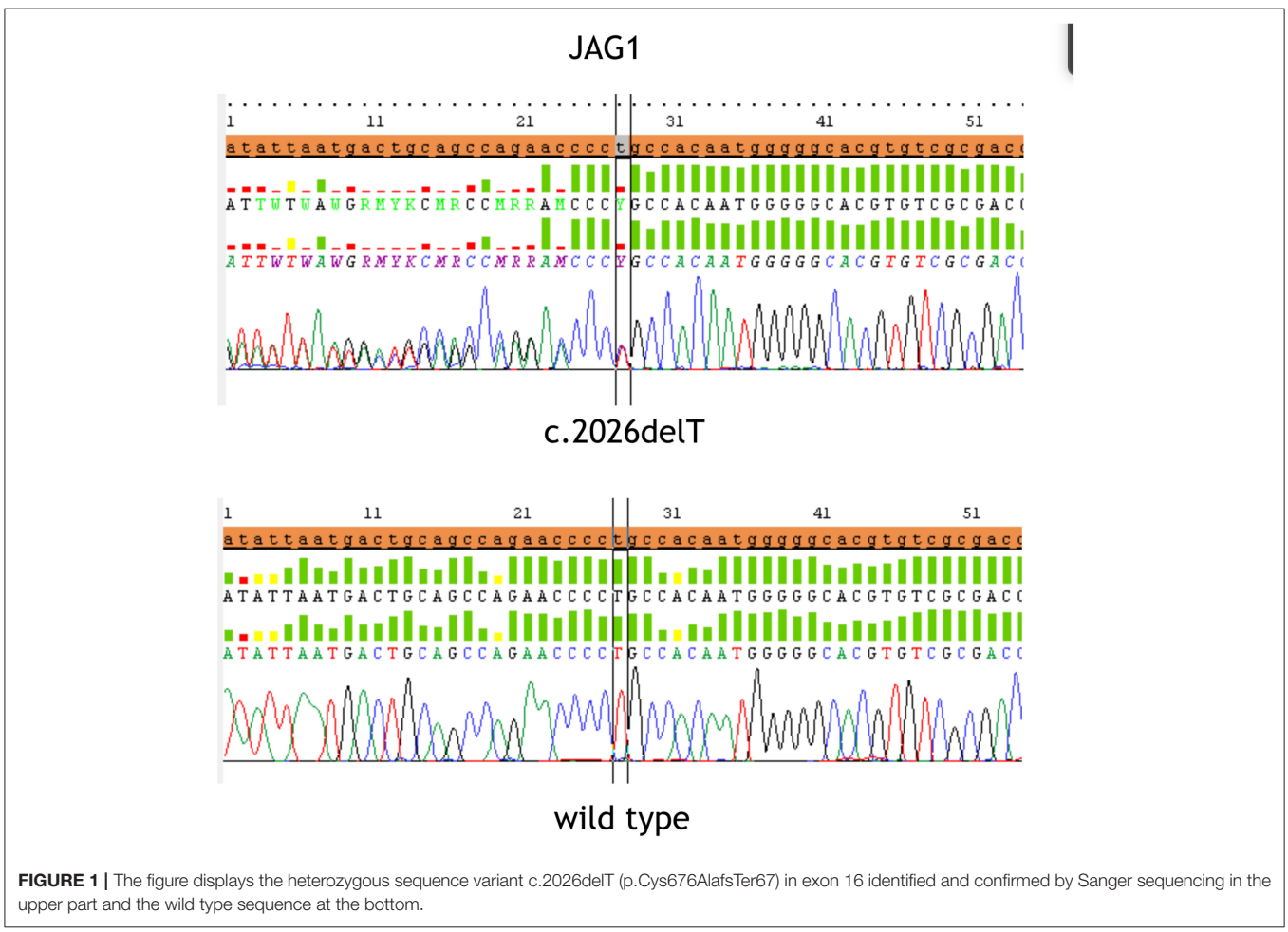


FIGURE 1 | The figure displays the heterozygous sequence variant c.2026delT (p.Cys676AlafsTer67) in exon 16 identified and confirmed by Sanger sequencing in the upper part and the wild type sequence at the bottom.

TABLE 1 | A summary of the clinical features and the frequency reported among individuals with ALGS.

Common system involved	Overall frequency	Frequency of Finding in *JAG1*+ ALGS	This case
Liver: Paucity of biliary duct, conjugated hyperbilirubinemia and liver failure	Up to 100%	100%	√
Heart: Structural changes, pulmonary stenosis, and tetralogy of Fallot	90–97%; 60–67%, and 7–16%	100%	√
Facial features: Prominent forehead, deep-set eyes with moderate hypertelorism, pointed chin, and saddle or straight nose with a bulbous tip	20–97%	97%	√
Eye: Posterior embryotoxon	78–89%	75%	X
Bones: Vertebral anomalies (butterfly vertebra)	33–93%	64%	Not available
Kidney: Ureteropelvic obstruction and renal tubular acidosis	39%	40%	X

Adapted from Saleh et al. (20). √: present; X: not present.

(Illumina, San Diego, CA, United States). Annotation and filtering of variants were performed with Illumina Variant Studio version 2.0, following recommended settings. To evaluate the completeness of the method for the screening of the targeted gene, the sequencing coverage of each amplicon was analyzed in detail using Integrative Genome Viewer version 2.3 (Broad Institute, Cambridge, MA). Variants and region with a depth coverage below 30x were confirmed by Sanger sequencing. The heterozygous sequence variant c.2026delT; p.Cys676AlafsTer67 in exon 16 was identified and confirmed using Sanger sequencing (**Figure 1**), also because the coverage in exon 16 was very low (<30 x). Primers sequences for PCR amplification of the exon 16 were designed using Primer3 software: Forward primer CCTGTCGTGAATGGTCCTG, Reverse primer CCAGGCCCAGAGAAATATCA. The variant was absent in both parents, arisen as a *de novo* variant, which determines the formation of a stop codon. Variant was checked for previously reported causative mutations in published works and mutation databases: Human Gene Mutation Database (HGMD) and Leiden Open Variation Database (LOVD) and it has never been described before. Moreover, variant was searched indbSNP, 1,000 Genomes and ExACdatbases, to exclude common single nucleotide polymorphism.

The child started symptomatic therapy with ursodeoxycholic acid and multidisciplinary follow-up, in particular rehabilitative psychomotor follow-up.

DISCUSSION

Alagille syndrome is a highly variable, autosomal dominant disorder, which involves multiple organ systems. **Table 1** shows a summary of the clinical characteristics and its frequency in patients with ALGS. Its management requires a multidisciplinary team, including mainly specialists in medical genetics, gastroenterology, hepatology, nutrition, cardiology, ophthalmology, and many others (20, 21). The main clinical and pathological features are chronic cholestasis, characteristic facial features, cardiac defect, minor abnormalities of vertebral segmentation, ophthalmologic abnormalities, and dysplastic kidneys (20). The liver disease typically causes severe debilitating pruritus and disfiguring xanthomas, which require treatment with ursodeoxycholic acid and other medications such as cholestyramine, rifampin, and naltrexone (22). Infants and children with ALGS are often diagnosed based on their clinical features: chronic cholestasis is the most common presenting attribute of patients, but dysmorphic facial features are considered one of the best ways to diagnose ALGS (5). The differential diagnosis included Progressive Familiar Cholestasis (PFIC), childhood primary sclerosing cholangitis, congenital hepatic fibrosis, childhood autoimmune hepatitis, childhood primary biliary cirrhosis, alpha-1 antitripsin deficiency, cystic fibrosis and other diseases in which paucity of bile ducts occurs (3).

ALGS is caused by heterozygous mutations in 1 of 2 genes that are fundamental components of the Notch signaling pathway, JAGGED1 and Notch 2. Mutations in *JAG1* gene were first identified more than 20 years ago, and above 500 mutations have been reported (1, 13, 16, 23, 24). The spectrum of *JAG1* mutations includes more frequently protein-truncating mutations (75%), and non-protein truncating mutations (25%) (**Tables 2A,B**) (1, 24). Sequencing all exons and the immediately

TABLE 2A | Type and frequency of *JAG1* mutations found in ALGS.

	Mutation type	Reported (n)	Frequency (%)
Protein truncating	Small deletions	150	28.4
	Small duplications/insertions	102	19.4
	Non sense	85	16.1
	Gross deletions	47	8.9
	Indels	12	2.3
Non-protein truncating	Missense	81	15.4
	Splice site	47	8.9
	Gross duplications/insertions	2	0.4
	Translocations	1	0.2
Total		527	100

Adapted from Mitchell et al. (1).

TABLE 2B | Exons more frequently involved per genetic mutation (Results for variants in the *JAG1* gene reported in the ClinVar database).

Exons more frequentlyinvolved per genetic mutation
Deletions: Exon 2,7,16,17,23,25
Duplications: Exon 3,8,11,13,17,18,23,24
Insertions: Exon 8, 23
Stop codon mutations: Exon 3,4,6,20,21,23,24
Missense: Exon 1,2,4,5,9,20
Splicing mutations: Exon 16

adjacent intronic regions to identify splice site mutations allows to identify the majority of *JAG1* and *NOTCH2* mutations. Because mutations in *JAG1* are predominant, sequencing of this gene occurs first followed by deletion or duplication analysis via multiplex ligation-dependent probe amplification, chromosomal microarray, or fluorescence *in situ* hybridization. Sequencing of *JAG1* identifies approximately 85% of ALGS mutations, and deletion/duplication analysis yields an additional approximately 9% of molecular diagnoses. In the absence of an identified mutation in *JAG1*, sequencing of *NOTCH2* identifies another 2 to 3% of mutations in ALGS. Up to date large deletion or duplication mutations in *NOTCH2* have not been reported. A causative mutation for the remaining 2 to 4% of clinically diagnosed ALGS patients has not been identified yet, and the application of various next-generation sequencing techniques could help identify a molecular origin in this population (1). Unfortunately no genotype-phenotype correlation exists between clinical manifestations and the specific *JAG1* pathogenic variant or the location of the mutation within gene (19). Although genetics of ALGS is well-defined, there is a very variable expressivity of the disease. Individuals with the same mutations, including patients belonging to the same family, show discordance in the phenotype (1). In support of this concept, the genotype-phenotype correlation studies did not identify a link between the mutation type and clinical manifestation or severity (18). In view of the absence of an identified environmental factor that influences the severity of the disease or presentation, scientists hypothesized that a second gene could work modifying the effects of a *JAG1* or *NOTCH2* mutation to worsen or improve the disease features. Several studies have identified putative genetic modifiers to help explain the variable expressivity of this disease (1).

The patient described in this paper was referred to our Unit because of psycho-motor delay, autistic pattern, facial dysmorphic features, and hypertransaminasemia. Thus, at referral beside the typical liver involvement which is the main feature of ALGS, the clinical features of our patient were facial features, heart disease, and severe neurological involvement; the less frequent clinical features in ALGS were not present in our case (**Table 1**).

The *JAG1* gene sequencing showed the novel*c.2026delT* variant in exon 16, which determines a stop codon (p.Cys676AlafsTer67) in the gene sequence. As a result, this mutation produces a truncated protein. Although exon 16 has already been described as a site of disease-causing mutations, this variant has not been reported yet in literature. Further studies could clarify the effect of this mutation, but the stop codon causes the premature truncation of the amino acids sequence. In the same position, it was previously reported the *de novo* c.2026T>G variant leading to the p.C676G missense mutation which was disease-causing according to the Mutation Tester and probably damaging according to the PolyPhen-2 (25). The mutation was found in a male with 4 clinical features (cholestasis, cardiac murmur, skeletal abnormalities, characteristic face). The majority of point and frameshift mutations could be detected by sequencing 11 exons (exons 3, 5, 6, 11, 14, 16, 18, 21, and 23–25). It has been reported that exon 16 is involved mostly by deletions and splicing mutations, due to a single nucleotide substitution (such c.2071T>A, c.2078G>A, and c.2091G>A) or small duplications (c.2070_2073dupCTGT) (25, 26). In this paper we describe a patient with Alagille syndrome carrying a frameshift mutation in the exon 16 of *JAG1* gene. This exon is frequently involved by disease-causing mutations in Alagille syndrome.

CONCLUDING REMARKS

Our report broadens the spectrum of mutations in the *JAG1* gene. Broadening the mutations spectrum may help genetists to better identify etiological mutations leading to ALGS and overall it could provide further insights to identify the hotspots exons where mutations happen more frequently.

ETHICS STATEMENT

Signed informed consent has been acquired from the patient's parents for the publication of this case report and any potentially identifying information was removed. Gene sequencing was performed after informed consent was signed by the patient's parents.

AUTHOR CONTRIBUTIONS

RiF and RuF were in charge of clinical follow-up of the patient. VP and MT drafted the paper. AG is the radiologist who performed the imaging. AM is the biologist who ran the gene sequencing. MD and PG reviewed the paper and gave substantial contribution to data interpretation.

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CASE REPORT
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A Novel 3q29 Deletion in Association With Developmental Delay and Heart Malformation—Case Report With Literature Review

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3q29 deletion syndrome is a rare disorder, causing a complex phenotype. Clinical features are variable and relatively non-specific. Our report aims to present an atypical, *de novo* deletion in chromosome band 3q29 in a preschool boy, first child of healthy non-consanguineous parents, presenting a particular phenotype (microcephaly, "full moon" face, flattened facial profile, large ears, auricular polyp, and dental dystrophies), motor and cognitive delay, characteristics of autism spectrum disorder and aggressive behavior. He also presented intrauterine growth restriction (birth weight 2,400 g) and a ventricular septal defect. SNP Array revealed a 962 kb copy number loss, on the chromosome 3q29 band (195519857–196482211), consistent with 3q29 microdeletion syndrome. FISH analysis using a RP11-252K11 probe confirmed the deletion in the proband, which was not present in the parents. Although the patient's deletion is relatively small, it partly overlaps the canonical 3q29 deletion (defined between *TFRC* and *DLG1* gene) and extends upstream, associating a different facial phenotype compared to the classic 3q29 deletion, nonetheless showing a similar psychiatric disorder. This deletion is different from the canonical region, as it does not include the *PAK2* and *DLG1* genes, considered as candidates for causing intellectual disability. Thus, narrowing the genotype-phenotype correlation for the 3q29 band, *FBX045* is suggested as a candidate gene for the neuropsychiatric phenotype.

Keywords: 3q29, cytogenetics, intellectual disability, cardiac malformation, behavior

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ESTABLISHED FACTS AND NOVEL INSIGHTS

Established Facts

- 3q29 deletion syndrome is characterized by a variable clinical presentation, with mild to moderate intellectual disability, autism, gait ataxia, microcephaly, cleft lip and palate, chest wall deformity.
- Affected people also show psychiatric disturbances, including aggression, anxiety, hyperactivity, and bipolar disorder with psychosis.
- Cardiac malformations, were reported in 11/42 of people with 3q29 del.

Novel Insights

- We report a *de novo* small deletion (<1Mb) on 3q29 in a patient with microcephaly, moon face, flat profile, global developmental delay, aggressive behavior, and ventricular septal defect.
- This cardiac malformation was reported previously in only two cases.
- This deletion is different from the canonical region, as it does not include the *PAK2* and *DLG1* genes, considered as candidates causing intellectual disability; thus, narrowing the genotype-phenotype correlation for the 3q29 band.
- *FBX045* is suggested as a candidate gene for the neuropsychiatric phenotype.

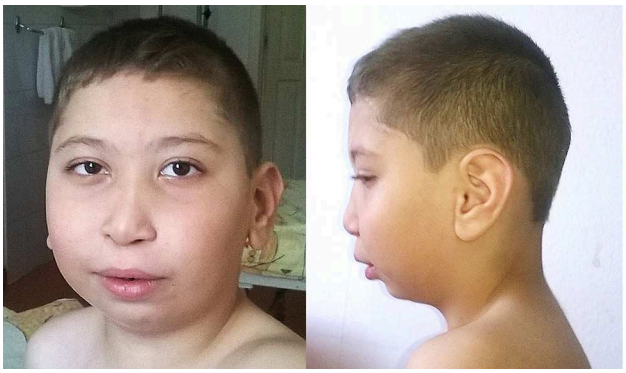


FIGURE 1 | Patient face and profile at the age of 7 years.

INTRODUCTION

Rare diseases are complex pathologies that require a multidisciplinary team and a very rigorous clinical evaluation. Some have a distinct, nonetheless, non-specific phenotype that makes the clinical diagnosis difficult. 3q29 deletion syndrome is a rare condition, reported in 2001 (1) and characterized by a variable clinical presentation, with mild to moderate intellectual disability, autism, gait ataxia, a chest wall deformity and additional features, including microcephaly, cleft lip and palate, horseshoe kidney and hypospadias observed in very few patients. Congenital anomalies associated with this condition are cardio-vascular defects, gastrointestinal abnormalities, failure to thrive, and teeth abnormalities (1, 2).

The canonical deleted region (1.6 Mb) includes several genes (∼22 genes) with important roles in brain and neurocognitive development (3). From those, *PAK2* and *DLG1* genes were considered as candidates for causing intellectual disability. The chromosome 3q29 band was identified as a risk factor for schizophrenia, autism, bipolar disorders (4), and other neuropsychiatric pathologies; however, the phenotype is not clearly defined (4–6).

3q29 deletion syndrome has an autosomal dominant transmission. The deletion is frequently associated with segregation of neuropsychiatric diseases in families (5, 6). The deletion presents with a variable phenotype; however, the neurocognitive abnormalities are presented in most patients (2).

We aim to present a case of atypical 3q29 deletion syndrome with a particular phenotype and intellectual disability in order to further refine the genotype phenotype relation.

CLINICAL PRESENTATION AND FAMILY HISTORY

The patient was born at 40 weeks of gestation, with a weight of 2,400 g, the first born from healthy non-consanguineous parents. The parents had normal intelligence and no reported behavioral issues. The patient's family history was unremarkable.

At 7 years of age, the patient had a particular phenotype (**Figure 1**) with a "full moon" face, strabismus, antimongoloid slants, flattened facial profile, deviated septum, long philtrum, microretrognathia, dental dystrophies, high palate, large ears, auricular polyp, and a nasally voice. His body mass index was 19.3 kg/m^2 (+2 standard deviations-SD) showing obesity. His height was 122.5 cm (+1 SD), within the normal range for boys at this age. At the age of 7 years he presented waddling gait and was not able to run. The clinical presentation of the patient in comparison to others reported in a 3q29 deletion registry (5), is presented in **Table 1**. The registry has the largest reported cohort in literature with 3q29 deletion.

The patient presented intellectual disability. At the age of 7 years, the patient's psychological development was comparable to that of a 2, 5 years old child. Additionally, he presented aggressive, sometimes uninhibited behavior and poor eye contact. Communication abilities included only hand gestures, and no words. He could interact with a mobile phone. The patient presented various anxieties (including a fear of dogs and stairs). He attended a normal kindergarten for 6 months, however, could not cope and thus, later attended services offered by a specialized center for intellectual disabilities.

His personal history showed delayed motor milestones. He started to walk around the age of 3 years and acquisitioned sphincter control after the age of 4 years. The history of illness included failure to thrive in the first 2 years, chronic constipation and repeated upper respiratory infections. He received treatment with Risperidone for his behavior, which showed a moderate response. Cardiologic evaluation at the age of 5 years, diagnosed the spontaneously closed ventricular septal defect.

The patient's imagistic brain evaluation, using MRI at 7 years of age was normal. Repeated EEG evaluations did not show significant changes.

METHODOLOGY

Clinical Evaluation

The patient was evaluated by clinical geneticists along with a multidisciplinary team. The mother completed a parent-report questionnaire (The Children Behavior Check List-CBCL) in order to evaluate her perception about the child's neuropsychiatric development.

The parents provided written informed consent to publish this case (including publication of images).

TABLE 1 | Clinical presentation of patient in comparison to phenotypic features, reported in a registry for 3q29 deletion (5) or by the literature review from Cox and Butler (6), arranged by decreasing frequency.

Clinical feature	Previous reported % (no. with feature/total no. people)	Present case
NEUROPSICHIATRIC DEVELOPMENT (5)		
Learning disability	98% (41/42)	+
Speech delay	59% (25/42)	+
Psychiatric disorder	28% (12/42)	+
Autism/autistic features	26% (11/42)	+
Anxiety disorder	19% (8/42)	
CRANIO-FACIAL DYSMORPHISM (6)		
High nasal bridge	72% (26/36)	–
Ocular abnormalities	58% (11/19)	+
Microcephaly	55% (18/55)	+
Short philtrum	51% (18/35)	–
Low-set, posteriorly rotated ears	43% (15/35)	–
Prominent or broad nasal tip/nose	40% (10/25)	–
Long narrow face	34% (12/35)	–
High-arched palate	33% (12/36)	+
Thin upper lip	30% (7/23)	–
Micrognathia	29% (7/24)	+
Abnormal brain MRI/CT	27% (3/11)	–
Brachycephaly	23% (6/26)	+
Frontal bossing	17% (4/23)	–
Facial asymmetry	17% (4/24)	+
Down-slanting palpebral fissures	17% (4/24)	–
Smooth philtrum	17% (4/23)	–
Cup-shaped ears	13% (3/24)	–
Large protruding ears	9% (2/23)	+
Broad nostrils	9% (2/23)	–
Long philtrum	9% (2/23)	+
Cleft lip/palate/submucous cleft	9% (3/32)	–
Up slanting palpebral fissures	8% (2/24)	+
MUSCULOSKELETAL ABNORMALITIES (6)		
Long/tapered fingers	37% (11/30)	–
Clinodactylous toes	32% (7/22)	–
Chest cavity deformity	29% (10/35)	+
Scoliosis	28% (5/18)	+
5th finger clinodactyly	19% (5/27)	–
Joint contractures	19% (4/21)	–
Abnormal palmar crease	17% (4/24)	–
Ligamentous laxity	11% (4/35)	–
Toe syndactyly	9% (2/23)	–
GASTROINTESTINAL ABNORMALITIES (5)		
Gastroesophageal reflux disease	43% (16/41)	–
Constipation	39% (9/41)	+
GENITOURINARY DEFECTS (6)		
Hypospadias	21% (3/14)	–
Horseshoe kidney	10% (2/21)	–
OTHER FEATURES (5)		
Abnormal teeth	66% (28/42)	+
Failure to thrive	39% (17/39)	+
Recurrent middle ear infections	32% (13/41)	–
Heart defects (PDA, ASD, and others)	26% (11/42)	–
OTHER FEATURES (6)		
Delayed walking	41% (14/34)	+
Low birth weight (<3rd percentile)	33% (10/30)	+
Ataxia gait/gait abnormality	38% (6/24)	+
Short stature	24% (7/29)	–
Abnormal skin pigmentation	14% (3/22)	–

PDA, patent ductus arteriosum; ASD, atrial septal defect; No., number.

Molecular Analyses

The genetic laboratory tests performed inlcuded: cytogenetic analysis (classic karyotype), SNP array using Infinium® OmniExpress-24 v1.2 Kit, ∼710,000 Markers (scan performed using Iscan Illumina and GenomeStudio v2.0 software) and FISH analysis for patient and parents using the RP11-252K11 probe (localized to chr3:195907410-196071593 on build hg19, includes the *ZDHHC19, PCYT1A, SLC51A, TCTEX1D2,* and *TM4SF19* genes).

The patient has a normal male karyotype (46, XY). SNP array identified a 962 kb copy number loss of chromosome 3q29, between 195519857 and 196482211 (**Figure 2**). That region includes the following morbid genes *TFRC, PCYT1A, RNF168, TCTEX1D2,* and *CEP19*. Non-morbid genes included in the deleted region are: *TNK2, ZDHHC19, SLC51A, TM4SF19, UBXN7, RNF168, SMCO1, WDR53, FBXO45, PIGX,* and *NRROS*. The identified copy number variation was associated with 3q29 Microdeletion syndrome (OMIM 609425). No other significant genomic imbalances were detected. The 3q29 deletion was confirmed in the patient by FISH analysis using a RP11-252K11 probe. The parental genetic evaluation using the same FISH probe, was negative, thus, showing a *de novo* origin of the deletion. The FISH analysis in the parents cannot exclude small deletion/duplication/insertion in the targeting region. Gonadal mosaicism cannot be excluded and was considered in genetic counseling.

A systematic literature search for similar reports was performed to have an overview of genotype and phenotype syndrome aspects. The findings are presented in **Figure 3** (ideogram) and **Table 2** (deleted limits).

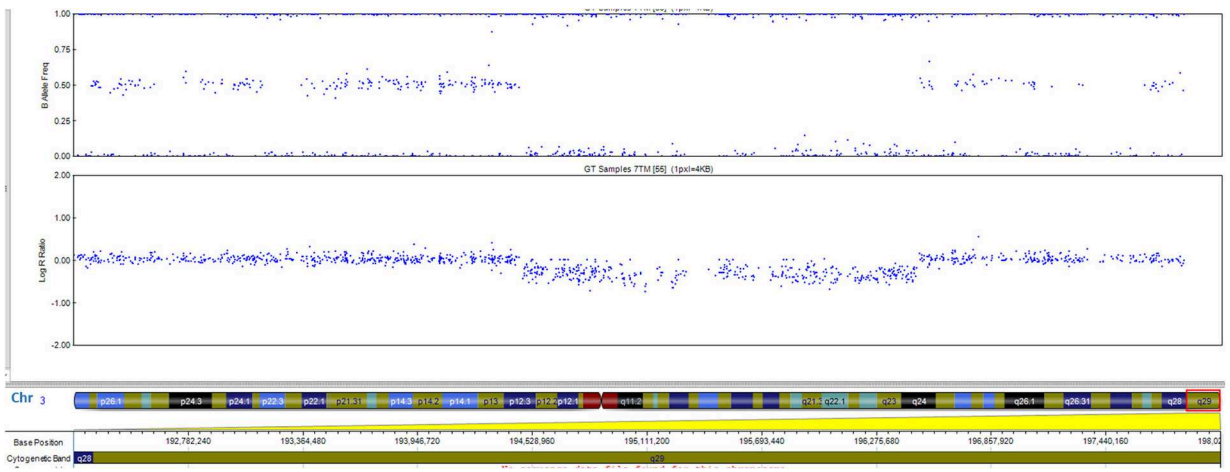


FIGURE 2 | The 0.96-Mb deletion in the 3q29 band, arr[GRCh37] 3q29(195519857_196482211)x1 detected by whole genome SNP-array analysis and ideogram of chromosome 3. Morbid genes are depicted in red for the 3q29 band.

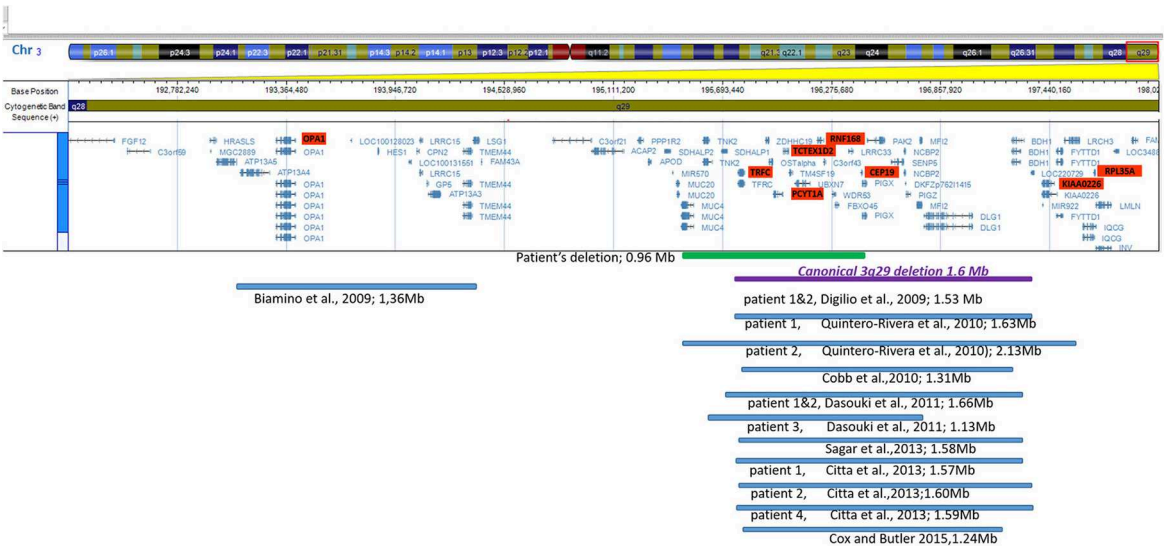


FIGURE 3 | Ideogram of chromosome 3 with zoomed-in details of the 3q29 band shown with a schematic view of the genes involved in the deletion. Horizontal lines in blue show proportional representation of deletion size in our patient and previously reported deletions in other patients (in order of publication year). Deletion limits were converted to GRCh37/hg19 reference (also shown in **Table 2**). The ideogram included the deletions reported in the literature which were detected by array. Cases reported using the FISH method were not included.

Discussion

Described initially as a 1.6 Mb deletion on THE long arm of chromosome 3 (1, 3), the 3q29 deletion syndrome was defined as a recurrent subtelomeric deletion (hg 19 coordinates 195,788,299–197,033,296) between *TFRC* and *DLG1* genes (6). Authors described families with 3q29 syndrome in several generations (11, 13). However, the present case is *de novo*, his parents do not present similar clinical or molecular abnormalities. The family history is not relevant for neuropsychiatric disorders.

The syndrome phenotype varies, from mild to severe, without very specific facial characteristics. Intellectual disability and psychiatric disorders are the most consistent findings (2). Glassford MR and collaborators have developed an online registry to summarize the phenotypic features of 3q29 deletion syndrome (5). To date, this is the largest cohort of patients with reported clinical data from 44 individuals. However, the data was self-reported/reported by family members and does not show deletion sizes to enable genotype-phenotype correlation. Authors highlighted the large clinical variability. Nonetheless, 64% of patients had a low birth weight and feeding problems, while ∼30% of cases had neuropsychiatric disorders (anxiety, panic attacks, depression or bipolar disorder, and schizophrenia). The present case showed various anxieties already present at an early age. The patients in the registry also associated organ abnormalities. Cardiac defects were reported in 26% of cases,

TABLE 2 | Deletion limits reported in literature using array platforms illustrated in **Figure 3**.

References	Deletion limits *GRCh37/hg19*		
	Start	End	Size Mb
Patient 1 and 2 (7)	195,777,965	197,310,451	1.532
Patient 1 (2)	195,710,112	197,338,701	1.629
Patient 2 (2)	195,455,944	197,558,893	2.103
One patient (8)	195,771,743	197,085,623	1.314
Patient 1 & 2 (9)	195,689,972	197,358,134	1.668
Patient 3 (9)	195,601,025	196,732,851	1.132
One patient (10)	195,740,402	197,320,103	1.580
Patient 1 (11)	195,740,357	197,310,392	1.570
Patient 2 (11)	195,731,956	197,339,270	1.607
Patient 4 (11)	195,747,856	197,339,270	1.591
One patient (6)	195,788,299	197,033,296	1.245
One patient and 6 family members (12)	193,046,853	194,407,385	1.360
Our patient	195,519,857	196,482,211	0.962

with the most frequently defect reported as ductus arteriosus and ventricular septal defect, a rare condition, reported only in two other cases (11, 14). The registry reported a high prevalence of gastrointestinal disorders (68%). In the present case constipation was one of the major issues for the family. The present case had rare clinical features (**Table 1**), reported in <20% of cases, such as large protruding ears (9%) and long philtrum (9%). Conversely, features frequently reported (in more than 40% of cases) were not identified, such as a high nasal bridge or long narrow face, however, these features could change with age.

The deleted region of the presented patient partly overlaps the canonical 3q29 deletion, associating a different facial phenotype compared to the classic 3q29 deletion, nonetheless showing a similar psychiatric disorder (**Figure 1**). *PAK2* and *DLG1* are autosomal homologs of the X-linked intellectual disability genes *PAK3* and *DLG3*. Haplosufficiency of *PAK3* or *DLG3* was previously associated with intellectual disability (14). Importantly, several transcription factors are mapped within this region, however their role in development is not completely understood. The patient reported by Krepischi-Santos et al. (15) had moderate intellectual disability and a particular phenotype caused by a small deletion of 1.0 Mb on the 3q29 chromosome, including gene *DIG1* (OMIM 601014) (5). Cobb's case, described in 2010 (8) was a 6 years old boy with a particular phenotype, autism spectrum disorders and ADHD but no intellectual disability, although the deleted region included morbid genes previously associated with intellectual disability (*DLG1* and *PAK2*) (8).

Out of the 11 non-morbid genes included in the deleted region of the patient, four (*TNK2, UBXN7, SLC51A, and FBX045)* have expressions in the brain. *TNK2* was reported (16), in a small family with autosomal recessive (AR), infantile onset epilepsy and intellectual disability. Functional studies were performed for the homozygous variant identified. Although our patient did not show seizures, this gene could potentially be considered relevant for the phenotype. The *SLC51A* gene was found to have a potential role of the organic solute transporter in brain dehydroepiandrosterone sulfate/pregnenolone sulfate homeostasis. This transporter was localized especially in steroidogenic cells of the cerebellum and hippocampus. The impact of transporters on neurosteroid homeostasis is poorly understood (17). The *UBXN7* gene was shown to be expressed in the brain, however its role is poorly understood at the moment (18). *FBX045* was suggested as a candidate gene for the neuropsychiatric phenotype by [Quintero-Rivera et al. (2)]. The *FBXO45* gene was also considered as a prominent candidate for mediating schizophrenia pathogenesis (19, 20), as the ubiquitin ligase F-box protein 45 is critical for synaptogenesis, neuronal migration, and synaptic transmission. While a genome-wide association study of coping behaviors suggests that FBXO45 is associated with emotional expression (21). Compared with other patient's deletion size and region involved in **Figure 3**, the closest is patient 3, described by [Dasouki et al. (9)], however patient phenotypic data was not available to the authors, except for the developmental delay and heart murmur, similar to our patient.

The function of the non-morbid *ZDHHC19, SMCO1, WDR53,* and *PIGX* genes is unclear or not relevant for the phenotype. Interestingly, the *NRROS, TM4SF19 (non-morbid)*, *RNF168,* and *TFRC (morbid, AR)* genes were found to be related to the immune system, yet there were no apparent immune disfunctions in our patient or in the literature.

An Italian group (12) reported a 1.36 Mb deletion of the 3q arm, 193,046,853–194,407,385, outside of the known specific region for the classical 3q29 deletion syndrome, inherited from the mother and reported in several siblings, presenting with gastroesophageal reflux and milk protein intolerance as first clinical symptoms, later developing mild intellectual disability and endocrinological abnormalities. An overarching characteristic, described by Biamino was overweight, which was also observed in our patient. However, they do not share similar deleted regions. Bioamino et al. (12) suggested that the *HES1* gene could be causative for the obesity phenotype, while in our patient the *CEP19* gene (associated with Morbid obesity and spermatogenic failure-AR) in haploinsufficiency could play a potential role in our patient.

Aside from *CEP19*, other AR genes in the deleted region of the patient were: *TFRC*, associated with immunodeficiency 46 (OMIM 616740), *PCYT1A*, associated with Spondylometaphyseal dysplasia with cone-rod dystrophy (OMIM 608940), *TCTEX1D2*, associated with Short-rib thoracic dysplasia 17 with or without polydactyly (OMIM 617405), and *RNF168*, associated with RIDDLE syndrome (OMIM 611943). Considering the lack of association between patient phenotype and the symptoms of the associated pathology, these genes were not considered relevant for the phenotype.

The reported case provides additional clinical and molecular features that complete the phenotype and genotype of 3q29 Deletion syndrome.

A second hypothesis was considered for this particular case, however, the other CNVs observed in the SNP array analysis were benign. Also, the parental study showed *de novo* deletion 3q29. These arguments are supportive for the phenotype-genotype correlation. Nonetheless, the possibility of a second event, at the molecular level, cannot be excluded.

CONCLUSION

We present the phenotype correlated with the smallest reported *de novo* chromosome 3q29 deletion (<1 Mb) in a patient with microcephaly, full moon face, flat profile, global developmental delay, aggressive behavior, and ventricular septal defect. This deletion is different from the canonical 3q29 deleted region, as it does not include the *PAK2* and *DLG1* genes, considered as candidates for causing intellectual disability, thus, narrowing the genotype-phenotype correlation for the region. *FBX045* is suggested as a candidate gene for the neuropsychiatric phenotype.

DATA AVAILABILITY

This manuscript contains previously unpublished data. The name of the repository and accession number are not available.

ETHICS STATEMENT

The parents provided the written informed consent to publish this case (including publication of images).

AUTHOR CONTRIBUTIONS

AC, AD, CP, MP, and SA contributed to patient clinical and genetic evaluation and the writing process. GD contributed to patient cardiological evaluation and the writing process. CH and DM contributed to patient genetic evaluation and the writing process.

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BRIEF RESEARCH REPORT
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Identification of Pathogenic Mutations and Investigation of the NOTCH Pathway Activation in Kartagener Syndrome

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Primary ciliary dyskinesia (PCD), a rare genetic disorder, is mostly caused by defects in more than 40 known cilia structure-related genes. However, in approximately 20–35% of patients, it is caused by unknown genetic factors, and the inherited pathogenic factors are difficult to confirm. Kartagener syndrome (KTS) is a subtype of PCD associated with situs inversus, presenting more complex genetic heterogeneity. The aim of this study was to identify pathogenic mutations of candidate genes in Chinese patients with KTS and investigate the activation of the heterotaxy-related NOTCH pathway. Whole-exome sequencing was conducted in five patients with KTS. Pathogenic variants were identified using bioinformatics analysis. Candidate variants were validated by Sanger sequencing. The expression of the NOTCH pathway target genes was detected in patients with KTS. We identified 10 KTS-associated variants in six causative genes, namely, *CCDC40*, *DNAH1*, *DNAH5*, *DNAH11, DNAI1,* and *LRRC6*. Only one homozygote mutation was identified in *LRRC6* (c.64dupT). Compound heterozygous mutations were found in *DNAH1* and *DNAH5*. Six novel mutations were identified in four genes. Further analyses showed that the NOTCH pathway might be activated in patients with KTS. Overall, our study showed that compound heterozygous mutations widely exist in Chinese KTS patients. Our results demonstrated that the activation of the NOTCH pathway might play a role in the situs inversus pathogenicity of KTS. These findings highlight that Kartagener syndrome might be a complex genetic heterogeneous disorder mediated by heterozygous mutations in multiple PCD- or cilia-related genes.

Keywords: Kartagener syndrome, compound heterozygous, *CCDC40*, *DNAH1*, *DNAH5, DNAI1*, NOTCH pathway

INTRODUCTION

Primary ciliary dyskinesia (PCD) is a sinopulmonary disease caused by dysfunctional immotile or dyskinetic respiratory cilia. It is an autosomal recessive disorder and clinically characterized by respiratory distress, tympanitis, sinusitis, bronchiectasis, and male infertility in approximately 50% of patients. Male infertility usually caused by aberrant sperm flagella function results from immotile or dyskinetic cilia. Further, PCD affects approximately 1 in 15,000 individuals of the general population (Bush et al., 2007). It was reported that patients with heterotaxy and airway ciliary dysfunction were enriched for mutations in the PCD genes, but interestingly, these were all heterozygous (Wilson et al., 2009). Kartagener syndrome (KTS;MIM# 244400), a subtype of PCD presenting with the phenotype situs inversus, might have a more complex genetic heterogeneity.

The nasal nitric oxide measurement and brush biopsy are typical diagnostic methods for PCD. The diagnosis of PCD is challenging due to phenotypic heterogeneity. Moreover, owing to the non-specificity of symptoms and lack of an early diagnostic method, the diagnosis of PCD is usually missed or delayed (Hosie et al., 2014; Lucas et al., 2014). Because of the lack of a reference standard for PCD diagnosis, a combination of tests is required (Lucas et al., 2016). Definitive diagnosis is usually confirmed through transmission electron microscopy (TEM) to visualize the ultrastructure of cilia, but approximately 30% of patients show no defect in TEM. Furthermore, there is a lack of evidence-based medicine in the management of PCD (Lucas et al., 2014). Therefore, the early diagnosis of PCD is important to select an appropriate management to prevent irreversible damage. With the rapid advances in next-generation sequencing, genetic-based studies have increased our understanding of the pathogenesis of even PCD with remarkable genetic heterogeneity. Diagnostic gene panel has been used for several diseases, and a genetic testing panel for PCD diagnosis is being developed (Paff et al., 2018; Takeuchi et al., 2018). As an autosomal recessive disorder, the inherited mutations of ciliary function-related genes are the main genetic risk factors of PCD. Most of the genetic mutations in PCD contribute to defective cilia structure. Some patients with PCD due to mutations also showed normal motility and ultrastructure, but reduced cilia numbers (Wallmeier et al., 2014). Mutations of ciliary function-related genes can cause immotile cilia due to congenital ultrastructural abnormalities. The prevalence of PCD has a relatively higher rate in consanguineous families (Onoufriadis et al., 2014). To date, genetic mutations in over 40 genes, including *DNAH5, CCD39*, and *DYX1C1*, account for an estimated 65% of individuals (Olbrich et al., 2002; Merveille et al., 2011; Tarkar et al., 2013). *DNAH5* mutations cause approximately 50% of outer dynein arm (ODA) defects, whereas SPAG1 mutations cause approximately 10% of inner dynein arm (IDA) and ODA defects (Hornef et al., 2006; Knowles et al., 2013; Lucas et al., 2014).

Abbreviations: PCD, primary ciliary dyskinesia; KTS, kartagener syndrome; ODA, outer dynein arm; IDA, inner dynein arm; TEM, transmission electron microscopy; ACMG, American College of Medical Genetics and Genomics; VUS, uncertain significance variants

The pathogenic genes reported previously have improved genetic-based diagnosis. Based on the pathogenic mutations of known genes involved in PCD, genetic testing presents a possibility to identify high-risk populations among familial siblings with PCD. However, the genetic factors in approximately 20–35% of patients with PCD are unknown, and the pathogenicity of novel mutations in known PCD genesis is also difficult to confirm based on a functional study. The genetic defect has not yet been well elucidated in some patients. Previously, one study demonstrated that *GALNT11* is a heterotaxy disease candidate gene (Fakhro et al., 2011). Further, another study showed that GALNT11 O-glycosylation modified NOTCH, which balances motile and immotile cilia in left–right patterning and affects the etiology of heterotaxy (Boskovski et al., 2013). However, whether the NOTCH pathway is associated with KTS remains unclear. In the present study, we aimed to identify pathogenic mutations and investigate the NOTCH pathway activation in Chinese patients with KTS. Our study would expand our knowledge on the spectrum of PCD- or cilia-related genes, thereby contributing to the genetic diagnosis and counseling of KTS patients.

Materials and Methods

Subjects

Five patients with KTS and nine familial control subjects were recruited for the present study conducted in the Second Clinical Medical College of Shenzhen People's Hospital and Institute of Guangzhou Respiratory Diseases (Guangdong, China) from December 2013 to July 2019. The control subjects included in the study were the parents of KT4 and LEI, father of KT2, mother of KT5, and the father, sister, brother, and uncle of KT1 (**Table 1**). Patients with KTS were diagnosed based on the presence of chronic sinusitis, situs inversus, bronchiectasis, and ciliary ultrastructural defects. Demographic characteristics and medical history were recorded, including age and clinical test results. Informed consent was obtained from each patient enrolled in the study. Research and ethics approval was obtained from the ethics committee of the Shenzhen People's Hospital.

Bronchoscopy and Electron Microscopy

High-resolution computed tomography and magnetic resonance imaging were performed to check for bronchiectasis or situs inversus. Nasal mucosa and bronchial cilia were obtained for electron microscopy. Transmission electron microscopic examination (Kingmed, China) was performed to identify the ultrastructural abnormality of cilia. Bronchoscopic examination was performed for PCD diagnosis.

Next-Generation Sequencing and Bioinformatics Analysis

High-quality genomic DNA was extracted from blood samples of patients, and exome capture was performed using the Agilent SureSelect V6 Capture Kit according to the manufacturer's protocols (Agilent, CA, USA). An enriched library targeting the exome was subjected to massive parallel sequencing using NovaSeq platform (Illumina). The variants were called using Genome Analysis Toolkit (GATK) and annotated using ANNOVAR (Wang et al., 2010). We

TABLE 1 | Demographics of the KTS participants.

KTS ID	Age	Gender	Inflammation	Situs inversus	Bronchiectasis	Bronchitis
KT1	7	Female	Respiratory infections	Yes	Yes	Yes
KT2	8	Male	Respiratory infections	Yes	Yes	Yes
LEI	6	Male	Rhinosinusitis	Yes	Yes	Yes
KT4	7	Female	Respiratory infections	Yes	Yes	Yes
KT5	3	Male	Normal	Yes	No	No

only focused on the genes with nonsynonymous, Indel, or deletion variants in the coding region. Later, all variants were filtered by region, frequency (MAF > 5%), and functional prediction. The following databases were used as the reference: 1,000 genome, dbSNP, and gnomAD (http://gnomad.broadinstitute.org/).

Candidate Gene Selection and Pathogenicity Assessment

Previous studies have reported that more than 40 genes are related to PCD. Based on previous panel analyses and genetic studies on PCD (Guo et al., 2017; Takeuchi et al., 2018; Yang et al., 2018), 43 genes were selected as candidate genes, which were highly associated with the pathogenesis of PCD (**Supplemental Table S1**). A gene-based filtering analysis was conducted among all variants of the five patients with PCD. To assess the pathogenesis of the variants, various protein functions were predicted using bioinformatic programs, including Sift, Polyphen-2, MutationTaster, and CADD (combined annotation dependent depletion). The interpretation of variants followed the American College of Medical Genetics and Genomics (ACMG) guideline (Richards et al., 2015). An evolutionary conservation analysis of the identified variants was conducted using CLUSTALW on orthologs of different mammal species.

Sanger Sequencing Validation

Genomic DNA was extracted from whole blood samples using the QIAamp DNA Blood Kit (QIANGEN, Hilden, Germany) according to the manufacturer's instruction. All primers were designed using IDT PrimerQuest Tool online software (https://sg.idtdna.com/Primerquest/Home/Index). All gene segments were amplified using the standard PCR. The candidate variants among patients with PCD and control subjects were genotyped using Sanger genotyping platform. The sequences were analyzed using Chromas software (Technelysium Pty Ltd).

Detection of NOTCH Signaling Pathway Activation

Blood samples of KTS and control subjects were collected into Na-citrate-containing Vacutainer tube. Total RNA was extracted from whole blood using animal tissue RNA Purification Kit (TRK-1002, LC Sciences, Houston, USA). To confirm whether the Notch pathway was abnormally activated in patients with PCD, real-time PCR was conducted for the Notch pathway–related and downstream target genes, including *ADAM17*, *Cyclin D3*, *p21*, *MYC*, and *HES1*. Primers were obtained from the database of PrimerBank (https://pga.mgh.harvard.edu/primerbank/) or designed using Integrated DNA Technologies online software (**Supplemental Table S2**).

Statistical Analysis

All statistical analyses were carried out using the software SPSS 19 (IBM, New York, USA). Chi square and Bonferroni (*post hoc*) tests were used for cross-table and multiple comparisons, respectively. The results with $P < 0.05$ were considered statistically significant.

Results

Characteristics of Patients and Diagnosis of KTS

All patients with KTS had a history of recurrent cough, expectoration, and rhinorrhea since the age of diagnosis at childhood (**Table 1**). Chest computed tomography images showed that all the patients had symptoms of KTS, including the classic phenotype of situs inversus (**Figure 1**). The family members (father) of two subjects showed slight bronchiectasis or sinusitis, but without situs inversus. These were taken as reference control subjects without KTS. Routine semen analysis and electron microscopic examination showed infertility in adult male patients and multiple ultrastructural abnormalities of cilia in both bronchial and nasal mucosa, respectively (**Figure 2**). The structural abnormalities of cilia included disarrangement, reduction, and absence of IDA and ODA structures of the subject LEI.

Candidate Gene Rare Variants Identified Using Filtering Analysis

To identify mutations in the Chinese population with KTS, 10 familial normal control subjects were used as annotation controls. We filtered variants to obtain rare mutations with the standard criteria as described in the methodology. With further filtering using integrated bioinformatics pipeline and software, we identified 30 variants of candidate genes (**Supplemental Table S3**). All KTS subjects carried coding mutations in *DNAH1* in the present study. Regarding the known gene variants, we changed the filtering cutoff frequency to 0.01 (gnomAD_all) to obtain all the rare coding variants in the known candidate genes of KTS. In total, 19 variants of the known candidate genes were identified (**Tables 2** and **3**). The pathogenicity of variants was assessed by four *in silico* software. All variants were interpreted based on the ACMG guideline (**Supplemental Table S3**). Nine variants were considered not associated with disease as they were benign or uncertain significance variants (VUS) based on the frequency in the Asian population and ACMG classification (**Table 2**). Six novel mutations were identified in four genes, which were not included in the dbSNP and gnomAD databases (**Table 3**). Two novel mutations of *DNAH5* were identified in the patient KT4. Only one homozygous variant was identified in *LRRC6*

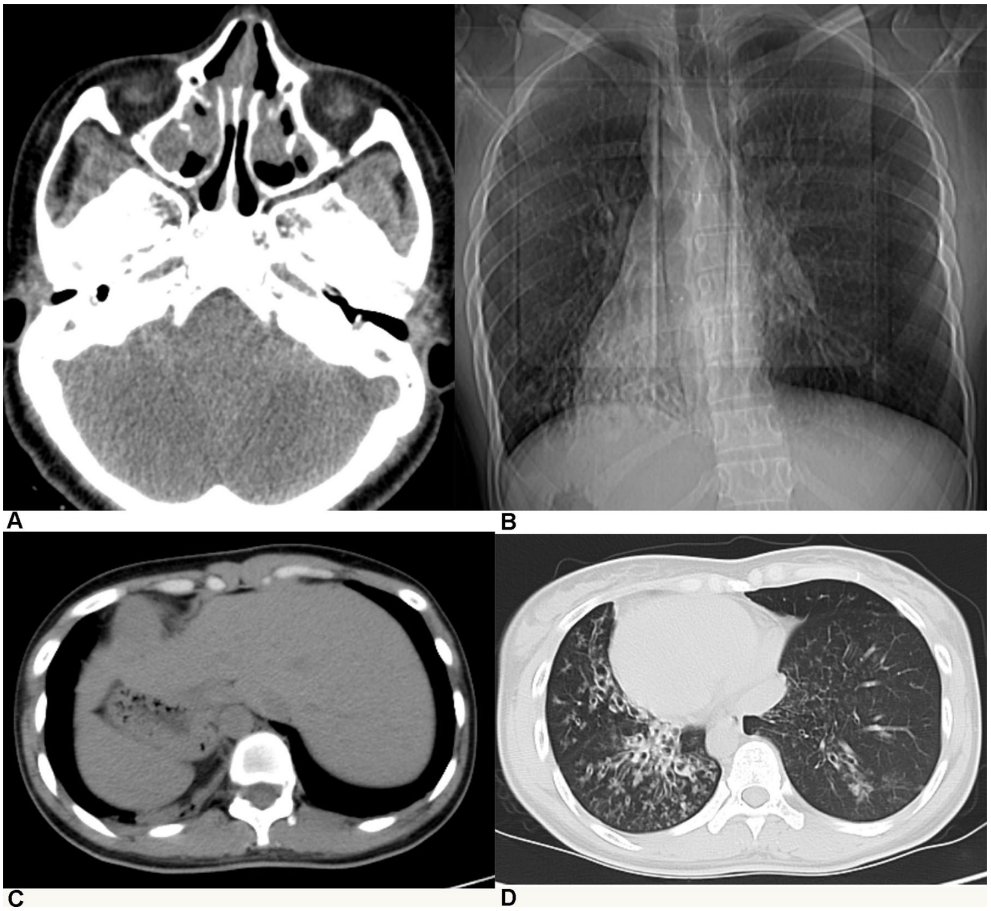


FIGURE 1 | Patients with Kartagener syndrome diagnosed by radiological examination: **(A)** chronic sinusitis, **(B**, **C)** situs inversus, and **(D)** diffuse bronchiectasis.

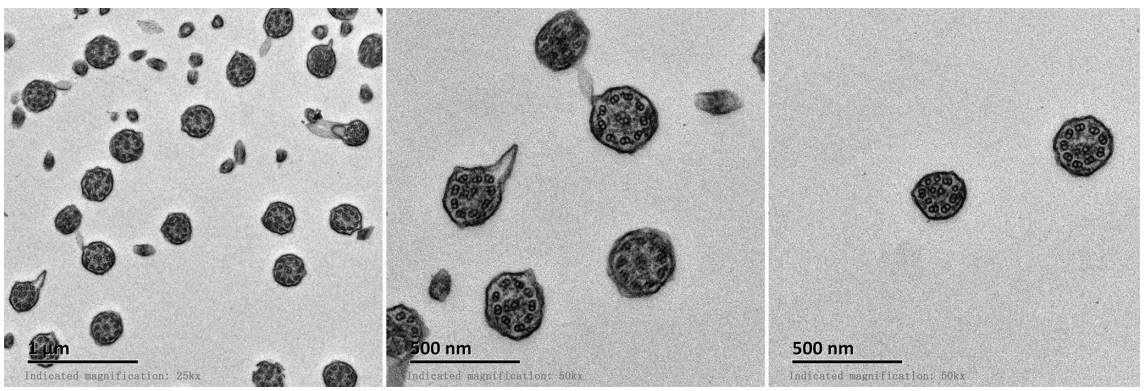


FIGURE 2 | Electron microscopic examination of bronchial cilia showed abnormal 9 + 2 structure and deletion of inner or outer dynein arms.

(c.64dupT), which is the likely cause for the genetic disorder in KT1 (**Table 3**). The two heterozygous mutations of *DNAH1* in KT1 were compound heterozygous. The variants in *DNAI1* and *DNAH1* might contribute to the phenotype of KT1. Advanced analysis showed no homozygous mutations in the known candidate genes among patients with KTS (data not shown).

Variants Validation and Pathogenicity Assessment

ACMG prediction and functional analysis showed that four variants are pathogenic, and six variants are of uncertain significance, which were considered KTS-associated variants (**Table 3**). Sanger sequencing validated all candidate variants including *LRRC6*, *DNAI1*, and *CCDC40* (**Figure 3A**). Our study

TABLE 2 | The identified likely benign variants of candidate genes in Kartagener syndrome (KTS).

Chr	Start	refGene	AAChange	KTS Carrier	gnomeAD_EAS	ACMG Classification
chr16	84189298	DNAAF1	NM_178452:exon5:c. 685C > T:p.H229Y	KT2, LEI	0.0198	Uncertain significance
chr14	50092598	DNAAF2	NM_001083908:exon2:c. 2032A > G:p.T678A	KT2	0.0185	Likely benign
chr3	52396410	DNAH1	NM_015512:exon31:c. 4987C > T:p.R1663C	KT2	0.0129	Likely benign
chr17	78061565	CCDC40	NM_017950:exon15:c.2609G > A:p.R870H	LEI	NA	Uncertain significance
chr17	72308221	DNAI2	NM_001172810:exon12:c.1538C > T:p.A513V	KT1	0.0093	Likely benign
chrX	38146295	RPGR	NM_001034853:exon15:c.1957G > A:p.G653S	KT1	NA	Likely benign
chr3	52426507	DNAH1	NM_015512:exon64:c.10080G > C:p.Q3360H	KT4	0.0136	Uncertain significance
chr17	78024067	CCDC40	NM_017950:exon7:c.1144G > A:p.E382K	KT5	0.0099	Likely benign
chr7	151797901	GALNT11	NM_001304514:exon2:c.71G > A:p.R24H	LEI,KT4,KT5	0.0426	Uncertain significance

TABLE 3 | Variants pathogenicity assessment and interpretation by the ACMG guideline.

Chr	Start	refGene	AAChange	KTS Carrier	gnomeAD_EAS	Segregation		ACMG Classification	Causative
						Paternal	Maternal		
chr3	52429325	DNAH1	NM_015512:exon69:c.10970C > G:p.T3657R	KT2	0.0037	Yes	Unavailable	Uncertain significance	Compound heterozygous
chr7	21641058	DNAH11	NM_001277115:exon18: c.3470T > G:p.L1157R	KT2	0.0025	Yes	Unavailable	Uncertain significance	
chr17	78071197	CCDC40	NM_017950:exon19:c.3175C > T:p.R1059X	LEI	0.0001	No	Yes	Pathogenic	Stopgain
chr3	52360191	DNAH1	NM_015512:exon4:c.442C > T:p.R148C	KT1	NA	Yes	Unavailable	Uncertain significance	Compound heterozygous
chr3	52387194	DNAH1	NM_015512:exon19:c.3103C > T:p.R1035C	KT1	0.0012	No	Unavailable	Uncertain significance	Compound heterozygous
chr9	34501139	DNAI1	NM_001281428:exon12: c.1035T > G:p.N345K	KT1	NA	Yes	Unavailable	Uncertain significance	
chr8	133673819	LRRC6	NM_001321961:exon2: c.64dupT:p.S22fs	KT1	NA	No	Unavailable	Pathogenic	Homozygote
chr5	13789010	DNAH5	NM_001369:exon51:c.8462T > C:p.L2821P	KT4	NA	No	Yes	Uncertain significance	Compound heterozygous
chr5	13830706	DNAH5	NM_001369:exon36:c.6061G > C:p.G2021R	KT4	NA	Yes	No	Likely pathogenic	Compound heterozygous
chr3	52380653	DNAH1	NM_015512:exon11:c.1822C > T:p.Q608X	KT5	NA	Unavailable	Yes	Pathogenic	Stopgain

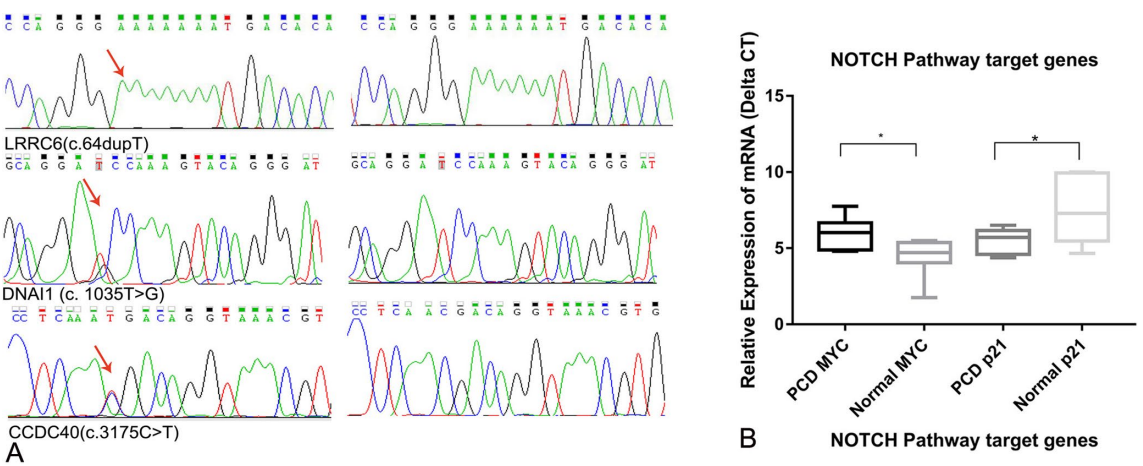


FIGURE 3 | (A) Sanger validation results of the variants *DNAI1* (c. 1035T > G), *CCDC40* (c. 3175 *C* > T), and *LRRC6* (c.64dupT). **(B)** Relative expression of the NOTCH pathway target genes *MYC* and *p21* (*p<0.05).

also showed the two rare variants in *DNAH5* of KT4 were inherited from the mother and father, respectively, indicating that they are compound heterozygous mutations (**Table 3**, **Supplemental Figure S1**). The variant of c.6061G > C in *DNAH5* changes the last nucleotide of exon 36 and completely abolishes the splicing signal, which indicated that it is more likely a pathogenic variant than a VUS. Only c.10970C > G of *DNAH1* was presented in the father of KT2, indicating that c.4987C > T and c.10970C > G are also highly likely to be compound heterozygous mutations. The variants in *DNAH11* might contribute to the phenotype of KT2. Hence, these identified compound heterozygous mutations were the genetic factors, which might cause the KT1, KT2, and KT4 phenotype disorder, but more evidence is needed.

The two heterozygous pathogenic variants were present in LEI and KT5, but this alone does not explain KTS in LEI and KT5, respectively. Since KTS is a recessive genetic disorder, the phenotypic defect in LEI and KT5 may be caused by trans-heterozygous interactions between the carried causative gene and other PCD genes (Li et al., 2016). In summary, in our study, 10 KTS-associated variants were identified among six causative genes of KTS, including four pathogenic variants.

Activation of the NOTCH Signaling Pathway by GALNT11

Real-time expression analysis showed that the expression of *MYC* and *p21* was significantly different among patients with KTS and normal individuals. The expression of *p21* was up-regulated by 5.03-fold in patients with KTS compared with that in normal individuals, but that of *MYC* was down-regulated by 0.29-fold (**Figure 3B**). Our study demonstrated the NOTCH1 pathway might be abnormally activated in patients with KTS, but the underlying mechanism is still unknown. No significant difference was found in terms of NOTCH1 pathway target gene expression among GALNT11 mutation carriers and controls.

Discussion

In this study, we investigated five Chinese patients with KTS and identified 10 KTS-associated variants that overlapped in six causative genes, namely, *CCDC40, DNAH1, DNAH5, DNAH11, DNAI1,* and *LRRC6*. Compound heterozygous mutations were found in *DNAH1* and *DNAH5*. Further analyses showed that the NOTCH pathway might be activated in patients with KTS. Our study showed that patients with KTS carried multiple PCD candidate genes with rare compound heterozygous mutations in causative genes of *DNAH1* and *DNAH5*.

Primary ciliary dyskinesia is remarkably heterogeneous although its inheritance mostly follows an autosomal recessive pattern (Djakow et al., 2016). To date, various gene mutations in over 40 causative genes have been reported, and approximately 35% of PCD remain genetically elusive (Kurkowiak et al., 2015; Yang et al., 2018). A series of monogenic mutations has been reported to contribute to the abnormal structure of motile cilia. The complete or partial deletion of IDAs or ODAs is a common abnormality. Ten KTS-associated variants in six causative genes were identified in our Chinese patients with KST. Defects in the *DNAH5* gene could cause ODA deletion (Hornef et al., 2006). Two novel heterozygous mutations in the *DNAH5* gene were observed in one patient with PCD, which might be highly deleterious, using *in silico* prediction methods. One stop–gain mutation was found in *CCDC40*. Pathogenic mutations in *CCDC40* cause IDA defects and microtubular disorganization (Becker-Heck et al., 2011). The frame-shift homozygous mutation of *LRRC6* might cause both ODA and IDA defects (Kott et al., 2012). The present study showed that most high-frequency mutations were present in the causative gene *DNAH1*. Six mutations in *DNAH1* were identified in all individuals, indicating that *DNAH1* might be a high-risk causative gene in Chinese patients with KTS. The other mutations were identified in PCD causative genes, namely, *DNAI2, DNAAF1, DNAAF2*, and *RPGR*, but with uncertain significance (**Supplementary Table S3**). Further functional investigation would aid in confirming the role of these mutations in the motile cilia structure of KTS.

GALNT11 is a polypeptide N-acetylgalactosaminyl transferase that can catalyze the initiation of protein O-linked glycosylation. O-Glycosylation of NOTCH1 promotes its activation, modulating the essential balance between motile and immotile cilia in the left–right organizer to determine laterality (Boskovski et al., 2013). In the present study, we found a mutation of *GALNT11* in three patients with KTS. The missense mutation (c.G71A) was located in the highly conserved domain of *GALNT11*, and it might disturb the protein function. Therefore, we further analyzed the activation of the NOTCH pathway between patients with KTS and normal controls. Our results showed that the expression of down-stream target genes of *p21* was significantly up-regulated in patients with KTS. *GALNT11* might contribute to the phenotype of situs inversus in KTS.

We identified compound heterozygous mutations in the causative genes *DNAH1* and *DNAH5*. We only identified one homozygous mutation in the known candidate gene *LRRC6* in one patient with KTS. Our study showed that most of the known candidate genes present heterozygous mutations, but each patient with KTS carried multiple gene mutations. One limitation of this study is that DNA samples were not available for all the parents of the recruited KTS patients to confirm the inheritance mode of these mutations. It has been shown that compound heterozygous mutations can also cause extreme phenotypic diversity in the cilia ultrastructure of patients with PCD (Yang et al., 2018). It has also been shown that trans-heterozygous interactions between *DNAH6* and other PCD genes can potentially cause heterotaxy and ciliary dysfunction (Li et al., 2016). This indicates that heterotaxy might have a non-monogenic etiology. In summary, KTS is a complex genetic heterogeneity disorder that might be mediated by heterozygous mutations in multiple PCD- or cilia-related genes.

In conclusion, we identified 10 KTS-associated variants in six causative genes by targeted next-generation sequencing, including compound heterozygous mutations in *DNAH1* and *DNAH5*. The potential pathogenic gene *GALNT11* activated the NOTCH pathway, which might cause situs inversus in KTS. The various pathogenic mutations in different genes involved in KTS indicate genetic diversity and heterogeneity among patients with KTS. The identification of genetic defects that cause KTS is of great importance for its clinical diagnosis and genetic counseling.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The project was approved by the ethics committee of the Ethics Committee of Shenzhen People's Hospital. Written informed consent was obtained from the patients for the publication of the patient's identifiable information. All procedures performed in studies involving human participants in accordance with the 1964 Declaration of Helsinki ethical standards.

AUTHOR CONTRIBUTIONS

YY, YF, and BL prepared the project proposal and study design. YY and PZ conducted bioinformatics and statistical analysis of sequencing data. QH, PanZ, and ShuL conducted sample collection and Sanger sequencing validation. XT, ShengL, LC, and YF conducted clinical diagnosis of Kartagener syndrome. XH and LC assisted with the prepared and revised manuscript. All the authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00749/full#supplementary-material.

SUPPLEMENTAL FIGURE S1 | Sanger validation results of the compound heterozygous variants c.8462 > C and c.6061 > C in *DNAH5* of KT4

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Biochemical, Clinical, and Genetic Characteristics of Short/Branched Chain Acyl-CoA Dehydrogenase Deficiency in Chinese Patients by Newborn Screening

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Short/branched chain acyl-CoA dehydrogenase deficiency (SBCADD) is an autosomal recessive disorder of impaired isoleucine catabolism caused by mutations in the *ACADSB* gene. There are limited SBCADD cases worldwide and to date no Chinese patients with SBCADD have been reported. The aim of this study was to investigate the biochemical, clinical information, and genotypes of twelve patients with SBCADD in China for the first time. The estimated incidence of SBCADD was 1 in 30,379 in Quanzhou, China. The initial newborn screening (NBS) results revealed that all patients showed slightly or moderately elevated C5 concentrations with C5/C2 and C5/C3 ratios in the reference range, which has the highest risk of being missed. All patients who underwent urinary organic acid analysis showed elevation of 2-methylburtyrylglycine in urine. All patients were asymptomatic at diagnosis, and had normal growth and development during follow-up. Eight different variants in the *ACADSB* gene, including five previously unreported variants were identified, namely c.596A > G (p.Tyr199Cys), c.653T > C (p.Leu218Pro), c.746del (p.Pro249Leufs*15), c.886G > T (p.Gly296*) and c.923G > A (p.Cys308Tyr). The most common variant was c.1165A > G (33.3%), followed by c.275C > G (20.8%). All previously unreported variants may cause structural damage and dysfunction of SBCAD, as predicted by bioinformatics analysis. Thus, our findings indicate that SBCADD may be more frequent in the Chinese population than previously thought and newborn screening, combined with genetic testing is important for timely diagnosis. Although the clinical course of Chinese patients with SBCADD is likely benign, longitudinal follow-up may be helpful to better understand the natural history of SBCADD.

Keywords: short/branched chain acyl-CoA dehydrogenase deficiency, 2-methylbutyryl-CoA dehydrogenase deficiency, *ACADSB* gene, newborn screening, isoleucine catabolism

Abbreviations: SBCADD, Short/branched chain acyl-CoA dehydrogenase deficiency; 2-MBCDD, 2-methylbutyryl-CoA dehydrogenase deficiency; NBS, Newborn screening; MS/MS, Tandem mass spectrometry; IVA, Isovaleric acidemia; DBS, Dried blood spots; NGS, Next-generation sequencing; PCR, Polymerase chain reaction; 3D, Three-dimensional; ACMG, American College of Medical Genetics Association of Clinical Genetics; 2-MBG, 2-methylburtyrylglycine; VUS, Variants of uncertain significance; LP, Likely pathogenic.

INTRODUCTION

Short/branched chain acyl-CoA dehydrogenase deficiency (SBCADD, OMIM # 610006), also known as 2-methylbutyryl-CoA dehydrogenase deficiency (2-MBCDD), is an autosomal recessive disorder of impaired isoleucine catabolism (Andresen et al., 2000; Gibson et al., 2000). The causative gene, *ACADSB* (MIM 600301), was mapped to chromosome 10q26.13, comprises 11 exons and encodes a SBCAD precursor protein of 432 amino acids (Arden et al., 1995). The clinical manifestations of SBCADD can be variable, including developmental delay, intellectual disability, seizures, muscular atrophy, hypotonia, autism and neonatal crisis (Akaboshi et al., 2001; Korman et al., 2001; Korman, 2006; Kanavin et al., 2007). However, patients with SBCADD ascertained *via* expanded NBS were reported to have normal development and no obvious health problems, especially in the Hmong population (Matern et al., 2003; Alfardan et al., 2010). Currently, therapeutic strategies for patients with SBCADD are poorly defined because there are no conclusive data. However, careful instructions regarding risks for metabolic stress and fasting avoidance, along with clinical monitoring are warranted (Knerr et al., 2012).

Most patients with SBCADD showed elevated concentrations of 2-methylbutyrylcarnitine (C5), which are detectable at NBS by tandem mass spectrometry (MS/MS). Nevertheless, MS/MS-based NBS may lack sensitivity as some patients with C5-acylcarnitine concentrations within the reference range cannot be detected through NBS. Furthermore, the C5 value was also elevated in isovaleric acidemia (IVA, OMIM # 243500) as isovalerylcarnitine and 2-methylbutyrylcarnitine presenting the same mass to charge are indistinguishable by MS/MS. Thus, urinary organic acid analysis and genetic testing are necessary for differential diagnosis (Madsen et al., 2006; Sass et al., 2008).

SBCADD is highly prevalent in the Hmong ethnic population, whereas only a small number of non-Hmong patients have been reported (van Calcar et al., 2007; Van Calcar et al., 2013). The majority of China's population is dominated with the Han nationality, however, no patients with SBCADD have been reported so far. Herein, we present for the first time the biochemical, clinical information, and genotypes of twelve patients with SBCADD from mainland of China, which may contribute to current research.

MATERIALS AND METHODS

Patients and Auxiliary Analysis

A total of 364,545 newborns (209,136 males and 155,409 females) were screened by MS/MS at Quanzhou Maternity and Children's Hospital between January 2014 and November 2018. Blood samples for NBS were collected between 72 h and 7 days after birth. The concentrations of C5-acylcarnitine, C5/acetylcarnitine (C2) and C5/propionylcarnitine (C3) on dried blood spot (DBS) filter paper cards were determined by using ACQUITY TQD (Waters, Milford, MA, USA) and a NeoBase non-derivatized MS/MS kit (PekinElmer, USA). Among the 364,545 newborns, 351 showed elevated C5-acylcarnitine concentrations in NBS, 44 newborns were involved with confirmatory testing and had positive results in the second test. Otherwise, twelve patients genetically diagnosed with SBCADD were followed up in our hospital. Nine patients' urine samples were then collected for urine organic acid analysis by gas chromatography-mass spectrometry (7890B/5977A, Agilent Technologies, Santa Clara, CA, USA). In addition, assessment of growth, development, and clinical manifestation was conducted, and the patients were followed and evaluated every 3–6 months. This study was approved by the Ethical Committee of Quanzhou Maternity and Children's Hospital. Written informed consent was obtained from all parents of the patients and control subjects, who agreed to participate in this study with the intent of using their medical data for scientific research and publication.

Next-Generation Sequencing (NGS) and Variants Analysis

DBS or peripheral whole blood of twelve patients and their parents, as well as 100 control subjects, were referred to the laboratory. Genomic DNA was extracted using Qiagen Blood DNA mini kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA samples of the probands were quantified using a Qubit® dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) and then were sequenced using NGS. The target sequencing panel of 94 known genes involved in inherited metabolic disorders including abnormal C5-carnitine related genes (*ACADSB* and *IVD*) was used. Genomic DNA was sheared to an approximate mean fragment length of 200-base pair (bp) using the Covaris LE220 ultrasonicator (Covaris, Woburn, MA). Sheared DNA was used for library preparation of targeted regions by multiplex polymerase chain reaction (PCR). The library concentration and amplicon size were determined using an Agilent High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA). The prepared sample libraries were sequenced using an Illumina NextSeq 500 platform (Illumina Inc., San Diego, CA, USA) in paired end mode, generating 150-bp paired end reads. The data were analyzed using NextSeq 500 Reporter and raw sequencing data were processed for subsequent bioinformatics analysis. The workflows of variant filtering and bioinformatics analysis were as previously described (Lin et al., 2018).

To explore the structural changes in the protein, homology modeling was used to build the three-dimensional (3D) models of ACADSB using Swiss Model Workspace with the PDB accession number 2JIF. The PDB files were submitted to the Swiss-pdb Viewer 4.0 to view the 3D-structure. The assessment of pathogenicity of these previously unreported variants was performed based on the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015). Additionally, one hundred healthy newborns from our center were randomly selected to assess the variant frequencies in normal controls. All control subjects with expanded NBS had results in the reference range.

Sanger Sequencing

The variants identified by NGS were further validated by Sanger sequencing of the patients and their parents. The exonic and flanking intronic sequences of the *ACADSB* gene were amplified by PCR. PCR products were sequenced directly on an ABI Prism 3500 automatic sequencer (Applied Biosystems, Foster City, CA, USA). All primers are listed in **Supplementary File 1: Table S1**.

Statistical Analysis

Statistical analysis was performed using SPSS 11.0 version (SPSS Inc., Chicago, IL, USA). A confidence interval of 95% was selected.

RESULTS

Biochemical and Clinical Characteristics

Twelve confirmed SBCADD cases (seven females and five males) were from twelve unrelated families, most from the Han ethic population. The initial NBS results revealed that all patients showed slightly or moderately elevated C5-carnitine concentrations (C5 cutoff value: 0.03–0.35 μmol/L), along with C5/C2 and C5/C3 ratios in the reference range (cutoff value: C5/C2: 0–0.04, C5/C3: 0.02–0.42). During follow-up, the patients' C5-carnitine concentrations presented variable levels but were always higher than the cut-off values, with the sole exception of one patient (no. 11) falling into the normal range at 31 days. Most of the C5/C2 and C5/C3 ratios were higher than the cut-off values. Increased concentrations of urinary 2-methylburtyrylglycine (2-MBG), in the absence of isovalerylglycine excretion were observed in all nine patients who underwent urinary organic acid analysis. All twelve patients in our study had normal growth and development and remained asymptomatic during follow-up (between ages 3–20 months). The biochemical, clinical features, and genotypes of all twelve patients are summarized in **Table 1**.

Genetic Studies and Variant Analysis

We applied our target sequencing panel, which included the abnormal C5-carnitine related genes (*ACADSB* and *IVD*) to all twelve patients with SBCADD and achieved high-quality results. On average, the mean coverage of the target regions was 530.14X. Coverage of targeted regions for >10X reads ranged from 99.75% to 99.91% and >20X reads from 99.50% to 99.91%. In total, eight different variants in the *ACADSB* gene were identified from 24 mutant alleles of twelve patients with SBCADD (**Supplementary File 2: Figure S1**). These patients have two allelic variants, eight of which were homozygotes and four were heterozygotes. Three variants have been reported previously, namely c.275C > G (p.Ser92*), c.655G > A (p.Val219Met), and c.1165A > G (p.Met389Val), the remaining five variants were previously unreported, including three missense variants c.596A > G (p.Tyr199Cys), c.653T > C (p.Leu218Pro), c.923G > A (p.Cys308Tyr), one nonsense variant c.886G > T (p.Gly296*) and one deletion variant c.746del (p.Pro249Leufs*15). The most frequently variant was c.1165A > G (p.Met389Val), with an allelic frequency of 8/24 (33.3%), followed by c.275C > G (p.Ser92*) 5/24 (20.8%). These previously unreported variants have not been recorded in disease databases such as HGMD, ClinVar, and LOVD, and were not detected in 100 control subjects. All previously unreported missense variants were predicted to be deleterious *in silico* by SIFT, Polyphen-2, PROVEAN, and the

TABLE 1 | Biochemical phenotype and genotype of twelve newborns with SBCADD.

Patient no.	Sex	Age[a]	Ethnic	NBS			Follow-up			Urine 2-MBG (mmol/mol creatinine)[e]	Genotype[f]	
				C5(μmol/L)[b]	C5/C2[c]	C5/C3[d]	C5(μmol/L)	C5/C2	C5/C3			
1	F	1 y, 8 m	Han	0.38	0.03	0.38	0.56/0.83 (20 d/34 d)	0.07/0.09 (20 d/34 d)	1.04/0.87 (20 d/34 d)	24.84	c.1165A > G c.1165A > G	p.Met389Val p.Met389Val
2	F	1 y, 6 m	Han	0.67	0.03	0.16	0.74 (15 d)	0.06 (15 d)	0.47(15 d)	31.73	c.655G > A **c.923G > A**	p.Val219Met **p.Cys308Tyr**
3	F	1 y, 3 m	Han	0.51	0.02	0.28	0.79/0.44 (19 d/7 m)	0.05/0.02 (19 d/7 m)	0.87/0.47 (19 d/7 m)	ND	**c.923G > A** **c.923G > A**	**p.Cys308Tyr** **p.Cys308Tyr**
4	F	1 y, 2 m	Hmong	0.69	0.04	0.26	1.66/0.86 (13 d/21 d)	0.17/0.09 (13 d/21 d)	0.97/0.66 (13 d/21 d)	40.55	c.1165A > G c.1165A > G	p.Met389Val p.Met389Val
5	M	1 y, 2 m	Han	0.37	0.02	0.23	0.88/1.01 (15 d/26 d)	0.09/0.08 (15 d/26 d)	0.54/0.5 (15 d/26 d)	15.19	c.275C > G **c.653T > C**	p.Ser92* **p.Leu218Pro**
6	F	1 y, 1 m	Han	0.38	0.03	0.39	0.53/0.5 (19 d/31 d)	0.05/0.07 (19 d/31 d)	0.48/0.56 (19 d/31 d)	79.49	**c.746del** **c.746del**	**p.Pro249Leufs*15** **p.Pro249Leufs*15**
7	F	10 m	Han	0.64	0.04	0.26	0.6/0.49 (17 d/30 d)	0.1/0.09 (17 d/30 d)	1.36/1.11 (17 d/30 d)	68.73	c.655G > A **c.886G > T**	p.Val219Metp. **Gly296***
8	M	8 m	Han	0.38	0.02	0.18	0.43 (25 d)	0.05(25 d)	0.3(25 d)	ND	c.655G > A **c.596A > G**	p.Val219Met **p.Tyr199Cys**
9	M	6 m	Han	0.44	0.02	0.28	0.64 (14 d)	0.09 (14 d)	1.08 (14 d)	ND	c.275C > G c.275C > G	p.Ser92* p.Ser92*
10	F	5 m	Han	0.41	0.03	0.35	0.55/0.58 (13 d/25 d)	0.06/0.08 (17 d/25 d)	0.71/0.92 (17 d/25 d)	39.69	c.275C > G c.275C > G	p.Ser92* p.Ser92*
11	M	5 m	Han	0.37	0.04	0.21	0.39/0.31 (14 d/31 d)	0.07/0.06 (17 d/31 d)	0.42/0.34 (14 d/31 d)	21.26	c.1165A > G c.1165A > G	p.Met389Val p.Met389Valw
12	M	3 m	Bouyei	0.36		0.17	0.51/0.38 (15 d/36 d)	0.08/0.05 (15 d/36 d)	0.76/0.43 (15 d/36 d)	18.47	c.1165A > G c.1165A > G	p.Met389Val p.Met389Val

[a]*As of December 2018, d: day, m: month, y: year.*

[b]*Reference range: 0.03–0.35 μmol/L;* [c]*reference range: 0–0.04;* [d]*reference range: 0.02–0.42;* [e]*2-MBG: 2-methylburtyrylglycine, reference range: < 0.2 mmol/mol creatinine.*

[f]*The previously unreported variants of this study are in boldface type.*

ND, not determined.

TABLE 2 | *In silico* prediction and analysis of the previously unreported *ACADSB* gene variants.

No	Location	Nucleotide change	Protein change	SIFT[a]	PolyPhen-2[b]	PROVEAN[c]	Mutation Taster[d]	HGMD[e]	ClinVar[f]	LOVD[g]	dbSNP[h]	Freq in ExAC[i]	Freq in 1000 Genome[j]
1	Exon 5	c.596A > G	p.Tyr199Cys	0	0.996	-7.57	0.999	ND	ND	ND	ND	ND	ND
2	Exon 5	c.653T > C	p.Leu218Pro	0	0.999	-6.04	0.999	ND	ND	ND	ND	ND	ND
3	Exon 6	c.746del	p.Pro249Leufs*15	N/A	N/A	N/A	1	ND	ND	ND	ND	8.24E-05	ND
4	Exon 7	c.886G > T	p.Gly296*	N/A	N/A	N/A	1	ND	ND	ND	ND	ND	ND
5	Exon 8	c.923G > A	p.Cys308Tyr	0	0.997	-7.87	0.999	ND	ND	ND	rs770456976	2.48E-05	ND

The reference sequence used in this study was based on the NCBI37/hg19 assembly of the human genome. NM_001609 was employed as reference sequence for ACADSB.
ND, no data.
N/A,t not available.
[a]SIFT: http://sift.jcvi.org/, [b]PolyPhen-2: http://genetics.bwh.harvard.edu/pph2/, [c]PROVEAN: http://provean.jcvi.org/index.php, [d]MutationTaster: http://www.mutationtaster.org/, [e]HGMD: http://www.hgmd.cf.ac.uk/ac/index.php, [f]ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/, [g]Leiden Open Variation Database http://www.lovd.nl/3.0/home, [h]dbSNP: https://www.ncbi.nlm.nih.gov/projects/SNP/, [i]ExAC: http://exac.broadinstitue.org/, [j]1000 Genome Project: http://www.1000genomes.org/.

Mutation Taster software (**Table 2**). Additionally, sequence alignments of different species revealed that the amino acid residues at positions 199, 218, and 308 in the SBCAD protein are strictly conserved (**Supplementary File 3: Figure S2**). Furthermore, although 3D-modeling analysis showed that the p.Tyr199Cys variant does not cause any significant change of the intramolecular hydrogen bonding (the corresponding 3D-modeling results are therefore not shown), since this variant substitutes a hydrophobic Tyrosine for a hydrophilic Cysteine, this might result in abnormal folding of ACADSB. The p.Leu218Pro variant may affect the ACADSB quaternary structure by losing intramolecular hydrogen bonding with 173-Ser. Thus, the protein product would be unstable and most likely be rapidly degraded (**Figure 1A**). The p.Cys308Tyr variant may alter the side chain conformations of the residues in the FAD cofactor binding cavity by inducing intramolecular hydrogen bonding with 382-Thr and 386-Ile, which may block the binding of FAD (**Figure 1B**). No further studies were performed on p.Gly296* and p.P249Lfs*15 since both variants would lead to truncated proteins that would potentially affect protein function. According to ACMG recommendations, three variants c.596A > G (p.Tyr199Cys), c.653T > C (p.Leu218Pro), and c.923G > A (p.Cys308Tyr) were classified as variants of uncertain significance (VUS), the c.886G > T (p.Gly296*) and c.746del (p.Pro249Leufs*15) were classified as likely pathogenic (LP) (**Supplementary File 4: Table S2**).

DISCUSSION

This study reported twelve patients with SBCADD in the Chinese population for the first time. The estimated incidence of SBCADD in the Chinese population was 1/30,379 (95% CI: 1/69,964-1/19,402) at a single screening center. Our findings indicate that SBCADD may be more frequent in the Chinese population than previously thought. It is notable that some patients with C5-carnitine concentrations below the current cutoff may go undetected, as previously described (Van Calcar et al., 2013). Hence the true incidence of SBCADD in our study is probably underestimated. In comparison, Van Calcar,SC *et al.* reported that the frequency of SBCADD in Hmong–American was up to 1:132, while the prevalence in non-Hmong population in Wisconsin was 1:540,780 (Van Calcar et al., 2013). Therefore, the prevalence of SBCADD shows regional and ethnic differences. Clearly, lowering the C5 cut-off value will increase the detection rate of SBCADD, but it will also increase the false positive rate. Since C5 is also the hallmark of IVA, a disorder of leucine catabolism, setting the C5 cut-off value is challenging.

All patients were identified *via* NBS with elevated concentrations of C5-carnitine, where the initial C5-carnitine concentrations ranged from an increase of 1–2 fold. In contrast, the C5 concentrations of patients with IVA increased more significantly, although the C5 values of IVA and SBCADD may overlap (Region 4 Genetics Collaborative MS/MS Project, 2012). It is worth noting that all the initial C5/C2 and C5/C3 ratios in our samples were within the reference range. This is in agreement with the previous notion that the absence of elevated C5 ratios on NBS are highly suggestive of SBCADD (Van Calcar et al., 2013). Thus our research shows once again that combining initial C5 concentrations with C5 ratios helps distinguish between these two disorders. All patients who underwent urinary organic acid analysis showed elevation of 2-MBG in the urine, indicating that 2-MBG may be a sensitive indicator, despite the limited number of patients presented.

Based on a recent literature review (Porta et al., 2019), SBCADD is symptomatic only in about 10% of reported patients. Notably, these patients' clinical manifestations cannot be attributed solely to SBCADD because there may be confounding factors such as the presence of other diseases or metabolic disorders that have not yet been diagnosed. Therefore, the small number of patients that have clinical symptoms appear to be coincidental due to the association between SBCADD and the presence of symptoms has not yet been established. In addition, most current evidence supports the idea that SBCADD is a biochemical phenotype rather than a disease, although a few reports claim that it is unsafe to consider SBCADD as a "non-disease" (Alfardan et al., 2010; Knebel et al., 2012; Van Calcar et al., 2013; Nochi et al., 2017).

The inclusion of SBCADD in NBS programs remains controversial. Nevertheless, it is noteworthy that SBCADD is extremely important for differential diagnosis of IVA, a metabolic disorder that can cause significant morbidity and mortality. Therefore, it is imprudent to completely disregard SBCADD from NBS programs. Given this, SBCADD is currently

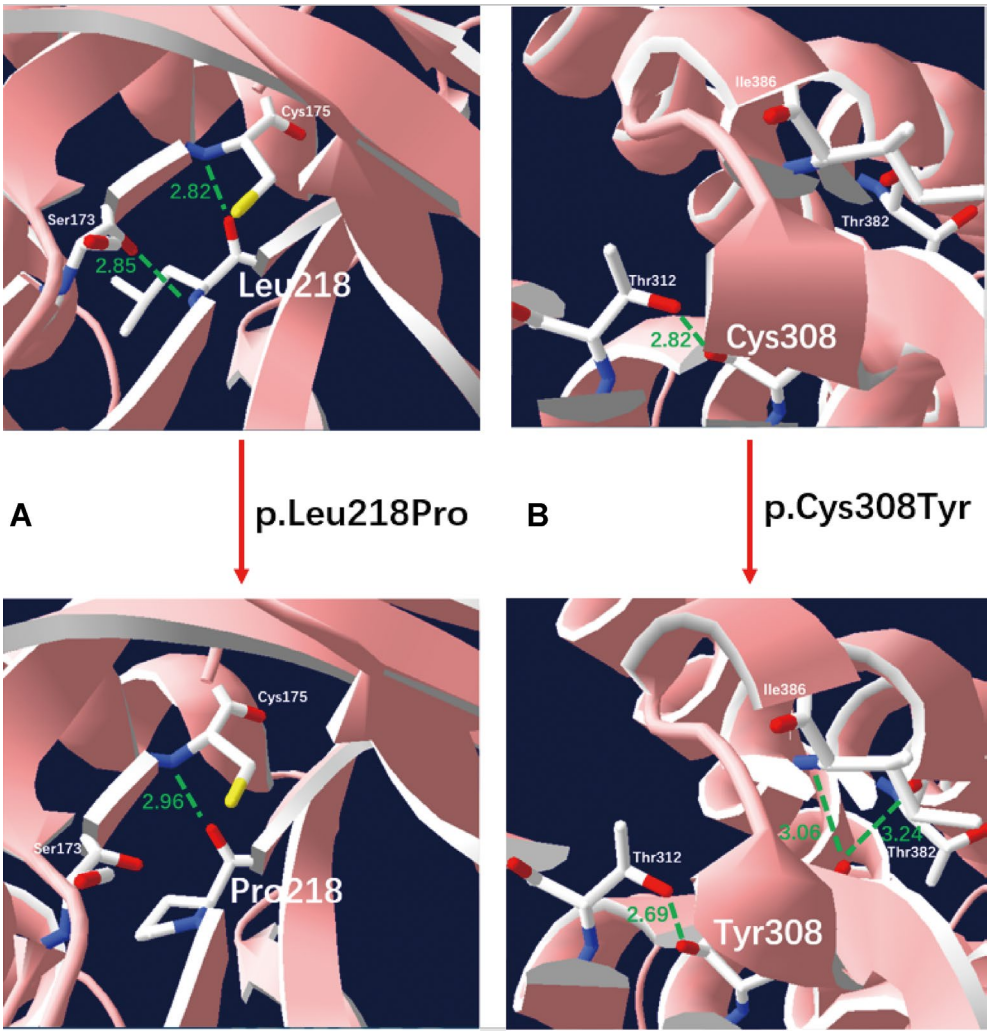


FIGURE 1 | Three-dimensional structure analysis modeling of wild-type and SBCAD mutant products. Green dashed lines represent hydrogen bonds and the green number shows the hydrogen bond distances. **(A)** The p.Leu218Pro variant may affect the *ACADSB* quaternary structure by losing intramolecular hydrogen bonding with 173-Ser. Thus, the protein product would be unstable and most likely be rapidly degraded. **(B)** The p.Cys308Tyr variant may alter the side chain conformations of the residues in the FAD cofactor binding cavity by inducing intramolecular hydrogen bonding with 382-Thr and 386-Ile, which may block the binding of FAD.

included in the recommended uniform screening panel (RUSP) as a secondary disorder. In contrast, SBCADD was not included in NBS programs of many countries when they considered cost-effectiveness and clinical utility, resulting in many patients with SBCADD not being diagnosed and less likely to be followed during development. Currently, we have retained SBCADD in our NBS programs. Similar to most SBCADD cases described so far, all patients in this study had normal growth and development and remained asymptomatic during follow-up. However, the clinical follow-up time was relatively short, we therefore cannot rule out the possibility of clinical manifestations in later life. Because of insufficient research and that the clinical course of SBCADD in the general population is not very predictable, longitudinal follow-up may be helpful to further define the natural history of SBCADD.

Until now, only fifteen variants in the *ACADSB* gene have previously been described in the literature to cause SBCADD (Andresen et al., 2000; Gibson et al., 2000; Matern et al., 2003; Korman et al., 2005; Madsen et al., 2006; Sass et al., 2008; Alfardan et al., 2010; Lanthaler et al., 2014; Deciphering Developmental Disorders Study, 2015; Porta et al., 2019). In the present study, eight different variants, including five previously unreported variants in the *ACADSB* gene were identified. The most common variant was c.1165A > G (33.3%), followed by c.275C > G (20.8%). The c.1165A > G variant was reported previously as a founder mutation in the Hmong ethnic group that was originally from southwestern China; the Hmong population ranks fourth among Chinese ethnic minorities. In this study, the c.1165A > G homozygous variant was found in two Han patients, one Bouyei patient and one Hmong patient, which suggest a possible founder effect in the Chinese population. The c.275C > G and c.655G > A variants were reported previously in an asymptomatic Taiwan infant identified by NBS (Liu et al., 2007). For previously unreported variants, the

main evidence for the classification of variants are as follows: (i) all variants were not present or have an extremely low allelic frequency in dbSNP, ExAC, and 1000 Genome, and are not listed in the disease databases such as HGMD, ClinVar, and LOVD; (ii) in all variants, a highly conserved amino acid of SBCAD was changed, and was predicted to have a damaging effect on the SBCAD protein using different bioinformatics analysis programs; and (iii) 3D-modeling analysis showed that these missense variants may affect the ACADSB quaternary structure and result in the production of an unstable ACADSB protein or block the binding of the FAD cofactor, which may affect enzyme activity. The limitation of our study is that further definite proof such as functional assays were not performed because of limited resources, although all three previously unreported missense variants were predicted as probably disease-causing by bioinformatics analysis and the other two previously unreported variants would lead to truncated proteins and potentially affect protein function.

In conclusion, this is the first report of biochemical, clinical, and genetic analysis of SBCADD in the Chinese population. Our findings indicate a high incidence of SBCADD among the Chinese population. Five previously unreported variants in the *ACADSB* gene were identified, which expanded the *ACADSB* variation spectrum. Although the clinical course of our patients are likely benign, longitudinal follow-up may be helpful to better understand the natural history of SBCADD.

AUTHOR CONTRIBUTIONS

YL designed the study, performed experimental work, paper writing and drafting; ML carried out the genetic tests, mutation analysis and paper editing; HG, SZ and XL participated in manuscript preparation; CL, YC, WL and ZZ followed the patients and collected the clinical data; QF was the mentor who designed and guided the research study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00802/full#supplementary-material

SUPPLEMENTARY FILE 1: TABLE S1 | List of primers used for Sanger sequencing.

SUPPLEMENTARY FILE 2: FIGURE S1 | Validation of *ACADSB* variants by Sanger sequencing (the variant is indicated with a red arrow).

SUPPLEMENTARY FILE 3: FIGURE S2 | Multiple sequence alignment using ClustalX. The amino acid residues at positions 199, 218, and 308 in the SBCAD protein (highlighted in box) are strictly conserved among various species.

SUPPLEMENTARY FILE 4: TABLE S2 | Evidence and classification of previously unreported variants.

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Expanded Newborn Screening for Inborn Errors of Metabolism by Tandem Mass Spectrometry in Suzhou, China: Disease Spectrum, Prevalence, Genetic Characteristics in a Chinese Population

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Expanded newborn screening for inborn errors of metabolism (IEMs) by tandem mass spectrometry (MS/MS) could simultaneously analyze more than 40 metabolites and identify about 50 kinds of IEMs. Next generation sequencing (NGS) targeting hundreds of IMEs-associated genes as a follow-up test in expanded newborn screening has been used for genetic analysis of patients. The spectrum, prevalence, and genetic characteristic of IEMs vary dramatically in different populations. To determine the spectrum, prevalence, and gene mutations of IEMs in newborns in Suzhou, China, 401,660 newborns were screened by MS/MS and 138 patients were referred to genetic analysis by NGS. The spectrum of 22 IEMs were observed in Suzhou population of newborns, and the overall incidence (excluding short chain acyl-CoA dehydrogenase deficiency (SCADD) and 3-Methylcrotonyl-CoA carboxylase deficiency (3-MCCD)) was 1/3,163. The prevalence of each IEM ranged from 1/401,660 to 1/19,128, while phenylketonuria (PKU) (1/19,128) and Mild hyperphenylalaninemia (M-HPA) (1/19,128) were the most common IEMs, followed by primary carnitine uptake defect (PCUD) (1/26,777), SCADD (1/28,690), hypermethioninemia (H-MET) (1/30,893), 3-MCCD (1/33,412) and methylmalonic acidemia (MMA) (1/40,166). Moreover, 89 reported mutations and 51 novel mutations in 25 IMEs-associated genes were detected in 138 patients with one of 22 IEMs. Some hotspot mutations were observed for ten IEMs, including *PAH* gene c.728G > A, c.611A > G, and c.721C > T for Phenylketonuria, *PAH* gene c.158G > A, c.1238G > C, c.728G > A, and c.1315+6T > A for M-HPA, *SLC22A5* gene c.1400C > G, c.51C > G, and c.760C > T for PCUD, *ACADS* gene c.1031A > G, c.164C > T, and c.1130C > T for SCAD deficiency, *MAT1A* gene c.791G > A for H-MET, *MCCC1* gene c.639+2T > A and c.863A > G for 3-MCCD, *MMUT* gene c.1663G > A for MMA, *SLC25A13* gene c.IVS16ins3Kb and c.852_855delTATG for cittrullinemia II, *PTS* gene c.259C > T and c.166G > A for

Tetrahydrobiopterin deficiency, and *ACAD8* gene c.1000C > T and c.286C > A for Isobutyryl coa dehydrogenase deficiency. All these hotspot mutations were reported to be pathogenic or likely pathogenic, except a novel mutation of *ACAD8* gene c.286C > A. These mutational hotspots could be potential candidates for gene screening and these novel mutations expanded the mutational spectrum of IEMs. Therefore, our findings could be of value for genetic counseling and genetic diagnosis of IEMs.

Keywords: expanded newborn screening, inborn errors of metabolism, tandem mass spectrometry, disease spectrum, prevalence, genetic characteristics, hotspot mutation

INTRODUCTION

Inborn errors of metabolism (IMEs) are a large group of monogenic diseases resulting in death and abnormalities of physical and neurological development at almost all stages of life. IMEs are always caused by the defect of an enzyme, its coenzyme, or a transporter leading to the accumulation of its substrate and/or the insufficiency of its downstream products. Nowadays, the introduction of tandem mass spectrometry (TMS) allows screening for more than 50 IMEs using dried blood spot in the neonatal period (Therrell et al., 2015). For the neonates screened to have IMEs, some serious clinical consequence could be prevented, including mild to severe irreversible mental retardation, lifelong disability, physical handicaps, coma, and early death, if early diagnosis and treatment were implemented (Therrell et al., 2015). Therefore, expanded newborn screening program covering dozens of diseases has been implemented in the majority of developed countries. For example, population-based data are available in the United States (Centers for Disease Control and Prevention, 2008; Gallant et al., 2012; Hsu et al., 2013), Canada (Karaceper et al., 2016), United Kingdom (Sanderson et al., 2006), Germany (Lindner et al., 2008), France (Dhondt, 2010), Egypt (Hassan et al., 2016), Greece (Loukas et al., 2010), Saudi Arabia (Alfadhel et al., 2017), Australia (Wiley et al., 1999; Webster et al., 2003), South Korea (Yoon et al., 2005), Singapore (Lim et al., 2014), and Japan (Shibata et al., 2018).

The advent of target capture and next generation sequencing (NGS) enables simultaneously sequence a large group of targeted genes accounting for numerous diseases, which has become the best choice for identification of genetic etiology of IMEs following expanded newborn screening program. The utility of NGS in expanded newborn screening has enriched our understanding of genetic etiology, genetic characteristics, and phenotype-genotype correlation of IMEs. Some hotspot variants resulting in the defect of enzymes have been identified in patients with IMEs, such as *ACADS* variants c.511C > T and c.625G > A for short chain acyl-CoA dehydrogenase deficiency (SCADD; MIM# 201470) (Tonin et al., 2016; Nochi et al., 2017), *PAH* variant c.728C > A for phenylketonuria (PKU; MIM# 261600) (Liu et al., 2017), and so on. Also, many IMEs have a dramatic variation of symptoms and the outcome of the affected patients was correlated with genotype, such as medium chain acyl-CoA dehydrogenase deficiency (MCADD; MIM# 201450) (Ensenauer et al., 2005; Maier et al., 2005), very long chain acyl-CoA dehydrogenase deficiency (VLCADD; MIM# 609016) (Andresen et al., 1999; Obaid et al., 2018), and so on. In addition, the spectrum, the incidence, and the genetic characteristics of IMEs vary dramatically in different regions and populations.

Expanded newborn screening was introduced in China in 2004, later than developed countries. In the milestone pilot study, a total of 371,942 newborns were screened in four centers, and the collective estimated incidence of overall IMEs was 1/3,795 in live births, with a sensitivity of 98.99% and a specificity of 99.83% (Shi et al., 2012). Recently, targeted sequencing of genes associated to more than 50 IMEs by NGS was used as a follow-up test for genetic diagnosis after the expanded newborn screening, and some novel variants were found in Chinese patients. In Suzhou, the expanded newborn screening program targeting 27 IMEs started in 2014. Until now, its screening rate is closed to 100% of live births and more than 400,000 newborns have been referred to expand newborn screening. A total of 22 kinds of IMEs were identified in Suzhou population and 153 infants were diagnosed with one of these IMEs. Almost all these patients were referred to genetic analysis *via* targeted NGS. 140 variants in 25 IMEs-associated genes were found in 138 patients. Some hotspot variants were also observed in Suzhou patients, including c.791G > A in *MATA1* gene for hypermethioninemia (MIM# 250850), c.158G > A in *PAH* gene for mild hyperphenylalaninemia (M-HPA; MIM# 261600), c.721C > T in *PAH* gene for PKU

Abbreviations: ASA, Argininosuccinate aciduria; BH4 deficiency, Tetrahydrobiopterin deficiency; BKT, beta-Ketothiolase deficiency; CACT, Carnitine-acylcarnitine translocase deficiency; CPT-Ia, Carnitine palmitoyltransferase I deficiency; CPY-II, Carnitine palmitoyltransferase II deficiency; EMA, Ethylmalonic encephalopathy; GA-I, Glutaric aciduria type I; H-ARG, Arginemia; HCY, Homocystinuria; H-MET, Hypermethioninemia; HMG, 3-Hydroxy-3-methylglutaric aciduria; H-ORN, Hyperornithinemia; H-PRO, Hyperprolinemia; H-TYR, Tyrosinemia; IBG, Isobutyrylglycinuria; IVA, Isovaleric acidemia; LCHADD, long-chain L-3-Hydroxy acyl-CoA dehydrogenase deficiency; MADD, mutiple Acyl-coa dehydrogenases deficiency; MAL, Malonic acidemia; MCADD, Medium chain acyl-CoA dehydrogenase deficiency; MCD, multiple Carboxylase deficiency (MCD); M-HPA, mild Hyperphenylalaninemia; MSUD, Maple syrup urine disease; MUT, Methylmalonic acidemia; NKHG, Nonketotic hyperglycinemia; OTC, Ornithine transcarbamylase deficiency; PCUD, Primary carnitine uptake defect; PKU, Phenylketonuria; PROP, Propionic acidemia; SCADD, Short chain acyl-CoA dehydrogenase deficiency; TFP, Trifunctional protein deficiency; TYR-I, Tyrosinemia type I; VLCADD, very long chain acyl-CoA dehydrogenase deficiency; 2M3HBA, 2-Methyl-3-hydroxybutyric aciduria; 2MBG, 2-Methylbutyrylglycinuria; 3-MCC, 3-Methylcrotonyl-CoA carboxylase deficiency; 3MGA, 3-Methylglutaconic aciduria.

(MIM# 261600), c.852_855delTATG in *SLC25A13* gene for citrullinemia type II (CTLN 2; MIM# 605814), c.639+2T > A in *MCCC1* gene for 3- methylcroton acyl coenzyme A carboxylase deficiency (3-MCCD; MIM# 210200 and 210210), c.1400C > G in *SLC22A5* gene for primary carnitine uptake defect (PCUD; MIM# 212140), and c1031A > G in *ACADS* gene for SCADD. These hotspot mutations could explain the relative high incidence of associated IMEs. As a result, it is critical to screen these mutations and prenatal genetic consulting for Suzhou population. These mutations are good candidates for further research on genetic characteristics in other Chinese populations.

MATERIAL AND METHODS

Subjects

A total of 401,660 newborns were referred to expand newborn screening. Informed and written consent was obtained from the parents of all screened newborns. Our screened protocol is consistent with other newborn screening centers in China, and was shown in **Figure 1**. The protocol was reviewed and approved by Ethic committee of the Affiliated Suzhou Hospital of Nanjing Medical University.

Expanded Newborn Screening Assay

Eleven amino acids, 30 acylcarnitines, free carnitine, and succinylacetone were tested using tandem mass spectrometry (**Supplementary Table 1**). Assays for screening inborn metabolism disorders were performed using screening kit (PerkinElmer, USA) and Waters HPLC-tandem mass spectrometry (TQD, Waters, USA). In brief, 100-ul extract liquor containing internal standards was added into U bottom plates. After incubating for 45 min at 45°C, 75-ul extract liquor was transferred into V bottom plates. After 2 h standing at ambient temperature, 25-ul liquor was injected into tandem mass spectrometry for metabolites analyses. Three levels of internal quality controls including blank, low, and high were used for quality control.

Positive Results for IMEs

In our screening panel, 26 kinds of IEMs were included. Each IEM had two or more indicators including metabolites and ratios, and their cut-off values. When DBS results met the positive rules of IEMs, they were considered as positive. All the positive rules of IEMs were shown in **Table 1**.

Genetic Analysis

High throughput sequencing was performed on all patients diagnosed with one kind of IEMs using the expanded edition panel of IMEs (Genuine Diagnostic, Hangzhou, China) including 306 genes related to IEMs. In brief, the target sequences were enriched using Agilent SureSelect Human Exon Sequence Capture Kit (Agilent Technologies, Inc, California, USA). Next, the captured products were purified using Agencourt AMPure XP beads (Beckman Coulter, Inc, Miami, USA). Then, the sequencing library was established using TruePrepTM DNA Library Prep Kit V2 (Vazyme Biotech, New Jersey, USA) and TruePrepTM Index Kit V2 (Vazyme Biotech, New Jersey, USA) and was examined by Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc, California, USA). Finally, the sequencing library was quantified by Illumina DNA Standards and Primer Premix Kit (KAPA Biosystems, Boston, USA), and massively parallel sequenced on Illumina HiSeq 2500 system.

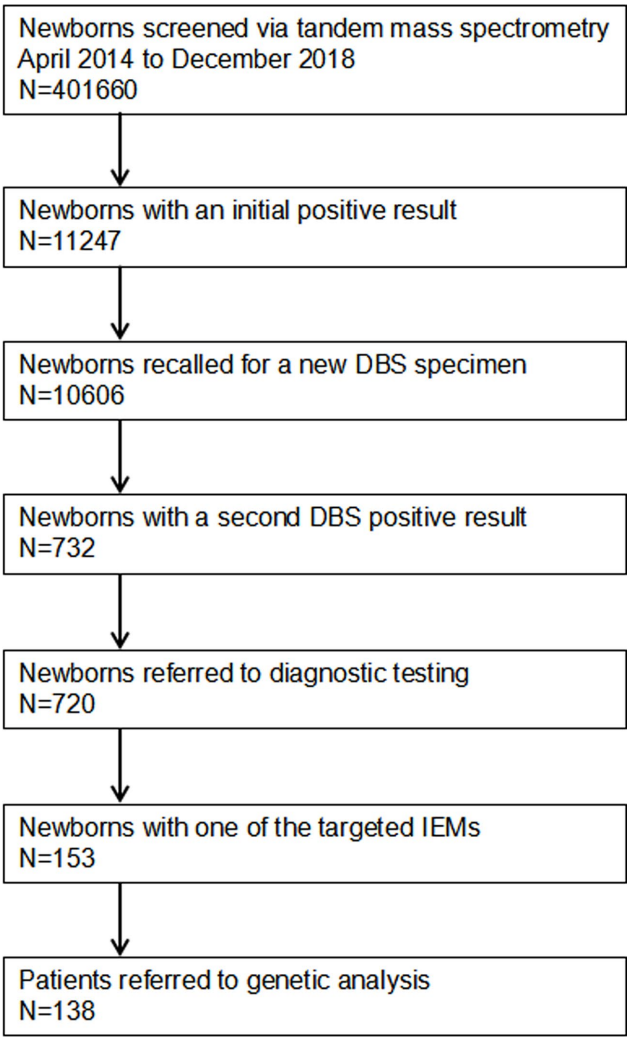


FIGURE 1 | Flowchart of expanded newborn screening for inborn errors of metabolism and genetic analysis of patients. DBS, dried blood spot; IEMs, inborn errors of metabolisms.

Statistical Analysis

Statistical analysis was performed using SPSS17.0 version. The difference of categorical data was compared using Chi-square test. The difference of measurement data was compared by analysis of variance. $p < 0.05$ was considered to be statistical significance.

TABLE 1 | Conditions and their positive rules in expanded newborn screening panel.

Conditions	Positive rule 1	Positive rule 2	Positive rule 3
PKU, M-HPA, BH4 deficiency	PHE > 100 nmol/L, PHE/TYR > 1.2	PHE > 130 nmol/L,	PHE/TYR > 2
HCY, H-MET	MET > 70 nmol/L	MET > 43 nmol/L, MET/PHE > 0.85	
ASA	CIT > 50 nmol/L	CIT > 35 nmol/L, ALA/CIT < 8.5	
MSUD	LEU+ILE+PRO-OH > 400 nmol/L	LEU+ILE+PRO-OH > 320 nmol/L, LEU+ILE+PRO-OH/PHE > 5.9, VAL > 250 nmol/L	
OTC	CIT < 5.5 nmol/L	CIT < 6.5nmol/L, CIT/PHE < 0.12	
H-ORN	ORN > 450 nmol/L	ORN > 340 nmol/L, ORN/CIT > 24	
H-TYR	TYR > 400 nmol/L	TYR > 350nmol/L, LEU+ILE+PRO-OH/TYR < 0.5, PHE/TYR < 0.15	
H-ARG	ARG > 65 nmol/L	ARG/PHE > 1.2, ARG > 50 nmol/L	
TYR-I	SA > 2 nmol/L	SA > 1.2 nmol/L, SA/PHE > 0.03	
H-PRO	PRO > 470 nmol/L		
MUT, PROP	C3/C0 > 0.3 nmol/L	C3/C2 > 0.21, C3 > 4.5nmol/L	C3 > 6.5
IVA, 2MBG	C5 > 0.8 nmol/L	C5 > 0.4 nmol/L, C5/C0 > 0.02	
3-MCC, MCD, 2M3HBA, 3MGA, HMG	C4DC+C5-OH > 0.7nmol/L	C4DC+C5-OH > 0.5nmol/L, (C4DC+C5-OH)/C0 > 0.025	
MADD	C5 > 0.4 nmol/L,C4 > 0.5 nmol/L		
BKT	C5:1 > 0.02nmol/L,C4DC+C5-OH > 0.5nmol/L		
MAL	C3DC+C4-OH > 0.8nmol/L	C3DC+C4-OH > 0.45nmol/L, (C3DC+C4-OH)/C10 > 5	
GA-I	C5DC+C6-OH > 0.4nmol/L	C5DC+C6-OH > 0.23nmol/L, (C5DC+C6-OH)/(C3DC+C4-OH) > 2, (C5DC+C6-OH)/(C4DC+C5-OH) > 1.38	
MCADD	C8 > 0.3nmol/L	C6 > 0.11nmol/L, C8 > 0.19nmol/L, C8/C2≥0.01, (C4DC+C5-OH)/C8 < 1	
VLCADD	C14:1 > 0.5nmol/L	C14:1 > 0.35nmol/L, C14:1/C16 > 0.14, C14:1/C2≥0.02	
LCHADD, TFP	C16-OH > 0.06nmol/L,C16-OH/C16 > 0.025,C18:1-OH > = 0.06 nmol/L,C18-OH > 0.03 nmol/L		
PCUD	C0 < 9.5 nmol/L		
CPT-Ia	C0 > 100 nmol/L	C0/(C16+C18) > 50, C0 > 55 nmol/L, (C16+C18:1)/C2 < 0.08	
CPY-II, CACT	C18 > 1.9 nmol/L, C18:1 > 3 nmol/L	C16> 12 nmol/L, C16 > 7 nmol/L, C18:1 > 3 nmol/L	
SCADD	C4 > 0.7 nmol/L	C4 > 0.5 nmol/L, C4/C2 > 0.03	
NKHG	GLY > 1100 nmol/L		
IBG, EMA	C4 > 0.7 nmol/L	C4/C3 > 0.45, C4/C2 > 0.03	

RESULTS

A total of 401,660 newborns were screened by expanded newborn screening program (**Figure 1**). After initial screening, 11,247 (2.80%) newborns, who had positive results, were recalled for a new specimen. However, only 10,606 (94.30%) newborns with an initial positive result were collected a new specimen. After a repeated test, 732 (6.90%) newborns with a second positive result were determined to be suspect positive, and 720 (98.36%) of them were referred to diagnostic testing. Finally, 153 infants were diagnosed with one of IMEs and treated, and 138 of them were referred to genetic analysis. As SCADD and 3-MCCD were diseases with questionable phenotype, the overall incidence (excluding SCADD and 3-MCCD) was 1/3163. The comparison of all characteristics between normal newborns and patients did not reach at significant difference, including age at testing ($p = 0.574$), gender ($p = 0.260$), gestational age ($p = 0.691$), birth weight ($p = 0.795$), number of fetus ($p = 0.988$), register region ($p = 0.571$), and household registration ($p = 0.166$). The characteristics of newborns screened by expanded newborn screening program were shown in **Table 2**.

Of 22 IEMs, 10 were amino acid metabolic disorders (AAMDs), 7 were organic acid metabolic disorders (OAMDs), and 5 were fatty acid metabolic disorders (FAMDs). The AAMDs were the most common diseases, accounting for 51.63% of patients, followed by FAMDs (19.61%) and OAMDs (28.76%). The overall prevalence of AAMDs, FAMDs, and OAMDs was 1/5,084, 1/11,814, and 1/10,041, respectively. HPA may be induced by PAH defect or tetrahydrobiopterin deficiency. A total of 48 infants with HPA were found, including 42 (87.5%) infants with PAH defect and 6 (12.5%) infants with tetrahydrobiopterin deficiency caused by PTPS (MIM* 612719) defect. The incidence of HPA, PAH defect, and PTPS defect were 1/8,368, 1/9,563, and 1/66,943, respectively. Furthermore, 42 infants with PAH defect were classified into two groups: 21 (50%) infants with PKU (≥360 μmol/L Phe) and 21 (50%) infants with M-HPA (120 μmol/L to 360 μmol/L Phe) (Chen et al., 2015). Of the 10 AAMDs, PKU and M-HPA were the most common diseases, accounting for 26.58% of patients, respectively, followed by hypermethioninemia (16.46%). The prevalence of single AAMD ranged from 1/401,660 to 1/19,127. Of the 7 OAMDs, 3-MCCD was the most common disease, accounting for 40.00% of patients, followed by methylmalonic acidemia (MMA; MIM# 251000) (33.33%). The prevalence of single OAMD ranged from 1/401,660 to 1/33,412. Of the 5 FAMDs, PCUD was the most common disease, accounting for 34.09% of patients, followed by SCADD (31.82%), VLCADD (13.64%), and MCADD (11.36%). The prevalence of single

TABLE 2 | Characteristics of newborns screened by expanded newborn screening program.

	Newborns without targeted IMEsN = 401,507	PatientsN = 153	*p*
Age at initial testing (days, mean ± SD)	7.35 ± 8.14	6.98 ± 6.08	0.574
Gender			
Male	210,273	86	0.260
Female	191,194	65	
No record	41	0	
Gestational age (weeks)			
<32	1,915	0	0.691
32~36	18652	7	
>37	379861	146	
No record	1232	0	
Birth Weight (g)			
<1,500	607	0	0.795
1,500–1,999	1,986	0	
2000–2499	9,904	4	
>2,500	377,858	149	
No record	11,305	0	
Number of fetus			
Singleton	398,649	152	0.988
Twins	2,992	1	
Triplet	19	0	
Register region			
Suzhou	229,793	91	0.571
Others	171,867	62	
No record	0	0	
Household registration			
Urban	245,863	102	0.166
Rural	155,797	51	
No record	0	0	

FAMD ranged from 1/100,411 to 1/26,777. All the above data were shown in **Figure 2** and **Table 3**.

With regard to genetic analysis, 89 reported mutations and 51 novel mutations were detected in 138 patients with one of IEMs (**Table 4**). All common IEMs affecting more than 10 patients had mutational hotspots. In 12 patients with PKU, 17 mutations were detected and the c.728G > A was the most common mutations in *PAH* gene, accounting for 20.8% of mutational alleles and 41.7% of patients, followed by c.611A > G (8.3% and 16.7%), c.721C > T (8.3% and 16.7%), and c.498C > G (8.3% and 8.3%). All the four hot mutations are pathogenic (www.ncbi.nlm.nih.gov/clinvar). In 18 patients with M-HPA, 19 mutations were detected and the c.158G > A was the most common mutations in *PAH* gene, accounting for 25.0% of mutational alleles and 50.0% of patients, followed by c.1238G > C (11.1% and 22.2%), c.728G > A (8.3% and 16.7%), and c.1315+6T > A (8.3% and 16.7%). However, the c.158G > A has a uncertain significance of pathogenicity (www.ncbi.nlm.nih.gov/clinvar). In 15 patients with PCUD, nine mutations were detected and the c.1400C > G was the most common mutation in *SLC22A5* (MIM* 603377) gene, accounting for 50.0% of mutational alleles and 80% of patients, followed by c.51C > G (13.3% and 26.7%) and c.760C > T (13.3% and 26.7%). All the three mutations are pathogenic and/or likely pathogenic (). In 14 patients with SCAD deficiency, nine mutations were detected and the c.1031A > G was the most common mutation in *ACADS* (MIM* 606885) gene, accounting for 42.9% of mutational alleles and 71.4% of patients, followed by c.164C > T (17.9% and 28.6%) and c.1130C > T (10.7% and 21.4%). The two mutation c.1031A > G and c.164C > T are likely pathogenic, and the c.1130C > T had conflicting interpretations of pathogenicity (www.ncbi.nlm.nih.gov/clinvar). Hypermethioninemia is able to be inherited by dominant transmission of *MAT1A* (MIM* 610550) gene. In 13 patients with hypermethioninemia, five mutations were detected and the c.791G > A was the most common mutation in *MAT1A* gene, accounting for 71.4% of mutational alleles and 76.9% of patients. The c.791G > A is pathogenic (www.ncbi.nlm.nih.gov/clinvar) and dominantly inherited. In 12 patients with 3-Methylcrotonyl-CoA carboxylase deficiency, 13 mutations and 2 mutations were detected in *MCCC1* (MIM* 609010) gene and *MCCC2* (MIM* 609014) gene. The most common mutation is c.639+2T > A of *MCCC1* gene, accounting for 16.7% of mutational alleles and 33.3% of patients, followed by c.863A > G of *MCCC1* gene (12.5% and 25.0%). The c.639+2T > A is pathogenic, but the c.863A > G has uncertain significance of pathogenicity (). Of 10 patients with MMA, 7 carried *MMUT* (MIM* 609058) gene mutations and 3 carried *MMACHC* (MIM* 609831) gene mutations. In all patients with MMA, the most common mutation was c.1663G > A of *MMUT* gene, accounting for 15.0% of mutational alleles and 30% of patients, followed by c.729_730insTT of *MMUT* gene (10.0% and 20.0%) and c.609G > A of *MMACHC* gene (10.0% and 20.0%).

Other IEMs including citrullinemia (MIM# 605814 and 603471), Tetrahydrobiopterin deficiency (MIM# 233910, 261640, 612716, 264070, and 261630), and Isobutyryl coa dehydrogenase deficiency (IBD; MIM# 611283) were also observed to have mutational hotspots. Of eight patients with Citrullinemia, seven were confirmed CTLN2 caused by mutations in *SLC25A13* (MIM* 603859) gene, and only one cittrullinemia I (CTLN 1; MIM# 215700) caused by mutations in *ASS1* (MIM* 603470) gene. In all patients with citrullinemia, the most common mutation was c.IVS16ins3Kb of *SLC25A13* gene, accounting for 25.0% of mutational alleles and 50.0% of patients, followed by c.852_855delTATG of *SLC25A13* gene (18.8% and 37.5%). Both c.IVS16ins3Kb and c.852_855delTATG of *SLC25A13* gene are pathogenic for CTLN2 (www.ncbi.nlm.nih.gov/clinvar). In six patients with Tetrahydrobiopterin deficiency, six mutations in *PTS* (MIM* 612719) gene were detected and the c.259C > T was the most common mutation, accounting for 41.7% of mutational alleles and 50.0% of patients, followed by c.166G > A (25.0% and 50.0%). Furthermore, the c.259C > T is pathogenic and the c.166G > A is likely pathogenic (www.ncbi.nlm.nih.gov/clinvar), and the two mutations account for 83.3% of patients. Interestingly, all the three patients with Isobutyryl coa dehydrogenase deficiency were heterozygous for the c.1000C > T variant of *ACAD8* (MIM* 604773) gene, and two patients were heterozygous for the c.286C > A variant, which has not been reported. The c.1000C > T of the *ACAD8* gene was reported to be pathogenic and likely pathogenic in patients with IBD (www.ncbi.nlm.nih.gov/clinvar). Obviously, the two mutations are hotspots and main causes for IBD.

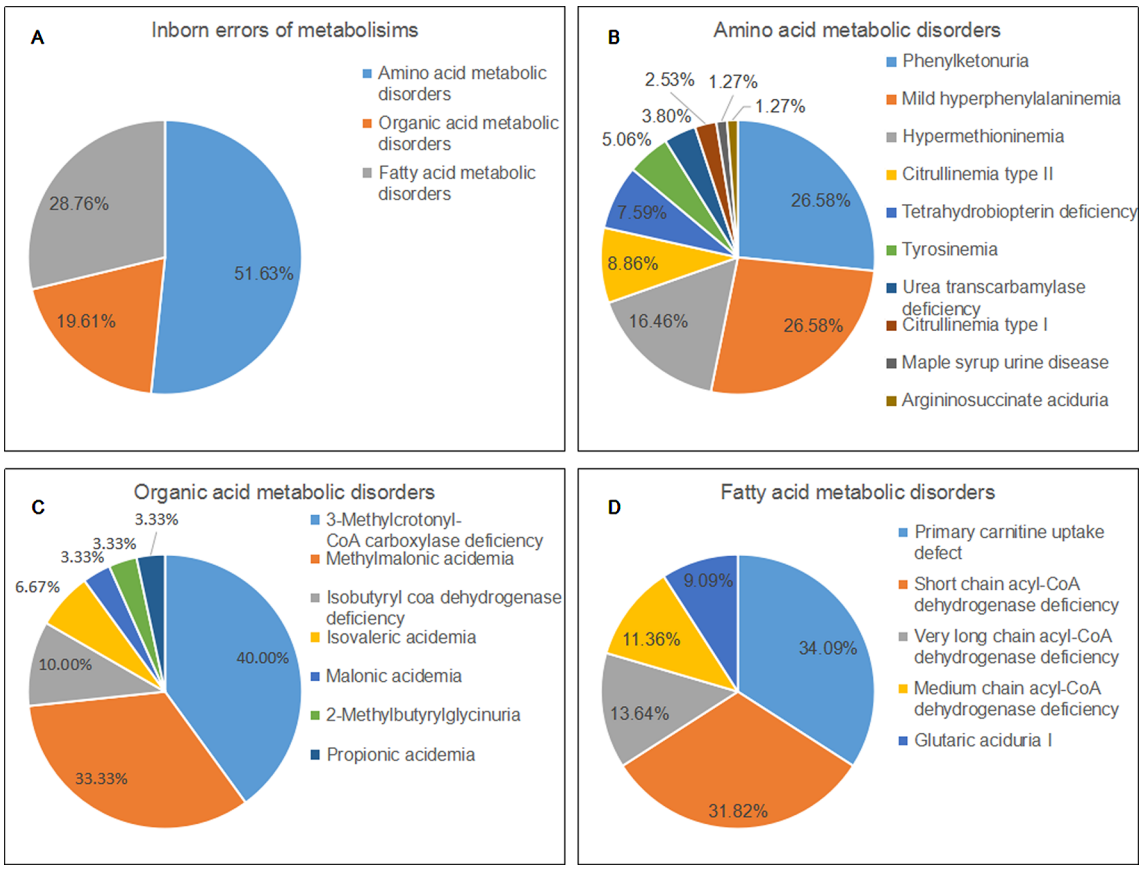


FIGURE 2 | Disease spectrum and distribution of inborn errors of metabolisms. **(A)** The percentage of three categories of inborn errors of metabolisms. **(B)** the percentage of different kinds of amino acid metabolic disorders. **(C)** the percentage of different kinds of organic acid metabolic disorders. **(D)** the percentage of different kinds of fatty acid metabolic disorders.

DISCUSSION

PKU is an autosomal recessive genetic AAMD caused by deficiency of phenylalanine hydroxylase (PAH) (Blau et al., 2010). They were the most common IMEs identified by expanded newborn screening program, and the incidence of both were about 1/20,000. Up to now, more than 800 *PAH* mutations have been identified in patients with deficiency of PAH (Zhang et al., 2018). Some hotspot mutations exist in *PAH* gene and vary in different populations. For example, the most common *PAH* mutation is c.1222C > T in American (Kaul et al., 1994), IVS10-11G > A in Iranian (Zamanfar et al., 2017; Esfahani and Vallian, 2018; Rastegar Moghadam et al., 2018) and Spanish (Bueno et al., 2013; Aldámiz-Echevarría et al., 2016), c.168+5G > C in western Iranian (Alibakhshi et al., 2014), c.1238G > C in Japanese (Okano et al., 2011; Dateki et al., 2016), c.728G > A in Chinese (Zhou et al., 2012; Li et al., 2015; Zhang et al, 2018), c.781C > T in Karachays (Gundorova et al., 2018), c.1068C > A and c.728G > A in south Korean (Lee et al., 2008), c.1162G > A in Brazilian (Vieira Neto et al., 2018), c.782G > A in Syrian (Murad et al., 2013), and c.1222C > T in Australian (Ho et al., 2014). These different hotspot mutations suggested different origins. Similar to other Chinese populations, in our cohort, the c.728G > A is the most common *PAH* mutation that account for 20.8% of mutational alleles and 41.7% of classical PKU patients. However, in patients with M-HPA, the c.158C > A is the most common mutation that account for 25.0% of mutational alleles and 50.0% of patients. In a Japanese population, the c.158C > A also exhibited a relative higher prevalence in patients with hyperphenylalaninemia compared with PKU (Dateki et al., 2016). It appears that PAH deficiency has a correlation between genotype and clinical phenotype, and the c.158C > A could be considered as a marker for differentiating hyperphenylalaninemia from classical PKU.

PCUD is the second common IME. It shows a large variation of prevalence in different populations. For example, the prevalence of PCUD is 1/297 in Faroese (Rasmussen et al., 2014), 1/120,000 in Australian (Wilcken et al., 2003), 1/40,000 in Japanese (Koizumi et al., 1999), and 1/20,000–70,000 in American (Magoulas and El-Hattab, 2012). According to the available data, the prevalence of PCUD ranges from 1/45,000 to 1/8,000 in different areas of China (Han et al., 2012; Sun et al., 2017; Zheng et al., 2017; Guo et al., 2018), while the incidence of PCUD is 1/26,777 in Suzhou population. PCUD is caused by deficiency of organic cation transporter 2 (OCTN2) that results from variants in *SLC22A5* gene. The symptomatic patients presented a variety of clinical symptoms, including muscle weakness, dilated cardiomyopathy, hepatomegaly, encephalopathy, sudden infant death, feeding

TABLE 3 | The spectrum and incidence of conditions from 401660 newborns screened by expanded newborn screening program.

Conditions	Patients	Estimated incidence	95%CI
Amino acid metabolic disorders	79	1/5,084	1/6,378–1/4,102
Phenylketonuria	21	1/19,128	1/30,093–1/12,729
Mild hyperphenylalaninemia	21	1/19,128	1/30,093–1/12,729
Hypermethioninemia	13	1/30893	1/55,556–1/18,532
Citrullinemia type II	7	1/57,372	1/131,199–1/29,011
Tetrahydrobiopterin deficiency	6	1/66,934	1/165,153-1/32,185
Tyrosinemia	4	1/100,411	1/316,056–1/41,632
Ornithine transcarbamylase deficiency	3	1/133,887	1/526,316–1/49,188
Citrullinemia type I	2	1/200,843	1/1,197,891–1/60,790
Maple syrup urine disease	1	1/401660	1/8,025,682–1/ 81,433
Argininosuccinate aciduria	1	1/401,660	1/8025682–1/81,433
Organic acid metabolic disorders	30	1/13,389	1/19,486–1/9,497
3-Methylcrotonyl-CoA carboxylase deficiency	12	1/33,412	1/61,767–1/19,689
Methylmalonic acidemia	10	1/40,166	1/79,051–1/22,533
Isobutyryl coa dehydrogenase deficiency	3	1/133,887	1/526,316–1/49,188
Isovaleric acidemia	2	1/200,843	1/1,197,891–1/60,790
Malonic acidemia	1	1/401,660	1/8,025,682–1/81,433
2-Methylbutyrylglycinuria	1	1/401,660	1/8,025,682–1/81,433
Propionic acidemia	1	1/401,660	1/8,025,682–1/81,433
Fatty acid metabolic disorders	44	1/9,129	1/11,669–1/6,863
Primary carnitine uptake defect	15	1/26,777	1/46,083–1/16,609
Short chain acyl-CoA dehydrogenase deficiency	14	1/28,690	1/50,403–1/17,516
Very long chain acyl-CoA dehydrogenase deficiency	6	1/66,934	1/165,153–1/32,185
Medium chain acyl-CoA dehydrogenase deficiency	5	1/80,332	1/219,250–1/36,245
Glutaric aciduria type I	4	1/100,411	1/316,056–1/41,632

difficulty, recurrent pneumonia, vomiting, abdominal pain, and diarrhea (Han et al., 2014). These symptoms might be caused by different genotypes of PCUD (Rose et al., 2012; Chen et al., 2013). To date, more than 110 *SLC22A5* mutations have been reported and hotspot mutations vary in different population (Han et al., 2014). For example, C.844T > C is always observed in Caucasian PCUD patients (Burwinkel et al., 1999; Vaz et al., 1999; Wang et al., 1999), and c.1400C > G is the hotspot mutation in Southeast Asian (Koizumi et al., 1999; Nezu et al., 1999; Tang et al., 1999). However, in California patients, no obvious hotspot mutation was observed in PCUD patients (Gallant et al., 2017). This could be explained by the fact that those patients are multi-ethnic. With regard to Chinese, the hotspot mutations are similar in different regions. Chen et al. found the most common mutations were c.760C > T (32.9%), c.1400C > G (21.1%), and c.51C > G (14.5%) in Taiwan PCD patients (Chen et al., 2013). Han et al. reported that c.760C > T is the most common mutation in patients with symptomatic, and c.51C > G in patients with asymptomatic in Shanghai (Han et al., 2014). Guo et al. observed that c.1400C > G were the most common mutation in five Jining PCUD patients (Guo et al., 2018). Sun et al. also noted that the c.1400C > G was the most common mutation in seven Nanjing PCUG patients (Sun et al., 2017). Tan et al. found the c.51C > G is the most common mutations in Liuzhou PCUD patients (Tan et al., 2017). In agreement with most studies, the c.1400C > G is the most common mutation, with a relative frequency of 50% and accounting for 80% of Suzhou PCUD patients.

SCADD is the third prevalent disease of IMEs and the most prevalent disease of fatty acid metabolic errors in Suzhou population. SCADD had a wide spectrum of symptoms, including hepatic dysfunction, bilateral optic atrophy, vomiting, dysmorphic facial features, feeding difficulties, metabolic acidosis, epilepsy, ketotic hypoglycemia, developmental delay, lethargy, seizures, dystonia, myopathy, and hypotonia (Kılıç et al., 2017; Nochi et al., 2017). However, almost all patients with SCADD identified by newborn screening present no symptom or significant health tissue (Jethva et al., 2008; Waisbren et al., 2008; Huang et al., 2016; Zheng et al., 2017). Therefore, SCADD was not included in expanded newborn screening panels in many newborn screening centers (Dietzen et al., 2009; Mak et al., 2013; Smon et al., 2018). The reported incidence of SCADD is 1/25,000~1/45,000 worldwide (Zytkovicz et al., 2001; Loukas et al., 2010; Lim et al., 2014). In consistence with the above reports, the incidence of SCADD in Suzhou population is 1/28,690. SCADD is caused by the deficiency of SCAD that is encoded by *ACADS* gene. Until now, about 70 variants have been reported to be pathogenic or likely pathogenic in *ACADS* gene, including two common variants, c.511C > T and c.625G > A (Tonin et al., 2016; Nochi et al., 2017). Most patients with SCAD deficiency carry two mutation alleles of the two common variants, or harbor one of them in combination with a rare variant in *ACADS* gene (van Maldegem et al., 2010), and the hotspot in Ashkenazi Jewish patients is a pathogenic c.319C > T mutation (Tein et al., 2008). However, 71.4% of Suzhou patients with SCAD deficiency carried the pathogenic mutation c.1031A > G, similar to Zhejiang SCADD patients (Huang et al., 2016), but different from Jining SCADD patients (Guo et al., 2018).

Several conditions, including deficiency in cystathionine β-synthase activity, tyrosinemia type I, and liver disease, could result in abnormal elevation of serum methionine. In this study, hypermethioninemia specially refers to abnormal elevated methionine caused by the abolished or reduced activity of hepatic methionine adenosyltransferase (MAT) I/III that are encoded by *MATA1* gene. More than 37 mutations described previously range from truncating mutations with no residual enzyme activity to mild missense mutations (Mudd., 2011; Chien et al., 2015). The prevalence of MAT I/III deficiency was reported to range from 1/110,000 to 1/20,000 in different newborn populations (Chien et al., 2005; Couce et al., 2008; Martins et al., 2012; Couce et al., 2013; Nagao et al., 2013). However, in mainland of China, the incidence is unreported.

TABLE 4 | Mutations detected in patients with inborn error of metabolism identified by expanded newborn screening.

Conditions (OMIM number)	Gene (OMIM number)	Mutation alleles number	Nucleotide variant	Amino acid variant	Reported	Pathogenic	RF%	Cases	Accounting for patients (%)
Phenylketonuria (261,600)	*PAH* (612,349)	24						12	
		5	c.728G > A	p.R243Q	Y	P	20.8	5	41.7
		2	c.611A > G	p.Y204C	Y	P/LP	8.3	2	16.7
		2	c.721C > T	P.R241C	Y	P	8.3	2	16.7
		2	c.498C > G	p.Y166X	Y	P	8.3	1	8.3
		1	c.125A > T	p.K42I	Y	NP	4.2	1	8.3
		1	c.158G > A	p.P53H	Y	US	4.2	1	8.3
		1	c.208_210delTCT	p.S70del	Y	P	4.2	1	8.3
		1	c.331C > T	p.R111T	Y	P	4.2	1	8.3
		1	c.442-1G > A	/	Y	P	4.2	1	8.3
		1	c.722delG	R241Pfs	Y	LP	4.2	1	8.3
		1	c.722G > A	p.R241H	Y	P	4.2	1	8.3
		1	c.740G > T	p.G247V	Y	P/LP	4.2	1	8.3
		1	c.827T > G	p.M276R	Y	NP	4.2	1	8.3
		1	c.929C > T	p.S310F	Y	P	4.2	1	8.3
		1	c.1223G > A	p.R408Q	Y	P	4.2	1	8.3
		1	c.1238G > C	p.R413P	Y	P	4.2	1	8.3
		1	c.1264G > A	p.E422K	Y	NP	4.2	1	8.3
Mild hyperphenylalaninemia (261,600)	*PAH* (612,349)	36						18	
		9	c.158G > A	p.P53H	Y	US	25.0	9	50.0
		4	c.1238G > C	p.R413P	Y	P	11.1	4	22.2
		3	c.728G > A	p.R243Q	Y	P	8.3	3	16.7
		3	c.1315+6T > A	/	Y	LP	8.3	3	16.7
		2	c.1174T > A	p.F392I	Y	NP	5.6	2	11.1
		1	c.208_210delTCT	p.S70del	Y	P	2.8	1	5.6
		1	c.310G > T	p.A104S	N	US	2.8	1	5.6
		1	c.331C > T	p.R111X	Y	P	2.8	1	5.6
		1	c.464G > A	p.R155H	Y	P	2.8	1	5.6
		1	c.721C > T	p.R241C	Y	P	2.8	1	5.6
		1	c.722G > A	p.R241H	Y	P	2.8	1	5.6
		1	c.754C > T	p.R252W	Y	P	2.8	1	5.6
		1	c.770G > T	p.G257V	Y	LP	2.8	1	5.6
		1	c.782G > A	p.R261Q	Y	P	2.8	1	5.6
		1	c.977G > A	p.W326X	Y	P	2.8	1	5.6
		1	c.1301C > A	p.A434D	Y	LP	2.8	1	5.6
		1	c.1123C > G	p.Q375E	Y	NP	2.8	1	5.6
		1	1197A > T	p.V399X	Y	P	2.8	1	5.6
		1	c.1199G > A	p.R400K	Y	LP	2.8	1	5.6
Primary carnitine uptake defect (212,140)	*SLC22A5* (603,377)	30						15	
		15	c.1400C > G	p.S467C	Y	P/LP	50.0	12	80.0
		4	c.51C > G	p.F17L	Y	LP	13.3	4	26.7
		4	c.760C > T	p.R254X	Y	P	13.3	4	26.7
		2	c.497+1G > T	/	N	US	6.7	2	13.3
		1	c.394-1G > T	/	Y	LP	3.3	1	6.7
		1	c.428C > T	p.P143L	N	US	3.3	1	6.7
		1	c.652+1G > A	/	Y	P	3.3	1	6.7
		1	c.1252C > T	p.Q418X	Y	P	3.3	1	6.7
		1	c.1462C > T	p.R488C	Y	US	3.3	1	6.7
Short chain acyl-CoA dehydrogenase deficiency (201,470)	ACADS (606,885)	28						14	
		12	c.1031A > G	p.E344G	Y	LP	42.9	10	71.4
		5	c.164C > T	p.P55L	Y	LP	17.9	4	28.6
		3	c.1130C > T	P377L	Y	CIP	10.7	3	21.4
		2	c.322G > A	p.G108S	Y	LP	7.1	2	14.3
		2	c.737G > A	p.C246T	N	US	7.1	2	14.3
		1	c.77A > G	p.H26R	N	US	3.6	1	7.1

(*Continued*)

TABLE 4 | Continued

Conditions (OMIM number)	Gene (OMIM number)	Mutation alleles number	Nucleotide variant	Amino acid variant	Reported	Pathogenic	RF%	Cases	Accounting for patients (%)
		1	c.973C > T	p.R325W	Y	CIP	3.6	1	7.1
		1	c.1054G > A	p.A352T	Y	US	3.6	1	7.1
		1	c.1055C > T	p.A352V	N	US	3.6	1	7.1
Hypermethioninemia (250,850)	MAT1A (610,550)	14*						13	
		10	c.791G > A	p.R264H	Y	P	71.4	10	76.9
		1	c.533C > T	p.P177L	N	US	7.1	1	7.7
		1	c.572_592dup		N	LP	7.1	1	7.7
		1	c.776G > T	p.A259V	Y	P	7.1	1	7.7
		1	c.790C > T	p.R264C	Y	P	7.1	1	7.7
3-Methylcrotonyl-CoA carboxylase deficiency (210,200 and 210,210)		24						12	
	MCCC1 (609,010)	4	c.639+2T > A	p.S164Rfs*3	Y	P	16.7	4	33.3
		3	c.863A > G	p.E288G	Y	US	12.5	3	25.0
		1	c.181G > T	p.A61S	N	US	4.2	1	8.4
		1	c.190G > A	p.V64M	N	US	4.2	1	8.4
		1	c.388G > A	p.G130S	Y	US	4.2	1	8.4
		1	c.416C > T	p.T139I	N	US	4.2	1	8.4
		1	c.490delA		N	US	4.2	1	8.4
		1	c.872C > T	p.A291V	Y	US	4.2	1	8.4
		1	c.1069G > T	p.E357X	N	LP	4.2	1	8.4
		1	c.1103delG	p.G368Vfs*70	N	LP	4.2	1	8.4
		1	c.1136G > A	p.G379D	N	US	4.2	1	8.4
		1	c.1381G > T	p.V461F	N	US	4.2	1	8.4
		1	c.1679dupA	p.N560Kfs*10	Y	P	4.2	1	8.4
	MCCC2(609,014)	2	c.577C > T	p.R193C	N	US	8.3	2	16.7
		1	c.592C > T	p.Q198X	N	LP	4.2	1	8.4
		3	undetectable	–	–	–	12.5	3	25.0
Methylmalonic acidemia (251,000)		20						10	
	MMUT (609,058)	3	c.1663G > A	p.A555T	Y	LP	15.0	3	30.0
		2	c.729_730insTT	p.D244Lfs	Y	P	10.0	2	20.0
		1	c.322C > T	p.R108C	Y	P	5.0	1	10.0
		1	c.454C > T	p.R152X	Y	P	5.0	1	10.0
		1	c.581C > T	p.P194L	N	US	5.0	1	10.0
		1	c.755dupAA	p.H252QfsX6	N	LP	5.0	1	10.0
		1	c.1280G > A	p.G427D	Y	P	5.0	1	10.0
		1	c.1677-1G > A	p.R559Sfs*14	Y	P	5.0	1	10.0
		1	c.2080C > T	p.R694W	Y	P	5.0	1	10.0
		1	c.2131G > T	p.E711X	Y	LP	5.0	1	10.0
	MMACHC (609,831)	2	c.609G > A	p.W203X	Y	P	10.0	2	20.0
		1	c.394C > T	p.R132X	Y	P	5.0	1	10.0
		1	c.567dupT	p.190Yfs*13	Y	P	5.0	1	10.0
		1	c.658_660del	p.L220del	Y	P	5.0	1	10.0
		2	undetectable	–	–	–	10.0	2	20.0
Citrullinemia (605814 and 603,471)		16						8	
	SLC25A13 (603,859)	4	c.IVS16ins3Kb	/	Y	P	25.0	4	50.0
		3	c.852_855delTATG	p.M285Pfs	Y	P	18.8	3	37.5
		1	c.851_854delGTAT	p.Met284fs	Y	LP	6.3	1	12.5
		1	c.1078C > T	p.R360X	Y	P	6.3	1	12.5
		1	c.1399C > T	p.R467X	N	LP	6.3	1	12.5
		4	undetectable	–	–	–	25.0	4	50.0
	ASS1 (603,470)	1	c.689G > C	p.G230A	Y	LP	6.3	1	12.5

(*Continued*)

TABLE 4 | Continued

Conditions (OMIM number)	Gene (OMIM number)	Mutation alleles number	Nucleotide variant	Amino acid variant	Reported	Pathogenic	RF%	Cases	Accounting for patients (%)
		1	c.1004G > A	p.R355H	Y	US	6.3	1	12.5
Very long chain acyl-CoA dehydrogenase deficiency (609,016)	ACADVL (609,575)	13#						6	
		2	c.887_888delCT	p.P296Rfs*17	Y	P/LP	15.4	1	16.7
		2	c.1349G > A	p.R450H	Y	P/LP	15.4	2	33.3
		1	c.278-31_278-18del	/	Y	US	8.3	1	16.7
		1	c.553G > A	p.G185S	Y	P/LP	8.3	1	16.7
		1	c.642_643delCT	p.F214Lfs*38	N	LP	8.3	1	16.7
		1	c.838A > G	p.T280A	N	US	8.3	1	16.7
		1	c.878+34G > A	/	N	US	8.3	1	16.7
		1	c.895A > G	p.K299E	N	US	8.3	1	16.7
		1	c.1077G > A	p.A359A	N	US	8.3	1	16.7
		1	c.1280G > A	p.W427X	Y	LP	8.3	1	16.7
		1	c.1345G > C	p.E449Q	Y	US	8.3	1	16.7
Tetrahydrobiopterin deficiency (233,910, 261,640, 612,716, 264,070, and 261,630)	PTS (612719)	12						6	
		5	c.259C > T	p.P87S	Y	P	41.7	3	50.0
		3	c.166G > A	p.V56M	Y	LP	25.0	3	50.0
		1	c.155A > G	p.N52S	Y	P	8.3	1	16.7
		1	c.272A > G	p.K91R	N	US	8.3	1	16.7
		1	c.277C > A	p.L93M	N	US	8.3	1	16.7
		1	c.286G > A	p.D96N	Y	P	8.3	1	16.7
Medium chain acyl-CoA dehydrogenase deficiency (201,450)	ACADM (607,008)	10						5	
		2	c.449_452delCTGA	p.T150Rfs	Y	P/LP	20.0	2	40.0
		1	c.589A > G	p.K197E	N	US	10.0	1	20.0
		1	c.790G > T	p.G264C	N	US	10.0	1	20.0
		1	c.970G > A	p.A324T	N	US	10.0	1	20.0
		1	c.1171A > G	p.M391V	N	US	10.0	1	20.0
		1	c.1238G > A	p.R413H	Y	US	10.0	1	20.0
		1	c.1247T > C	p.I416T	Y	CIP	10.0	1	20.0
		1	c.1248T > G	p.I416M	N	US	10.0	1	20.0
		1	undetectable	–	–	–	10.0	1	20.0
Tyrosinemia (276,700, 276,600, 276,710)		8						4	
	FAH (603,859)	1	c.5C > T	p.T2M	N	US	12.5	1	25.0
		1	c.236G > A	p.G79E	N	US	12.5	1	25.0
		2	undetectable	–	–	–	25.0	2	50.0
	HPD (609,695)	1	c.784G > A	p.A262T	N	US	12.5	1	25.0
		1	c.916C > T	p.R306X	N	LP	12.5	1	25.0
	TAT (613,018)	1	c.1162G > A	p.A388T	N	US	12.5	1	25.0
		1	c.1210G > A	p.A404T	N	US	12.5	1	25.0
Glutaric aciduria I (231,670)	GCDH (608,801)	8						4	
		2	c.1064G > A	p.R355H	Y	P	25.0	2	50.0
		1	c.158C > G	p.P53R	N	US	12.5	1	25.0
		1	c.554G > A	p.G185E	N	US	12.5	1	25.0
		1	c.892G > A	p.A298T	Y	P/LP	12.5	1	25.0
		1	c.916G > A	p.E306K	N	US	12.5	1	25.0
		1	c.1186G > C	p.D396H	N	US	12.5	1	25.0
		1	c.1240G > A	p.E414K	Y	P	12.5	1	25.0

(*Continued*)

TABLE 4 | Continued

Conditions (OMIM number)	Gene (OMIM number)	Mutation alleles number	Nucleotide variant	Amino acid variant	Reported	Pathogenic	RF%	Cases	Accounting for patients (%)
Isobutyryl coa dehydrogenase deficiency (611,283)	ACAD8 (604,773)	6						3	
		3	c.1000C > T	p.R344C	Y	P/LP	50.0	3	100.0
		2	c.286C > A	p.G96S	N	US	33.3	2	66.7
		1	c.568-3C > G	/	N	US	16.7	1	33.3
Isovaleric acidemia (243,500)	IVD (607,036)	4						2	
		1	c.241C > T	p.R81X	N	LP	25.0	1	50.0
		1	c.466-29A > G	/	N	US	25.0	1	50.0
		1	c.1216A > G	p.T406A	N	US	25.0	1	50.0
		1	undetectable	–	–	–	25.0	1	50.0
Argininosuccinate aciduria (207,900)	ASL (608,310)	2						1	
		2	c.331C > T	p.R111W	N	LP	100.0	1	100.0
Maple syrup urine disease (248,600)	DBT (248,610)	2						1	
		2	c.1132C > T	p.Q378X	N	LP	100.0	1	100.0
Ornithine transcarbamylase deficiency (311,250)	OTC (300,461)	2						1	
		2	c.829C > T	p.R277Y	Y	P	100.0	1	100.0
Malonic acidemia (248,360)	MLYCD	2						1	
		2	c.482T > C	p.L161P	Y	US	100.0	1	100.0
2-Methylbutyrylglycinuria (611,283)	ACADSB (600,301)	2						1	
		2	C.1165A > G	p.M389V	N	LP	100.0	1	100.0
Propionic acidemia (606,054)	PCCA (232,000)	2						1	
		1	c.229C > T	p.R77W	Y	LP	50.0	1	100.0
		1	c.2002G > A	p.G668R	Y	P/LP	50.0	1	100.0

CIP, conflicting interpretations of pathogenicity; LP, likely pathogenic; N, no; P, pathogenic; RF, relative frequency; US, uncertain significance; Y, yes.
**one case carry two mutational alleles.*
#, one case carry three mutational alleles.

Our study reported a prevalence of 1/30,893 in Suzhou population of newborns. Of 13 Suzhou hypermethioninemia patients, 10 cases carried the dominant mutation c.791G > A (Pérez Mato et al., 2001; Muriello et al., 2017). Previous studies reported that the c.791G > A was the most prevalent mutation in Asian populations, such as Japanese, Chinese in Taiwan, and so on (Chien et al., 2005; Nagao et al., 2013). MATA1 deficiency is inherited either as autosomal-recessive or autosomal-dominant. Most *MAT1A* mutations give rise to autosomal recessive phenotypes, but several autosomal dominant mutations have also been observed, including c.776C > T, c.791G > A (Muriello et al., 2017), c.746G > A, and c.838G > A (Kim et al., 2016). With the exception of a few individuals with hypermethioninemia who present with abnormal neurological symptoms, most patients generally are free of major clinical manifestation. Hypermethioninemia shows clinical symptoms correlated to genotypes (Chou, 2000), while the c.791G > A could lead to mild hypermethioninemia. Of all *MATA1* mutations related to hypermethioninemia, the c.791G > A was the most common mutation identified in patients screened by expanded newborn screening (Couce et al., 2008; Martins et al., 2012; Couce et al., 2013). The c.791G > A mutation was the most prevalent mutation in Asian populations, including Japanese (Nagao et al., 2013) and Taiwan population (Chien et al., 2005). As expected, the c.791G > A was the most prevalent (80%) mutation in Suzhou newborns. In addition, another autosomal dominant mutation c.776G > T was found in one patient, and t one patient carried a novel heterozygous c.533C > T mutation. It appears that *MATA1* deficiency is mainly inherited *via* autosomal dominant mode in Suzhou population. Furthermore, we found no Suzhou patients exhibit obvious clinical abnormality. There is a wide range of clinical manifestations in individuals with mutations in *MAT1A* gene, from completely asymptomatic to neurological problems associated with brain demyelination (Furujo et al., 2012). As a result, we speculated that the extent of clinical manifestations is associated with the inherited mode, which needs further research.

3-MCCD is an autosomal recessive inborn error of leucine metabolism, resulting in leukodystrophy, developmental delays, hypoglycemia, acidosis, failure to thrive, lactic acidosis, and hyperammonemia (Elpeleg et al., 1992; de Kremer et al., 2002; Forsyth et al., 2016). Despite cases with 3-MCCD identified by expanded newborn screening are more than previous expected, a growing number of reports have shown that the majority of cases

are in fact asymptomatic (Stadler et al., 2006; Arnold et al., 2008; Arnold et al., 2012; Lam et al., 2013; Ye et al., 2014; Rips et al., 2016). This suggests this condition might represent a biochemical phenotype, but not a disease, and therefore should be excluded from newborn screening panels (Wilcken, 2008; Forsyth et al., 2016; Rips et al., 2016). The 3-MCCD is classified into type I (MIM# 210200) and type II (MIM# 210210), caused by *MCCC1* gene and *MCCC2* gene, respectively. Until now, at least 66 *MCCC1* and 83 *MCCC2* mutations have been reported (Yang et al., 2015). The 3-MCCD was the most prevalent organic acid metabolic error and showed a large variation of incidence from 1/27,000 to 1/110,000 in different countries (Yang et al., 2015; Fonseca et al., 2016). Similar to another Chinese population (Yang et al., 2015), the incidence of 3-MCCD is about 1/33,412 in Suzhou population. Some previous reports revealed that *MCCC2* mutations were the main etiology of 3-MCCD (Uematsu et al., 2007; Cho et al., 2012; Grünert et al., 2012; Fonseca et al., 2016). However, in Zhejiang population, almost all (5/6) 3-MCCD patients carried one or two *MCCC1* mutations (Yang et al., 2015). Similarly, of 12 Suzhou patients with 3-MCCD, 75% (9/12) were caused by *MCCC1* mutations, which suggested the *MCCC1* mutations might be prevalent in China (Yang et al., 2015). It is worth mentioning that further genetic testing for more Chinese patients should be conducted to confirm the above conclusion. Several mutations were observed to have a relative high frequency in 3-MCCD patients, including c.838G > T (4/12), c.295G > A (3/56), c.1574+1G > A (3/56) in *MCCC2* gene and c.1155A > C (4/56) in *MCCC1* gene (Dantas et al., 2005; Cho et al., 2012). However, most studies did not observe mutational hotspot of the two genes (Stadler et al., 2006; Yang et al., 2015; Smon et al., 2018). Contrary to a previous report on Zhejiang patients, a mutational hotspot c.639+2T > A, that was observed in only one Zhejiang patient and predicted to be pathogenic (Yang et al., 2015), had a high prevalence (4/12) in Suzhou patients. This inconsistency could be caused by the diversity of races or a small sample size of patients. As a result, the mutational hotspot should be confirmed by further research based on a large number of patients.

MMA is a family of lethal, severe, and multisystems organic acid metabolic errors, which has a wide clinical spectrum, including anorexia, failure to thrive, hypotonia, developmental delay, progressive renal failure, functional immune impairment, optic nerve atrophy, and hematologic abnormalities. MMA is classified into two main forms according to phenotype, including isolated methylmalonic acidurias and combined methylmalonic aciduria and homocystinuria, and caused by the defects of 10 genes, including *MUT, MMAA, MMAB, CD320, MMADHC, LMBRD1, HCFC1, ABCD4, MCEE,* and *SUCLA2*. According to previous reports, the incidence of MMA was 1/50,000 in Japan (Shigematsu et al., 2002), 1/85,000 in Taiwan of China (Cheng et al., 2010), and 1/250,000 in Germany (Schulze et al., 2003). However, in mainland China, the incidence of MMA ranged from 1/3,920 to 1/26,000 (Tu, 2011; Han et al., 2016; Yin, 2016; Zhao et al., 2016). In Suzhou, the incidence of MMA is about 1/40,000, obviously higher than that in the above countries, but lower than that in Shandong, Henan, Beijing, Shanghai, and Taiwan. Recently, a study with large sample size, containing 1,003 MMA patients derived from 26 provinces or cities of China, demonstrated that MMA cblC and MMA mut were the two major types in China. Similar to the report by Liu et al., the MMA cblC and the MMA mut were also the most prevalent types in Suzhou. However, contrary to Liu et al. report, in Suzhou population, the MMA mut (six patients) was more prevalent than MMA cblC (three patients). Several hotspot mutations were reported in *MUT* gene and *MMADHC* gene. Han et al. reported that the c.729_730insTT of the *MMUT* gene was the most common mutation in Shanghai patients (Han et al., 2015). Liu et al. reported that the c.609G > A and the c.658_660delAAG of the *MMACHC* gene were the most common mutations in 70 unrelated MMA cblC patients (Liu et al., 2010). Yu et al. reported that the c.609G > A and the c.658_660delAAG were the most common mutations detected in 13 (81%) out of 16 MMA cblC patients (Yu et al., 2015). However, in Suzhou patients with MMA, the mutations c.1663G > A and c.729_730insTT of the *MMUT* gene and c.609G > A of the *MMACHC* gene were the most common mutations. Therefore, the hotspot mutations in Chinese patients with MMA might be c.609G > A and c.658_660delAAG of the *MMACHC* gene and might be c.1663G > A and c.729_730insTT of the *MMUT* gene.

Citrullinemia is an autosomal recessive disorder and a urea cycle disease leading to a wide spectrum of phenotypes, from life-threatening neonatal hyperammonemia to adult onset with mild symptoms, and even no manifestation (Saheki and Kobayashi, 2002; Gao et al., 2003; Häberle et al., 2003; Enns et al., 2005; Dimmock et al., 2008; Komatsu et al., 2008; Häberle et al., 2009; Salek et al., 2010). This disease is classified into CTLN 1 and CTLN 2, caused by mutations of *ASS1* gene and *SLC25A13* gene, respectively. The estimated prevalence of CTLN 1 and CTLN 2 is 1 in 44,300–200,000 (Kasper et al., 2010; Niu et al., 2010) and 1 in 7,100–230,000 (Yamaguchi et al., 2002; Kobayashi et al., 2003; Lu et al., 2005; Tabata et al., 2008; Kikuchi et al., 2012) based on expanded newborn screening, respectively. However, most CTLN2 cases were identified in countries of East Asia, especially in Japan. More than 137 mutations in ASS1 gene have been identified in worldwide patients (Diez-Fernandez et al., 2017). The c.1168G > A mutation is the most common mutation in several ethnic groups, including Germans, Spaniards, and Turks, but rare in Asians (Gao et al., 2003; Engel et al., 2009; Diez-Fernandez et al., 2017). Whereas, the c.421-2A > G is the most frequent mutation in East Asians (Kobayashi et al., 1995; Lee et al., 2013; Woo et al., 2013). However, in this study, only one CTLN 1 patient and two mutations of the *ASS1* gene were identified in Suzhou citurillinemia patients, which could be caused by ethnic specificity. With regard to CTLN2, a higher prevalence is observed in Suzhou patients compared to CTLN1. In previous studies, mutation detection of the *SLC25A13* gene was very high, greater than 90% of CTLN2 in East Asians (Yasuda et al., 2000; Saheki and Kobayashi, 2002; Yamaguchi et al., 2002; Kobayashi et al., 2003; Saheki et al., 2004; Lu et al., 2005). In contrast to CTLN1, the CTLN2 had a narrow spectrum of mutations in *SLC25A13* gene and highly clustered mutations (Woo et al., 2014). The c.851_854del mutation in *SLC25A13*, which was suspected to have a founder effect, was identified in CTLN2 patients throughout East Asian countries, such as China, Japan, Korea, and so on (Kobayashi et al., 1999; Tanaka et al., 2002; Imamura et al., 2003; Tabata et al., 2008). According to targeted

mutation analysis, the frequency of overall mutations in CTLN2 is estimated to be 1/65–79 in Chinese, 1/69–73 in Japanese, 1/50–112 in Korean, and 1/70–97 in Taiwanese (Yamaguchi et al., 2002; Saheki and Kobayashi, 2002; Kobayashi et al., 2003; Saheki et al., 2004; Lu et al., 2005). In above countries, c.1177+1G>A and c.851_854del are the most common mutations in CTLN 2 patients. In Japanese patients, the c.1177+1G>A mutation (up to 43.1% of detected alleles) had the highest frequency, followed by c.851_854del mutation (up to 38.9%) (Saheki and Kobayashi, 2002; Yamaguchi et al., 2002). In Korea patients, the IVS16ins3kb and the c.851_854del were the most common mutations and were found at very high frequencies (100%) (Ko et al., 2007). In Hong Kong CTLN2 patients, the c.851_854del GTAT, IVS16ins3kb and c.852_855delTATG were the most common mutations of *SLC25A13* gene (Hui et al., 2014; Chong et al., 2018). In another Chinese population, the c.851_854del GTAT was the most common mutation and was observed in 100% CTLN2 patients (Lin et al., 2017). Some other studies also observed c.851_854del GTAT was the most common mutation in Chinese populations (Xing et al., 2010; Fu et al., 2011; Song et al., 2011). In our study, we found the c.852_855delTATG and the IVS16ins3kb were the most common mutations and accounted for 75.0% of cases. In addition, another reported hotspot mutation c.851_854del GTAT was also observed in one Suzhou patient. As a result, c.851_854del, c.852_855delTATG, and IVS16ins3kb might be the most common mutations and should draw more attention in genetic analysis of Chinese CTLN2 patients.

Tetrahydrobiopterin deficiency (or BH4 deficiency) is a rare inborn metabolic disorder characterized by the deficiency of tetrahydrobiopterin or BH4 and caused by mutations in one of the four genes, including *GCH1*, *PCBD1*, *PTS*, and *QDPR*. This condition is inherited by autosomal recessive pattern and has a wide spectrum of symptoms, including intellectual disability, progressive problems with development, movement disorders, difficulty swallowing, seizures, behavioral problems, and inability to control body temperature. The total prevalence of this condition is estimated 1/500,000 to 1/1,000,000 worldwide and was relative high in Asian populations. In mainland of China, BH4 deficiency accounted for 8.55% of patients with HPA (Ye et al., 2009), significantly higher than 1%–3% of HPA worldwide (Blau et al., 1996). In our study, the prevalence of BH4 deficiency in Suzhou newborns was about 1/67,000, higher than 1/140,000 in the mainland of China (Ye et al., 2009; Li et al., 2018). Two teams reported the mutations in *PTS* gene were the main cause of BH4 deficiency, accounting for more than 95% of Chinese patients (Ye et al., 2009; Ye et al., 2013; Li et al., 2018). Similar to the above reports, all these six Suzhou patients with BH4 deficiency were caused by mutations in *PTS* gene. To date, more than 90 mutations in *PTS* gene have been reported in different populations. There are several mutational hotspots in different regions. Wang and coworkers investigated 204 PTPS deficiency patients and found the c.259C > T (38.2%) in *PTS* gene was the most common mutation, followed by c.84-291A > G (11%) (Wang et al., 2018). Ye and coworkers investigated 136 Chinese patients with PTPS deficiency and found c.259C > T (42.9%) in *PTS* gene was the most common mutation, followed by c.286G > A (13.4%) (Ye et al., 2013). Similar to the report by Ye et al., Li and coworkers reported that the c.259C > T (31.82%) in *PTS* gene was the most common mutation, followed by c.286G > A (13.64%) (Li et al., 2018). In Suzhou BH4 deficiency patients, we also found the c.259C > T was the most common mutation, accounting for 41.7% of mutation alleles. However, the second common mutation was c.166G > A, accounting for 25% of mutation alleles. The two most common mutations accounted for 83.3% of BH4-deficiency patients. The remaining four mutation alleles were c.155A > G, c.272A > G, c.277C > A, and c.286G > A, respectively, and all of these mutations were reported previously. However, c.155A > G, c.272A > G, and c.286G > A were reported as common mutations in Chinese patients with BH4 deficiency (Ye et al., 2013; Li et al., 2018; Wang et al., 2018). This difference could be caused by a small sample size and different populations. As a result, these mutational hotspots are potential candidates for genetic analysis of Chinese patients with BH4 deficiency.

IBD deficiency is a very rare disorder characterized by disrupting the breakdown of Val. This condition is an autosomal recessively inherited disease and caused by mutations in the *ACAD8* gene. To the best of our knowledge, only 27 patients with IBD deficiency were reported in literature, and 28 mutations in the *ACAD8* gene were detected in these patients (Yun et al., 2015; Santra et al., 2016; Lin et al., 2018). Most patients with IBD deficiency were asymptomatic in neonatal period, and a few had developed features such as dilated cardiomyopathy, hypotonia, developmental delay, and speech delay (Yun et al., 2015; Lin et al., 2018). In Suzhou population of newborns, three patients with IBD deficiency were identified from more than 400,000 newborns, and all patients remained asymptomatic during treatment and follow-up. Two reported common mutations were detected, including c.1000C > T and c.286C > A in the *ACAD8* gene. Recently, Lin and coworker reported six Chinese patients with IBD deficiency and found the c.286C > A (7/14) was the most common mutation (Lin et al., 2018), followed by c.1000C > T. However, we found that the c.1000C > T was the most common mutation accounting for 50.0% (3/6) of mutational alleles in Suzhou patients with IBD deficiency, followed by c.286C > A (33.3%). As a result, the two mutations c.286C > A and c.1000C > T in the *ACAD8* gene could be considered as mutational hotspots resulting in IBD deficiency in Chinese population. In addition, a novel heterozygous mutation c.568-3C > G was found in one patient with IBD deficiency. Our results characterized the mutational hotspots in the *ACAD8* gene in Chinese patients with IBD deficiency and broaden the mutational spectrum of the *ACAD8* gene.

There were five patients who were affected with Argininosuccinic aciduria (ASA; MIM# 207900), Maple syrup urine disease (MSUD; MIM# 248600), ornithine transcarbamylase deficiency (OTD; MIM# 311250), 2-methylbutyrylglycinuria (MBG; MIM# 248360), and Malonic acidemia (MA; MIM# 248360), respectively. Despite these five kinds of IEMs are extremely rare in Suzhou population, the cases affected with one of these IEMs are homozygous for one of the mutations, including c.331C > T in *ASL,* c.1132C > T in *DBT*, c.829C > T in *OTC*, 1165A > G in *ACADSB*, and c.482T > C in *MLYCD*. Therefore, these mutations might be hotspots causing the above five IEMs. In Suzhou patients, the remaining six IEMs were not observed to have mutational hotspots, including MCAD deficiency, VLCAD deficiency, glutaric acidemia type I (GA-I; MIM# 231670), tyrosinemia (MIM# 276700, 276600, and 276710),

isovaleric acidemia (IVA; MIM# 243500), and propionic acidemia (PROP; MIM# 606054). In 32 mutation alleles of 16 patients with one of above six IEMs, 24 reported mutations and 6 novel mutations were detected. This might be caused by a small sample size and further research is needed.

In summary, we have detected a few mutational hotspots and some novel mutations that account for most Suzhou patients with IEMs identified by expanded newborn screening that might be pathogenic. These mutational hotspots could be potential candidates for gene screening and these novel mutations expanded the mutational spectrum of IEMs. Our findings could be of value for genetic counseling and genetic diagnosis of IEMs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in Sequence Read Archive using the accession number PRJNA566217.

ETHICS STATEMENT

The protocol was reviewed and approved by Ethic committee of the affiliated Suzhou hospital of Nanjing Medical University.

AUTHOR CONTRIBUTIONS

BW and JX conceived and designed the research, analyzed data, and wrote the manuscript. TW, JM, and BW conducted experiments and reviewed the manuscript. QW conducted experiments. HL analyzed data and reviewed the manuscript. QZ and AG took part in diagnosis and treatment of infants with IME. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01052/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Diagnostic Yield of a Targeted Next-Generation Sequencing Gene Panel for Pediatric-Onset Movement Disorders: A 3-Year Cohort Study

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In recent years, genetic techniques of diagnosis have shown rapid development, resulting in a modified clinical approach to many diseases, including neurological disorders. Movement disorders, in particular those arising in childhood, pose a diagnostic challenge. First, from a purely phenomenological point of view, the correct clinical classification of signs and symptoms may be difficult and require expert evaluation. This is because the clinical picture is often a mixture of hyperkinetic and hypokinetic disorders, and within hyperkinetic movement disorders, combined phenotypes are not unusual. Second, although several genes that cause movement disorders in children are now well-known, many of them have only been described in adult populations or discovered in patients after many years of disease. Furthermore, diseases that alter their mechanisms from childhood to adulthood are still little known, and many phenotypes in children are the result of a disruption of normal neurodevelopment. High-throughput gene screening addresses these difficulties and has modified the approach to genetic diagnosis. In the exome-sequencing era, customized genetic panels now offer the ability to perform fast and low-cost screening of the genes commonly involved in the pathogenesis of the disease. Here, we describe a 3-year study using a customized gene panel for pediatric-onset movement disorders in a selected cohort of children and adolescents. We report a satisfying diagnostic yield, further confirming the usefulness of gene panel analysis.

Keywords: dystonia, chorea, neurodegeneration with brain iron accumulation disorders, genetics, next-generation sequencing, children, myoclonus, neurotransmitters

INTRODUCTION

Movement disorders (MDs) are a heterogeneous group of neurological conditions characterized by the production of abnormal voluntary or involuntary movements (Sanger et al., 2010). In children, MDs frequently occur in complex presentations, with different MDs appearing simultaneously or sequentially in the same patient. Frequently, MDs can coexist with other neurological disorders,

resulting in complex neurodevelopmental conditions (Cordeiro et al., 2018). A systematic approach is needed to reach the correct diagnosis, and the first step usually relies on the phenomenological classification of the disorder (Sanger, 2003; Abdo et al., 2010; O'Malley and Gilbert, 2018).

Many pediatric-onset MDs are monogenic diseases, and their genetic landscape has been widely explored in recent years due to the introduction and spread of next-generation sequencing (NGS) technology. This has had a significant impact on the definition of phenotypes and syndromes. NGS denotes a group of technologies that allow the sequencing of a large amount of nucleic acid, representing an entirely new paradigm in sequencing technology that follows Sanger sequencing (Sanger et al., 1977; Lohmann and Klein, 2014). Using targeted resequencing, a subset of regions of interest on the genome can be sequenced by NGS. This strategy is widely used to sequence selected genes involved in the pathogenesis of specific disease (Reale et al., 2018). Few studies have investigated the diagnostic yield of gene panel analysis in MDs (van Egmond et al., 2017; Montaut et al., 2018; Reale et al., 2018), and molecular investigations in pediatric-onset MDs are highly variable, according to the availability of local resources and the methods of molecular genetics laboratories.

In this study, we retrospectively analyzed the diagnostic performance of a customized, targeted NGS panel specifically designed for pediatric-onset MDs in a large cohort of children referred to our institution.

MATERIALS AND METHODS

Participants

This retrospective study was conducted in a cohort of pediatric patients referred to the Movement Disorders Clinic of Bambino Gesù Children's Hospital in Rome, a tertiary referral hospital for rare neurological diseases in children. All patients underwent genetic tests for MDs with the customized NGS panel available at the molecular medicine laboratory in our department. All data available from January 2015 to November 2018 were collected. Electronic patient files were reviewed by pediatric neurologists experienced in MDs, and the phenomenology of the MDs was classified by reviewing video-recorded examinations. Furthermore, the clinical features of MDs, neuroimaging tests, biochemical work-up, and neurophysiological tests (when performed) were reviewed. Inclusion criteria were diagnosis of an MD (including dystonia, chorea, myoclonus, and tremor) with onset before 18 years of age and/or positive family history of an MD and ruling out of secondary causes (such as perinatal asphyxia or cerebral infection) during the diagnostic work-up. Patients with tic disorders or Tourette's syndrome, pure cerebellar ataxia, or hereditary spastic paraplegia were excluded, as were patients with incomplete medical records. In addition, patients who underwent the same gene panel analysis for a clinical suspicion of familial hemiplegic migraine (fHM) or childhood periodic syndromes (e.g., paroxysmal torticollis) were not included in the analysis.

The patients were grouped into five categories according to their most prominent MD: isolated dystonia, combined dystonia (when dystonia was associated with other MDs), paroxysmal MDs (PMDs), chorea, and tremor. The PMD group was divided into subgroups using phenomenology and the trigger factors of the attacks. DNA samples of index patients and both parents (trios) were collected from peripheral blood leukocytes using standard procedures. Written informed consent for genetic testing was obtained from patients' legal guardians or directly from the patient for those aged 18 years or older. During the diagnostic work-up, all patients underwent brain Magnetic Resonance Imaging (MRI), and neurophysiological exams, neuropsychological tests, and biochemical studies on blood, urine, and cerebrospinal fluid were performed following a case-by-case clinical assessment.

Targeted Sequencing

Targeted sequencing of index cases' DNA was performed using a mean of a customized panel (Nextera Rapid Custom Enrichment, Illumina, San Diego, CA), including 102 genes, selected in accordance with the results of a systematic literature review of studies of MD-associated genes. The panel was designed with the Illumina Design Studio tool. The region of interest was the coding sequence of each gene with a ±20 bp intronic flanking region to include splicing mutations. The 3′ and 5′ untranslated regions (UTRs) were included in the sequenced region only in genes with previously described pathogenic variants in the UTRs. Gene libraries were obtained from a Nextera Rapid Capture Target Enrichment Kit and sequenced on a *MiSeq* platform (Illumina). The generated reads were aligned to human genome assembly hg38 (December 2013, GRCh38). Variants were called using the HaplotypeCaller tool of GATK software, version 4.3 (Cambridge, MA, USA) and annotated with the ANNOVAR software tool (Wang et al., 2010). Annotated data were filtered to exclude intronic and synonymous variants that were not predicted to affect splice sites, as well as variants with reported minor allele frequency (MAF) ≥ 0.01 in publicly available resources on human variation, such as dbSNP ver. 144, 1000 Genomes, Exome Aggregation Consortium (ExAC), NHLBI Exome Sequencing Project Exome Variant Server (EVS). Missense variants of suspected pathogenicity were investigated using *in silico* prediction tools, including PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), MutationTaster (http://www.mutationtaster.org/), and Alamut (https://www.interactive-biosoftware.com/). Changes affecting the splice site were investigated with Human Splicing Finder (http://www.umd.be/HSF/). Novel variants were considered to be variants of unknown significance when these were rare (MAF < 0.01%) in population databases and/or were predicted not to be pathogenic by prediction tools. Putative pathogenetic variants were validated by Sanger sequencing and investigated in the parents to assess intrafamilial segregation. Possible copy number

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; CSF: cerebrospinal fluid; DYT, dystonia; EA, episodic ataxia; EPD, exercise-induced paroxysmal dyskinesia; fHM, familial hemiplegic migraine; HSP, hereditary spastic paraplegias; MAF, minor allele frequency; MD, movement disorders; MRI, magnetic resonance imaging; NBIA, neurodegeneration with brain iron accumulation; NGS, next generation sequencing; NT, inherited disorders of neurotransmitter metabolism; PKD, paroxysmal kinesigenic dyskinesia; PMD, paroxysmal movement disorders; XLD, X-linked; WES, whole exome sequencing.

variations were investigated using multiplex ligation-dependent probe amplification or real-time PCR techniques.

RESULTS

Clinical Characterization of the Cohort

Overall, 204 patients underwent gene panel analysis for MD and 38 were excluded because they were referred with a clinical diagnosis of fHM or paroxysmal torticollis. Then, 18 patient files were excluded due to incomplete chart documentation. Thus, we included 148 patients in the study, of whom 82 were male (55%) and 66 were female (45%). The median age of onset of disease was 8 (range 0–17) years old, and the median age at the time of gene panel analysis was 9.5 (range 0–20) years. A total of 134 cases (93%) were sporadic (meaning that no other relatives were affected), and the remaining 14 (7%) had a family history of disease. Of the total, 59 patients were investigated for combined dystonia (40%), 31 for isolated dystonia (21%), 34 patients for (PMDs) (23%), 20 for chorea (13%), and 4 for tremor (3%) (**Figure 1**). Among the 34 patients referred for PMD, 10 were investigated for episodic ataxia (EA), 18 for paroxysmal kinesigenic dyskinesia (PKD), 2 for exercise-induced paroxysmal dyskinesia (EPD), 4 for hemiplegic attacks, and 1 for paroxysmal myoclonus (**Figure 1**). In all, 102 patients (68.9%) presented neurological features other than MDs (**Figure 2A**).

Diagnostic Yield

Pathogenic variants were detected in 42 out of 148 patients, leading to a diagnostic yield of 28% (**Table 1** in the **Supplementary Materials**), and 106 patients were left without a definite molecular diagnosis after gene panel analysis (**Figure 2B**). We detected pathogenic variants in 20 of 59 patients in the combined dystonia group (34%), 5 of 31 in the isolated dystonia group (16%), 5 of 20 in the chorea group (25%), and 12 of 34 in the pMD group (35%, **Figure 2B**). Among the pMD patients, the diagnostic rate was 50% for PKD and EPD patients, 25% for patients with hemiplegic attacks, and 10% in those with EA. No pathogenic variants were identified in the only patient with paroxysmal, non-epileptic myoclonic attacks or in the patients investigated for tremor (**Figure 2C**).

The distribution of the pathogenic variants detected in specific genes is shown in **Figure 2D**. A total of 28 patients exhibited *de novo* variants; in the remaining patients, autosomal dominant and autosomal recessive inheritance was found in 11 and 2 patients, respectively. One case was X-linked. Nine patients had a variant in the *PRRT2* gene, and eight of those nine carried the classical PRRT2 mutation variant (Chen et al., 2011); in the *ATP1A3*, *SLC2A1*, and *GNAO1* genes, pathogenic variants were detected six, five, and three times, respectively. *NKX2-1*, *ADAR1*, *ADCY5*, *ATP1A2*, and *KMT2B* were found to be mutated twice each, and *CACNA1B*, *TOR1A*, *KCTD17*, *PANK2*, *PLA2G6*, *SCN1A*, *SCN2A*, *STXBP1*, and *WDR45* were found to be mutated one time each. **Table 1** summarizes all pathogenic variants detected.

Peculiar Findings

The application of gene panel analysis allowed previously known phenotypes to be expanded and new genotypes to be detected for several pediatric-onset MDs.

A *de novo*, previously unreported, heterozygous variant in the *GNAO1* gene (c.535A>G, p.Arg179Gly) was found in a male subject with a progressive upper body dystonia with onset in the second decade, first involving the arms and later spreading to the cranio-cervical region. He presented with a moderate intellectual disability and was suffering from partial epilepsy, controlled by therapy with carbamazepine. He had never experienced an episode of acute dystonia exacerbation. Taken together with the findings of a recent report (Kelly et al., 2019), this finding expands the spectrum of *GNAO1*-related neurological conditions beyond the two most frequently

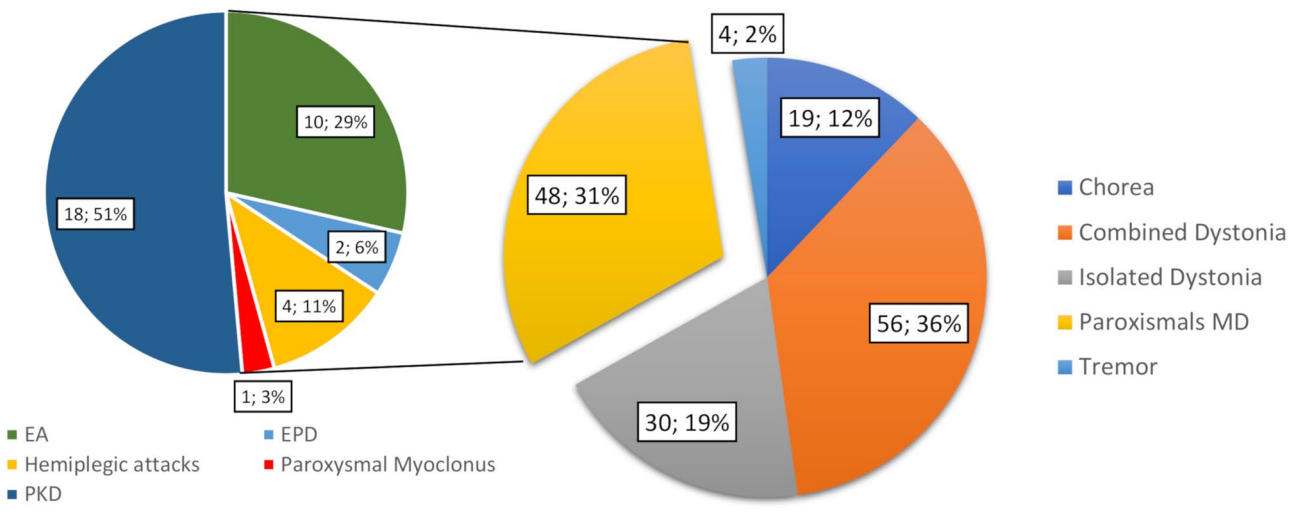


FIGURE 1 | Number and percentage of the total patients by classification according to prominent MD phenomenology. Following the phenomenology of the attacks, the paroxysmal MD group is further classified into episodic ataxia (EA), paroxysmal kinesigenic dyskinesia (PKD), and exercise-induced paroxysmal dyskinesia (EPD), hemiplegic attacks, and paroxysmal myoclonus groups. MD, movement disorder.

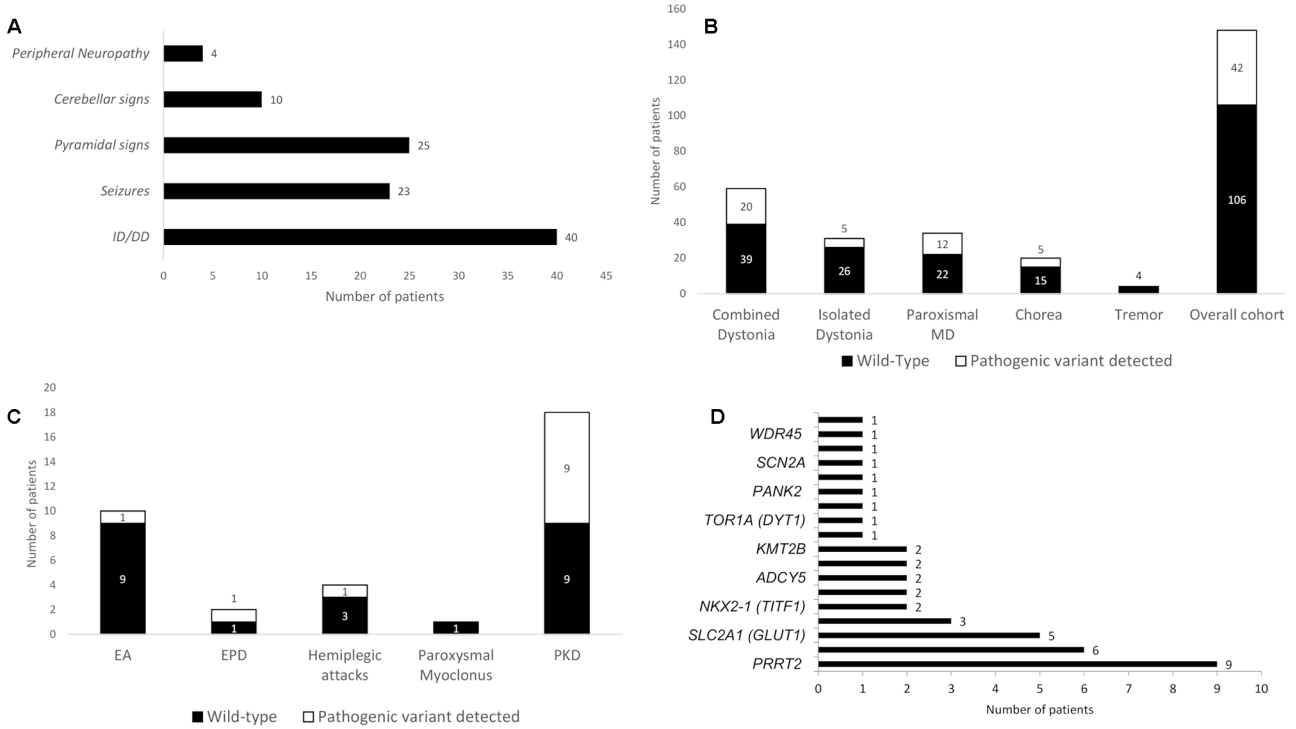


FIGURE 2 | Associated neurological features of the cohort and diagnostic yield of the gene panel analysis. **(A)** Associated neurological features in the overall cohort. **(B)** Diagnostic yield in the overall cohort and in the different groups, expressed as total number of patients receiving a definite molecular diagnosis through gene panel analysis. **(C)** Diagnostic yield of gene panel analysis in paroxysmal MD according with their phenomenological classification. **(D)** Distribution of pathogenetic variants encountered per gene (see **Appendix 2** in the **Supplementary Materials** for the complete list of genes sequenced in the panel). DD, developmental delay; EA, episodic ataxia; EPD, exercise-induced paroxysmal dyskinesia; ID, intellectual disability; MD, movement disorder; PKD, paroxysmal kinesigenic dyskinesia.

reported and severe phenotypes, namely, early infantile epileptic encephalopathy (EIEE17, OMIM 615473) and early-onset hyperkinetic phenotype (OMIM 617493) (Schirinzi et al., 2018a).

In addition, we detected a novel heterozygous variant in the *KCTD17* gene (c.508-2A>T) in a girl with a childhood-onset complex MD, mainly characterized by myoclonic dystonia. This variant affects the acceptor splice site of the exon 5, unveiling an alternative cryptic site 34 bp downstream and determining the skipping of the first 35 nucleotides of the exon. This results in a shift of the reading frame with a premature stop codon introduction in exon 7, which causes reduced protein expression (Graziola et al., 2018). This finding was further confirmed by an independent group that described a boy with childhood-onset myoclonus-dystonia harboring a novel *KCTD17* variant in an adjacent nucleotide (c.508-1G>C) affecting the same splice site (Marcé-Grau et al., 2019).

Finally, we found a novel, heterozygous missense variant affecting the *STXBP1* gene (c.1324A>G, p.N442D) in a 12-year-old boy with a severe neurodevelopmental disorder characterized by infantile-onset, drug-resistant epilepsy (with generalized myoclonic and focal motor seizures), an intellectual disability, and a peculiar complex MD phenotype with hypokinetic-rigid and dystonic features, with marked fluctuations in severity from day to day. Cerebrospinal fluid (CSF) neurotransmitter analysis revealed low levels of homovanillic and 5-hydroxyindoleacetic acids. First described as causative of a severe early infantile epileptic encephalopathy (EIEE4, OMIM 612164) (Saitsu et al., 2008), the *STXBP1* gene is now known to cause a severe neurodevelopmental disorder with almost constant intellectual disability, a high prevalence of epilepsy, and frequent occurrence of various MDs (Stamberger et al., 2016). This previously unreported variant falls within the 3b domain of the protein, with missense variants affecting the adjacent codon being already reported in patients with EIEE4 (Stamberger et al., 2016). Despite the numerous reports of *STXBP1*-encephalopathy, the clinical and biochemical picture of related MD is largely unknown.

DISCUSSION

Over the last decade, NGS techniques have increasingly been used as a diagnostic tool for rare genetic diseases (Neveling et al., 2013; van Egmond et al., 2017; Cordeiro et al., 2018; Montaut et al., 2018; Reale et al., 2018). To date, few studies have investigated the diagnostic yield of molecular testing in MD (Neveling et al., 2013; van Egmond et al., 2017; Cordeiro et al., 2018; Montaut et al., 2018; Reale et al., 2018). The overall rate of molecular diagnosis in previous studies ranges from 11% to 51%. However, the results of previous studies are not generalizable, due to heterogeneous study designs, sample sizes, inclusion

TABLE 1 | All variants detected in our cohort classified on the basis of main phenotype.

Phenotype	Gene (#MIM number)	Pattern of inheritance	DNA variation	Amino acid change	Allele transmission	Segregation	Reference
Chorea	*NKX2.1 (#600635)*	AD	c.344delG	p.G115AfsX9	het	Sporadic	Novel
		AD	c.391C>T	p.Q131X	het	Familial	Novel
	ADCY5 (#600293)	AD	c.2083C>G	p.R695G	het	Sporadic	Novel
		AD	c.1253G>A	p.R418Q	het	Sporadic	Chen et al. (2015)
	GNAO1 (#139311)	AD	c.709G>A	p.E237K	het	Sporadic	Schirinzi et al., 2018a
Dystonia	*ADAR1 (#606601)*	AR	c.557C>G+c.2894C>T	p.P193A+ p.P965L	comp het	Sporadic	Rice et al. (2012) Wang et al. (2010)
		AD	c.3019G>A	p.G1007R	het	Sporadic	Suzuki et al. (2005)
	ATP1A2 (#182340)	AD	c.2335A>C	p.S779R	het	Sporadic	Novel
		AD	c.889G>A	p.A297T	het	Sporadic	Novel
	ATP1A3 (#182350)	AD	c.2227_2229delGAC	p.D743del	het	Sporadic	Schirinzi et al., 2018b
		AD	c.2266C>T	p.R756C	het	Sporadic	Kanemasa et al. (2016)
		AD	c.2266C>T	p.R756C	het	Familial	Kanemasa et al. (2016)
		AD	c.2452G>A;	p.E818K	het	Sporadic	Demos et al. (2014)
		AD	c.2767G>A	p.D923N	het	Sporadic	Anselm et al. (2009)
		AD	c.2838G>C	p.G947R	het	Sporadic	Heinzen et al. (2012)
	CACNA1B (#601012)	AD	c.5381C>T	p.T1794M	Het	Sporadic	Novel
	TOR1A (#605204)	AD	c.907_909delGAG	p.E303del	Het	Familial	Ozelius et al. (1997)
	GNAO1 (#139311)	AD	c.535A>G	p.R179G	Het	Sporadic	Novel
		AD	c.607G>A	p.G203R	Het	Sporadic	Schirinzi et al., 2018a
	KCTD17 (#616386)	AD	c.508-2A>T			Sporadic	Graziola et al. (2018)
	KMT2B (#606834)	AD	c.1664dupC	p.V557GfsX4	Het	Sporadic	Novel
		AD	c.649dupC	p.R217fsX34	Het	Sporadic	Novel
	SCN2A (#182390)	AD	c.4951T>G	p.F1651V	Het	Sporadic	Novel
	SLC2A1 (#138140)	AD	c.152G>A	p.R51H	Het	Familial	Novel (rs201815571)
		AD	c.458G>C	p.R153P	Het	Sporadic	Novel
	SGCE (#604149)	AD	c.386T>C	p.I129T	Het	Sporadic	Tedroff et al. (2012)
	STXBP1 (#602926)	AD	c.1324A>G	p.N442D	Het	Sporadic	Novel
	PANK2 (#606157)	AR	c.1151C>A+c.444_446delG	p.P384H+ p.E148fsX55	comp het	Sporadic	Novel Novel
	PLA2G6 (#603604)	AR	c.607C>T+dupEx 6-7	p.Q203X+ dup6-7	comp het	Sporadic	Novel –
	WDR45 (#300526)	XLD	c.7C>T	p.Q3X	Het	Sporadic	Novel
Paroxysmal MD	*PRRT2 (#614386)*	AD	c.577G>T	p.E193X	Het	Sporadic	Novel
		AD	c.649dupC	p.R217fsX8	Het	Sporadic	Chen et al. (2011)
		AD	c.649dupC	p.R217fsX8	Het	Familial	Chen et al. (2011)
		AD	c.649dupC	p.R217fsX8	het	Familial	Chen et al. (2011)
		AD	c.649dupC	p.R217fsX8	het	Familial	Chen et al. (2011)
		AD	c.649dupC	p.R217fsX8	het	Familial	Chen et al. (2011)
		AD	c.649dupC	p.R217fsX8	het	Familial	Chen et al. (2011)
		AD	c.649dupC	p.R217fsX8	het	Familial	Chen et al. (2011)
		AD	c.649dupC	p.R217fsX8	het	Familial	Chen et al. (2011)
	SLC2A1 (#138140)	AD	c.275+3A>T	p.R92fsX26	het	Sporadic	Novel
		AD	c.972+1G>T	-	het	Sporadic	Wang et al. (2000)
		AD	c.1097_1100delATCT	p.Y366X	het	Sporadic	Novel

AD, autosomal dominant; AR, autosomal recessive; XLD, X-linked; het, heterozygosis; comp het, compound heterozygosis.

criteria, and specific diagnostic techniques (Neveling et al., 2013; van Egmond et al., 2017; Cordeiro et al., 2018; Montaut et al., 2018; Reale et al., 2018). To the best of our knowledge, this is the first study to specifically address the diagnostic yield of a customized gene panel for MD in a large pediatric cohort.

Our diagnostic rate of 28% is slightly higher than the rates reported in previous studies that feature gene panel analysis for MD, where diagnostic rates have ranged from 11% to 22% (van Egmond et al., 2017; Montaut et al., 2018; Reale et al., 2018). Even if these differences can be attributed to heterogeneous cohort sizes and variant panel design, this finding suggests that the application of MD gene panels to selected target populations (such as children) can improve their diagnostic yield. Higher diagnostic rates have been reported only in studies with combinations of different genetic investigation approaches, such as direct testing, multiple gene panel analysis, and whole-exome sequencing (Cordeiro et al., 2018). A higher diagnostic rate was found in the PMD group (35%), similar to the rate reported by Montaut et al. (2018) in a smaller cohort (35% in 20 patients).

In contrast to previous reports (Montaut et al., 2018; Reale et al., 2018), a relatively high diagnostic rate was found in patients with dystonia (28%, where both isolated and combined forms are considered), probably reflecting a wider genetic landscape of onset in childhood and adolescence relative to adult-onset dystonia. In addition, the diagnostic rate in combined dystonia was considerably higher than in isolated forms of dystonia (34% vs. 16%), suggesting a higher yield for gene panel analysis in complex dystonia-plus syndromes.

A diagnostic rate of 25% was found in samples analyzed for suspicion of genetic chorea, very similar to the report of Montaut et al. (2018). By contrast, the diagnostic rate in patients investigated for tremor was null. There may be two reasons for this finding. First, the smaller sample of patients analyzed for tremor syndromes might drive this result. Second, no gene included in the panel specifically targeted isolated tremor syndromes.

A surprising result for a pediatric cohort was that no pathogenic variant was found in genes involved in the synthesis or transportation of neurotransmitters. No patient undergoing CSF neurotransmitter analysis showed a metabolite profile that could be clearly diagnosed as a primary defect of monoamine or folate metabolism. Only nonspecific changes were found. Secondary neurotransmitter abnormalities are frequently found in children with MDs (García-Cazorla et al., 2007; Tonduti et al., 2015; Burlina et al., 2017), and abnormal patterns suggestive of specific conditions other than primary defects have been recently described (Peall et al., 2017; Papandreou et al., 2018). Nevertheless, most of the commonly encountered secondary neurotransmitters abnormalities still lack a specific diagnostic value. This finding suggests that gene panel analysis has a limited diagnostic yield for the genetic diagnosis of inherited disorders of neurotransmission when biochemical studies are inconclusive. **Table 2** summarizes the relevant literature on the topic and shows the divisions in phenotypic groups of the different cohorts.

With regard to the specific findings we encountered, gene panel analysis proved to be a valid method to expand the

TABLE 2 | Summary and comparison of published papers on genetic diagnosis of pediatric movement disorders. For each cohort, number of genes analyzed, sample size, genetic techniques and overall diagnostic yield are reported. All diagnostic categories of patients included in each cohort are also reported.

Study	Genes analyzed (No.)	Sample size (No.)	Technique(s)	Diagnostic Rate	DYT	PMD	Myoclonus	Chorea	NBIA	NT	Tremor	Hyperkinetic MD	Parkinsonism	Ataxia	HSP
Neveling et al. (2013)	151	50	WES	20%	X	–	–	–	–	–	–	–	–	X	X
van Egmond et al. (2017)	94	61	NGS panel	14%	X	–	–	–	–	–	–	–	–	–	–
Montaut et al. (2018)	127	378	NGS panel	22%	X	X	X	X	–	–		–	X	–	–
Reale et al. (2018)	65	221	NGS panel	11%	X	–	–	–	X	X	–	–	X	–	–
Cordeiro et al. (2018)	26	51	NGS, WES, Direct Seq	51%	X	–	–	X	–	–	X	X	–	–	–
Our cohort	102	148	NGS	28%	X	X	–	X	–	–	X	–	–	–	–

WES, whole exome sequencing; NGS, next generation sequencing; DYT, dystonia; PMD, paroxysmal movement disorders; NBIA, neurodegeneration with brain iron accumulation; NT, inherited disorders of neurotransmitter metabolism; HSP, hereditary spastic paraplegias.

phenotypic and genotypic spectra of MD-associated genes, helping to expand the rapidly growing body of knowledge on recently discovered genes. Taken together, our results underline the extent to which the diagnostic yield of targeted gene panels for MD depends on the selection of patients and the investigation strategies adopted by each diagnostic facility. Nevertheless, targeted panels are a high-throughput sequencing strategy, which is efficient and cost-effective, with a good diagnostic rate. It is recommended that a multiple-strategy approach be adopted to improve the overall diagnostic rate for pediatric-onset MDs. This is necessary, considering the inability to detect copy number variants for these diseases, the low level of mosaicism, and the triplet-repeat expansions through NGS and the occurrence of disorders due to recently discovered or ultra-rare causative genes. A multiple-strategy diagnostic approach should combine target gene sequencing, (multiple) gene panel analysis, molecular cytogenetics, and whole exome or whole genome sequencing in a patient-tailored strategy (Cordeiro et al., 2018). However, the large number of patients with pediatric-onset MD and without a genetic diagnosis for all cohorts reported so far suggests the likely existence of several, still undiscovered, responsible genes (van Egmond et al., 2017; Cordeiro et al., 2018; Montaut et al., 2018; Papandreou et al., 2018; Reale et al., 2018).

From this perspective, gene panel analysis should be proposed as the first diagnostic tool for the genetic investigation of pediatric-onset MD, and the information obtained may be relevant for both diagnostic and research purposes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bambino Gesù Local Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardians or next of kin.

AUTHOR CONTRIBUTIONS

FG, GG, FS, and AC contributed to the conception and design of the study; FG and LB organized the database; FG, GG, and AC performed the statistical analysis; FG wrote the first draft of the manuscript; GG, FS, LB, and LT wrote sections of the manuscript; and FV, PC, EB, and AC revised the final draft. All authors contributed to the manuscript revision and read and approved the submitted version.

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accumulation; NGS, next generation sequencing; NT, inherited disorders of neurotransmitter metabolism; PKD, paroxysmal kinesigenic dyskinesia; PMD, paroxysmal movement disorders; XLD, X-linked; WES, whole exome sequencing

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01026/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pediatrics

CASE REPORT
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Two Novel Mutations (c.883-4_890del and c.1684C>G) of *WDR62* Gene Associated With Autosomal Recessive Primary Microcephaly: A Case Report

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Background: Autosomal recessive primary microcephaly (Microcephaly Primary Hereditary, MCPH) is a rare disorder, affecting 1 in 10,000 children in areas where consanguineous marriages are common. *WDR62* gene mutations are the second most common cause of MCPH. Herein, we report a case of primary microcephaly caused by two novel *WDR62* mutations, which is, to our knowledge, the first such case report in East Asia.

Case presentation: A 6-year-old girl visited our outpatient clinic as a result of microcephaly and delayed development. The patient was born at 36 weeks 4 days through cesarean section. Her birth weight was 1.8 kg (<1st percentile), and she was noted to have microcephaly (head circumference at birth was 28 cm, < −3SD). On examination, delayed speech development and microcephaly with an occipitofrontal head circumference of 43.5 cm (< −3SD) were noted. The patient's gross and fine motor development was normal. Her intelligence quotient was 43 (<0.1 percentile), the same as a 27-month-old child, and her social intelligence quotient was 76.92. Brain imaging revealed simplified gyral patterns of the cerebral cortex; however, laboratory findings, including organic acids, were normal. Multiplex ligation-dependent probe amplification technique for microdeletion syndrome and chromosomal microarray, showed no abnormality. Clinical exome sequencing test revealed two novel heterozygous variants in the *WDR62* gene at two different sites: in the boundary of intron 7 and exon 8 (NM_001083961.1: c.883-4_890del) and in exon 13 (NM_001083961.1: c.1684C>G). The patient's parents were identified as heterozygous carriers for each variation.

Conclusion: We report on two novel heterozygous mutations in East Asia. Our data expand the understanding of *WDR62* mutations.

Keywords: autosomal recessive primary microcephaly (MCPH), exome sequencing test, novel mutation, *WDR62* gene mutation, neurodevelopment

INTRODUCTION

Microcephaly is a condition characterized by a head circumference measuring at least three standard deviations below the mean for a given population, gender, and age (1). Primary microcephaly is a congenital developmental defect in which the cerebral cortex may be thickened or disorganized and in which the gyral pattern may be normal or simplified (1–5). Autosomal recessive (AR) primary microcephaly (Microcephaly Primary Hereditary, MCPH) is a rare form of primary microcephaly, characterized by a marked reduction in brain size, and intellectual disability, which affects 1 in 30,000 children in Japan (6) and 1 in 10,000 children in areas where consanguineous marriages are common (1, 4).

MCPH has previously been described as a genetically heterogeneous disorder influenced by mutations in at least 20 genes including (*MCPH1, WDR62, CDK5RAP2, KNL1, ASPM, CENPJ, STIL, CEP135, CEP152, ZNF335, PHC1, CDK6, CENPE, SASS6, MFSD2A, ANKLE2, CIT, AGMO, RTTN,* and *PGAP2*) (2, 7). However, mutations in two particular genes are thought to be primarily responsible, with *ASPM* mutations being observed in over half of cases (1, 7, 8), and *WDR62* mutations accounting for around 10% of MCPH cases (3, 5, 7, 8).

WDR62 is known to encode the WD repeat-containing protein 62, playing a significant role in neuronal progenitor cell proliferation and spindle formation (2, 5, 9, 10). Children with *WDR62* mutations often present with intellectual disability alongside delayed development of speech and language skills and prominent microcephaly (2–5, 10, 11), whereas some also present with seizures (2, 3, 5, 11). Mutations in this gene are also reported to cause a range of other cortical malformations including polymicrogyria, pachygyria with cortical thickening, lissencephaly, schizencephaly, corpus callosum hypoplasia, simplified gyral patterns, cerebral hypoplasia, and band heterotopias (2–5, 11).

Herein, we report a case of primary microcephaly with two novel mutations in the *WDR62* gene. This is, to the best of our knowledge, the first such case report in East Asia. In this case, two novel heterozygous variants were found in the *WDR62* gene at two different sites, reflecting cultural characteristics of South Korea, which prohibits consanguineous marriages and therefore results in a low incidence of rare AR diseases.

CASE PRESENTATION

Clinical Presentation

A 6-year-old girl visited our outpatient clinic as a result of microcephaly and delayed development. Despite exhibiting delayed acquisition of language and cognitive milestones, her gross and fine motor functions were in correspondence with her age. The child was born by cesarean section at 36 weeks 4 days, with good Apgar scores. Her birth weight was 1.8 kg (<1st percentile), and microcephaly was detected (her head circumference at birth was 28 cm, <−3SD). Intrauterine growth restriction was detected on a prenatal ultrasonography test. She was capable of walking independently at the age of 14 months.

The patient had one 10-year-old brother who had a normal head circumference and showed no signs of developmental delay. Neither parent had any developmental abnormalities.

Upon examination, delayed speech development and microcephaly with an occipitofrontal head circumference of 43.5 cm (<−3SD, **Figure 1**) were noted. Her gross and fine motor development was found to be normal. She was capable of independent outdoor gait, running, jumping, hopping,walking up and down the stairs, touching the ground, and block building. However, she could perform only simple errands and had

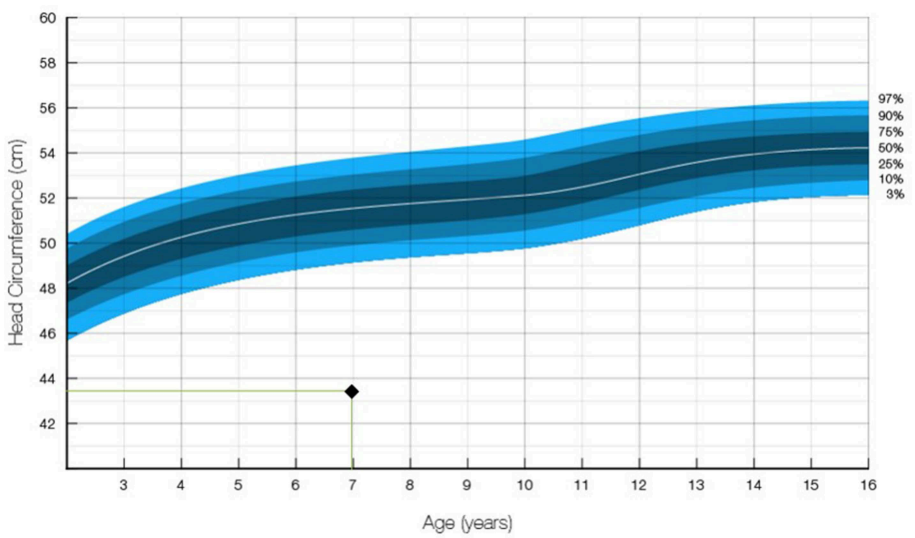


FIGURE 1 | Occipitofrontal head circumference of the patient. The measurement of 43.5 cm at age 7 was in the 1.00 percentile and below −3 SD (12).

dysarthria. She found reading almost impossible, and she could not write at all. On the Preschool Receptive-Expressive Language Scale, the patient was determined to have the receptive and expressive language of a 41-month-old. On the Korean–Leiter International Performance Scale-Revised examination, the patient's Full-Scale Intelligence Quotient was 43 (<0.1 percentile), corresponding to the level of a 27-month-old child. Her social maturity scale score was 35.5, and her social intelligence quotient was 76.92.

Brain magnetic resonance imaging revealed simplified gyral patterns of the cerebral cortex (**Figure 2**); however, laboratory tests, including organic acids, revealed no abnormal results.

Cytogenetic and Molecular Analyses

Chromosomal study revealed a karyotype of 46, XX, inv (9) (p12q13), which had no clinical significance. Furthermore, multiplex ligation-dependent probe amplification (MLPA) technique for microdeletion syndrome showed no abnormality. MLPA analysis of patient was performed using the SALSA MLPA P064-C1 Reference Kit (MRC Holland, Amsterdam, The Netherlands). Chromosomal microarray (performed using an Affymetrix Cytoscan 750K array, CytoScan® 750K Cytogenetics Solution, Keppel Logistics Building, Singapore 629563, genome build: Hg19 method) was normal.

Genomic DNA was extracted from the peripheral blood of the child and her parents. Genomic DNA was enriched using the xGen Inherited Disease Panel (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). Two compound heterozygous variants were observed in the *WDR62* gene: a heterozygous deletion variant in the boundary of intron 7 and exon 8 (NM_001083961.1: c.883-4_890del) and a heterozygous missense variant in exon 13 (NM_001083961.1: c.1684C>G).

Sanger sequencing revealed that the parents were heterozygous carriers for each variant, occurring in trans configuration (**Table 1**). The patient's mother possessed mutations in the *WDR62* gene boundary of intron 7 and exon 8 (NM_001083961.1: c.883-4_890del), as shown in **Figure 3A**, while her father possessed variant in the *WDR62* gene at exon 13 (NM_001083961.1: c.1684C>G), as shown in **Figure 3B**.

TABLE 1 | *WDR62* mutations of the patient and her parents.

Mutation	Patient affected	Mother unaffected	Father unaffected
c.883-4_890del	+	+	−
c.1684C > G (p.His562Asp)	+	−	+

DISCUSSION

Based on clinical and molecular findings, the proband was diagnosed with AR primary microcephaly type 2. According to ClinVar, a public sequence database (https://www.ncbi.nlm.nih.gov/clinvar/), 36 pathogenic mutations have been identified in the *WDR62* gene to date. *WDR62* variants have previously been reported in people of Northern European descent and in Saudi Arabian, Indian, Mexican, Turkish, Iranian, Arabic, and Pakistani families. In a large consanguineous Saudi family, there were two affected siblings born to a consanguineous union in the family of heterozygous carriers of parents (2). In a non-consanguineous family of Northern European descent, compound heterozygous mutations in the *WDR62* was reported as the cause of recurrent polymicrogyria in a sibling pair (13). Contrastingly, this is the first report of a *WDR62* mutation in East Asian countries including China, Japan, and South Korea.

Consanguineous marriages are banned in South Korea, reducing the possibility of AR disease incidence. In the patient in this study, a *WDR62* mutation phenotype might be expressed due to novel heterozygous mutations.

Functional Analysis of Two Novel Variants

We found compound heterozygous variants in the patient's *WDR62*, the deletion variant (c.883-4_890del) from the mother and the missense variant (c.1684C>G/p.His562Asp) from the



FIGURE 2 | Brain magnetic resonance imaging (MRI) of the patient, showing microcephaly and simplified gyrus of the cerebral cortex. **(A)** Axial T1-weighted image; **(B)** sagittal T1-weighted image; **(C)** coronal T1-weighted image.

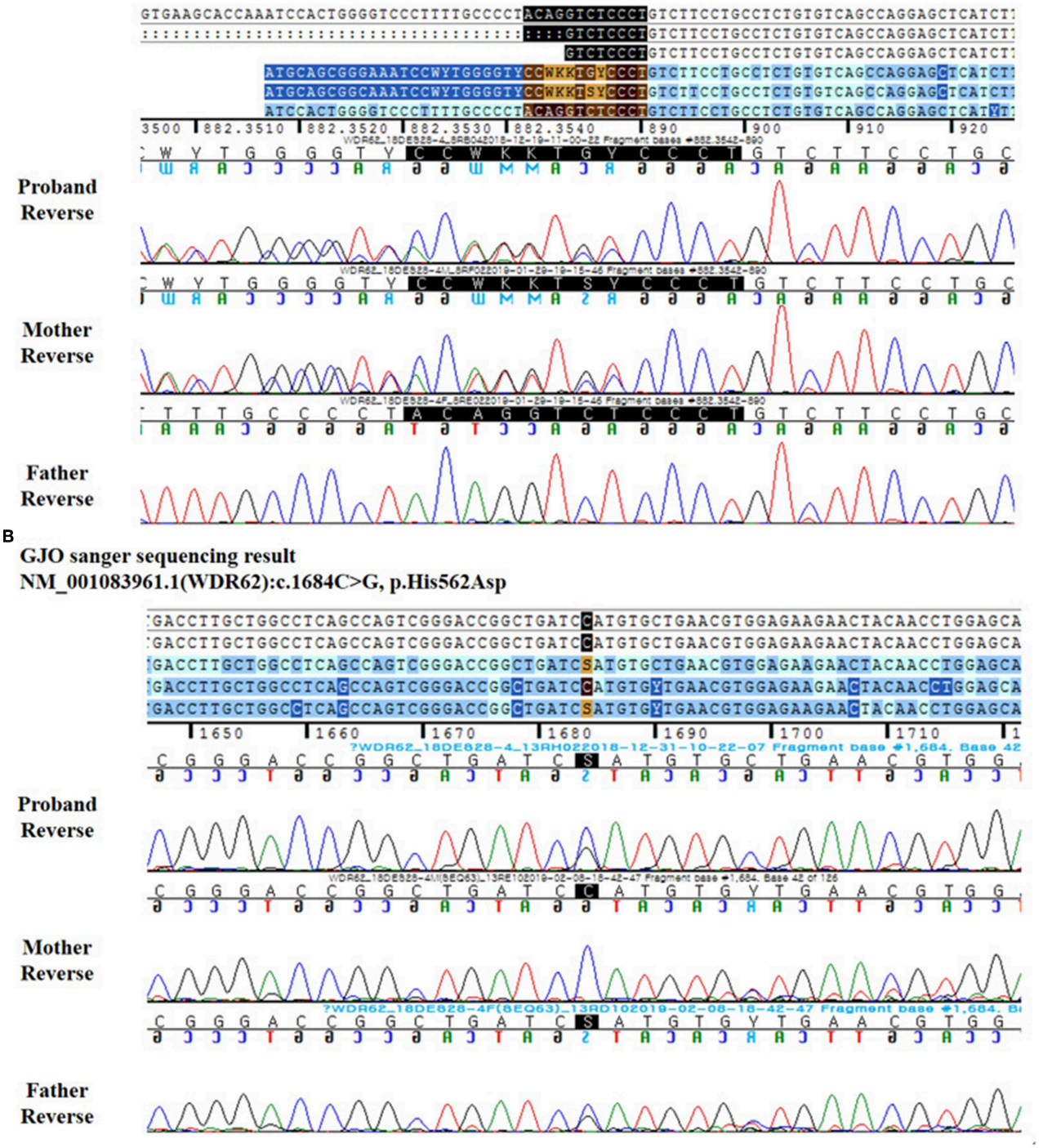


FIGURE 3 | Reverse DNA sequence chromatography for the patient and her parents. **(A)** c.883-4_890del. **(B)** c.1684C>G.

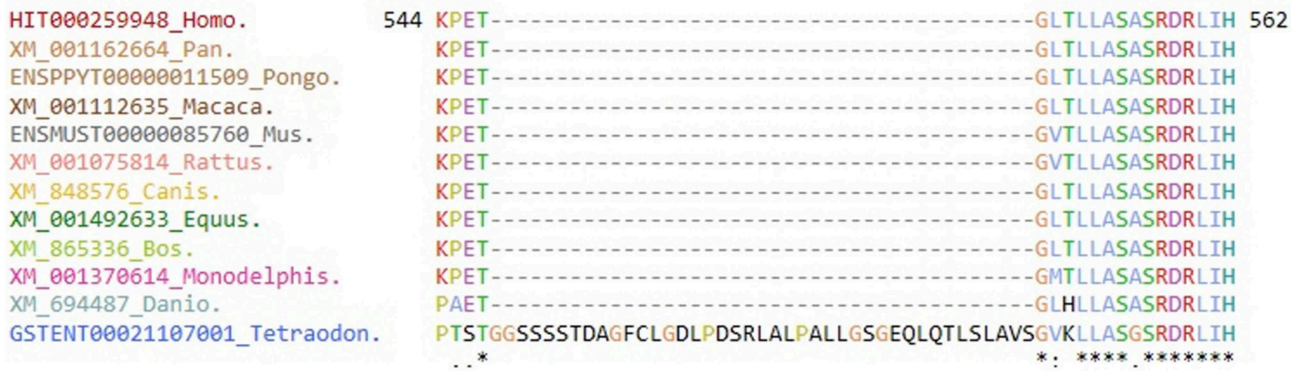


FIGURE 4 | Evolutionary conservation of the amino acid residues for mutant site. Multiple sequence alignment shows the amino acid site of a novel variant (c.1684C>G/p.His562Asp) is highly conserved among different species [Evola version 7.5 (www.h-invitational.jp/evola/)].

father. Neither of these variants has previously been reported in control databases, such as the 1,000 Genomes Project, Exome Variant Server, Exome Aggregation Consortium, or the dbSNP Database.

The c.883-4_890del variant involves splice acceptor site between intron 7 and exon 8, which might result in aberrant splicing, and exon 8 is not an alternatively splice exon among the transcript isoforms (PMID 20729831). This novel canonical splicing variant is suggested as the "likely pathogenic" variant, based on the American College of Medical Genetics and Genomics guidelines regarding the interpretation of sequence variations (PSV1+PM2) (14).

The c.1684C>G variant is located in conserved sequences across species (GERP++_RS 4.45 and phyloP20way_mammalian 0.852, **Figure 4**) and has been predicted to be deleterious by several *in silico* analysis tools [SIFT damaging (0.004), PolyPhen-2 deleterious (1), and MutationTaster (1)]. This novel missense variant is suggested as the possible pathogenic element (PM2 + PM3 + PP3 + PP4) (14). However, we were unable to perform some functional assay using *in-vitro* cell culture to further confirm the pathologic effects of those mutations.

Clinical Features of *WDR62* Mutations

In cases featuring *WDR62* mutations, a broad range of clinical phenotypes have been observed by several independent research groups. These include microcephaly (2–5, 7–11, 15–21), intellectual disability (ID) (2–5, 10, 11, 19–22), speech delay (2, 3, 11, 16, 20), motor delay (3, 5, 16), spasticity (3, 7, 23), infantile spasm (3, 5), epilepsy (2–5, 7, 8, 11), behavior abnormalities (3, 4, 7, 11, 16), high-arched palate (3, 7, 16), dysmorphic face (3, 5, 11, 24), spastic quadriparesis (3, 7), micrognathia (3), dysconjugate gaze (25), and dysarthria (25). Brain imaging in these patients has variously revealed normal findings (26), simplified gyrus (2–5, 9, 10, 16, 20), pachygyria (2–5, 16), cortical thickening (2–5, 16), corpus callosum abnormalities (3–5, 7, 16), lissencephaly (2, 3, 11, 16, 20), schizencephaly (2–4, 16, 20), polymicrogyria (2–4, 16), and heterotopia (3, 4). In our patient, a simplified gyrus was observed alongside definite microcephaly, ID, and speech delay, which are all typical features of *WDR62* mutation. However, the patient presented with no evidence of epilepsy, dysmorphic face, or spasticity.

Molecular and Neurodevelopmental Etiology of *WDR62* Mutation

WDR62 is the second most frequently mutated gene in MCPH, accounting for ∼10% of cases (8). This gene is located within the MCPH2 candidate junction of chromosome 19q13.12, with 32 functional exons and a genome size of 50230 bp (4). This gene encodes a protein with several WD40 domains, which mediate protein–protein interactions (5, 15, 20).

WDR62 functions to preserve centrosome and spindle pole integrity after bipolar spindle formation (2, 16). Loss of the *WDR62* encoded protein leads to the dispersal of pericentriolar matrix components, including pericentrin, γ-tubulin, and CDK5 regulatory subunit-associated protein 2 (1, 7, 15, 20, 22, 24). These components interact with one another in metaphase centrosomes (1, 23, 24). In patients with heterozygous *WDR62* mutations, lymphoblastoid cells displayed mitotic spindle defects and abnormal centrosome protein localization (8, 10, 24).

WDR62 exhibits remarkable cell cycle dependence (20). *WDR62* is mainly associated with the nucleus during interphase, accumulating in the spindle pole, but not in the midbody in cytokinesis during mitosis (5, 8, 11, 16). Therefore, *WDR62* mutations can result in various abnormal phenotypes of cerebral cortical development (20, 23).

Correlation Between Phenotypes and Genotypes in the Patient

The frameshift, missense, non-sense, and splice site mutations in the *WDR62* are randomly distributed (5). Although missense mutations may cause a defect in neurogenesis resulting in primary microcephaly, it has been suggested that non-sense mutations may cause a more severe microcephaly phenotype by adding a cerebral cortex lamination defect (27). However,

subsequent studies have not reported genotype–phenotype correlation. In the present study, splice site mutations in the *WDR62* might be attributable to the pachygyria in addition to microcephaly, which is in line with previous studies (20, 28). Further cumulative data and molecular approaches are required to accurately identify genotype–phenotype correlations in *WDR62*.

CONCLUSION

We reported two novel heterozygous mutations in East Asia. Our data helped in the understanding of *WDR62* mutations.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Clinvar: SCV000924581, SCV000924580.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YY: acquisition of data, analysis and interpretation of data, writing, and critical revision of manuscript. S-ML, J-HJ, D-WL, and JK: acquisition of data, analysis and interpretation of data. D-HJ: study concept and design, acquisition of data, analysis and interpretation of data, study supervision, and critical revision of manuscript for intellectual content.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Genetics

CASE REPORT
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Rare *De Novo IGF2* Variant on the Paternal Allele in a Patient With Silver–Russell Syndrome

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Silver–Russell syndrome (SRS) is a rare, well-recognized disorder characterized by growth restriction, including intrauterine and postnatal growth. Most SRS cases are caused by hypomethylation of the paternal imprinting center 1 (IC1) in chromosome 11p15.5 and maternal uniparental disomy in chromosome 7 (UPD7). Here, we report on a Chinese family with a 4 year old male proband presenting with low birth weight, growth retardation, short stature, a narrow chin, delayed bone age, and speech delays, as a result of a rare molecular etiology. Whole-exome sequencing was conducted, and a novel *de novo IGF2* splicing variant, NM_000612.4: c.157+5G > A, was identified on the paternal allele. *In vitro* functional analysis by RT-PCR and Sanger sequencing revealed that the variant leads to an aberrant RNA transcript lacking exon 2. Our results further confirm the *IGF2* variant mediates SRS and expand the pathogenic variant and phenotypic spectrum of *IGF2*-mediated SRS. The results indicate that, beyond DNA methylation and UPD7 and *CDKN1C* variant tests, *IGF2* gene screening should also be considered for SRS molecular diagnoses.

Keywords: *IGF2*, *de novo*, splicing variant, Silver–Russell syndrome, whole-exome-sequencing

BACKGROUND

Silver–Russell syndrome (SRS) (MIM number: 180860) is a rare disorder characterized by intrauterine growth retardation accompanied by postnatal growth deficiency. The clinical features include proportionately short stature, normal head circumference, fifth-finger clinodactyly, typical facial features (triangular faces characterized by broad forehead and narrow chin), and limb-length asymmetry potentially resulting from hemihypotrophy with diminished growth of the affected side (Saal, 1993).

SRS is considered an etiologically and clinically heterogeneous disease, thus complicating clinical and molecular diagnosis. An SRS scoring system, the Netchine–Harbison Clinical Scoring System (NH-CSS), was developed for the clinical diagnosis of SRS. This scoring system is recognized in

an international consensus statement on the diagnosis and management of SRS (Azzi et al., 2015; Wakeling et al., 2017).

IGF2, located in 11p15.5, encodes a member of the insulin family of polypeptide growth factors, which are involved in development and growth (Cassidy and Charalambous, 2018). It is an imprinted gene, expressed only from the paternal allele, and epigenetic changes at this locus are associated with several disorders, including Wilms tumors, Beckwith–Wiedemann syndrome, rhabdomyosarcoma, and SRS (Anderson et al., 1999; Md Zin et al., 2011; Azzi et al., 2014). Previous studies have reported that 30–60% of SRS cases are caused by hypomethylation of the paternal imprinting center 1 (IC1) of chromosome 11p15.5, whereas approximately 10% of cases have maternal uniparental disomy for chromosome 7 (UPD7) (Wakeling et al., 2017). Examination of rare cases has resulted in the identification of pathogenic *CDKN1C* variants as part of the SRS etiology (Wakeling et al., 2017). The molecular etiology of 30–40% of SRS patients with a clinical diagnosis of this condition remains unknown. Recently, a pathogenic *IGF2* variant in a multigenerational family with growth restriction and a phenotype similar to that of SRS was identified (Begemann et al., 2015). Subsequently, five reports revealed SRS-associated *IGF2* variants. However, the variants and phenotypic spectrum remain very limited (Liu et al., 2017; Yamoto et al., 2017; Abi Habib et al., 2018; Poulton et al., 2018).

Here, we report a *de novo IGF2* splicing variant occurring on the paternal allele in an SRS patient born small for his gestational age, and presenting with growth retardation, short stature, and facial abnormalities. The newly identified *IGF2* variant indicates another molecular etiology for SRS beyond the hypomethylation of IC1, UPD7, and *CDKN1C* variants, which should be screened in SRS patients with negative regular molecular diagnosis results.

CASE PRESENTATION

The proband is a 4 year old male who is the only child of a Chinese family (**Figure 1**). The parents are non-consanguineous and clinically normal. The heights of the father and mother are 178 cm and 163 cm, respectively. No family members or relatives have developmental delay. During pregnancy, oligohydramnios was observed. The proband was born at 40 weeks' gestation with a small placenta, and he was relatively small for his gestational age (weight 1,850 g, <3%; length 42 cm, <3%; head circumference 27 cm, <3%). The patient had mild feeding difficulties: he was under-eating and required Enfamil Enfacare. His height and weight at 24 months were 75 cm (<3%) and 7,500 g (<3%), respectively, and his BMI was 10.5 (<3%). In addition, a prominent forehead, fifth-finger clinodactyly, a triangular face, micrognathia, low-set ears, delayed bone age, low muscle mass, delayed motor, and speech development were identified (**Table 1**). No other obvious abnormalities were found. According to growth restriction, the patient was subjected to GH therapy, which was efficacious; the patient grew 3 cm after 16 GH injections administered over the course of 114 days.

According to the NH-CSS, the patient was diagnosed with SRS. Because the patient was born small for his gestational age and did not show body asymmetry, we initially speculated that there might be a genetic, rather than an epigenetic, defect causing the patient's phenotype. Peripheral blood samples from the family members and a healthy control member were collected. The family members and the healthy control member provided written informed consent to participate in the study. This study was approved by the ethics committee of Shengjing Hospital of China Medical University. The patient's samples were subjected to next generation sequencing-based copy number variation assays, and WES trio testing was performed according to our rare disorder diagnosis process.

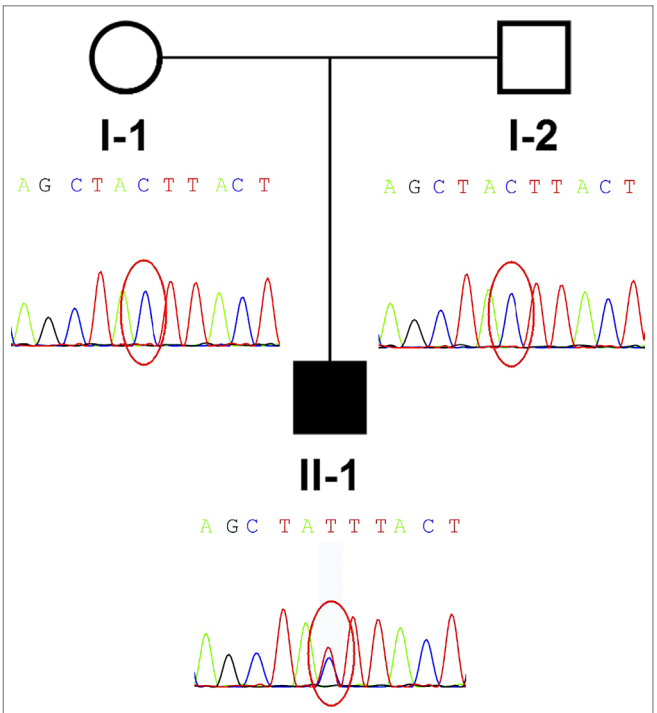


FIGURE 1 | Pedigree and *de novo IGF2* variant identified in the family. The proband (II-1) was the only patient in the family and carried a de novo *IGF2* variant. The parents of the patient were wild type.

WHOLE-EXOME SEQUENCING

Low depth whole genome sequencing based on next generation sequencing (NGS) and analysis of copy number variant was performed in the family. TMAP software (Version 4.6), Picard software (Version 2.18.17), and the analytic method of LOWESS regression and circular binary segmentation were used to analyze copy number variant. Only a single maternal 0.41Mb duplication (17q11.2: 28920000-29330000) was found. Only RNF135 within this interval has been previously associated with abnormal phenotype, including overgrowth, mild learning disability, strabismus, dysmorphism, and advanced bone age (Douglas et al., 2007). This variant alone could not explain the phenotype of the patient. Subsequently, whole-exome sequencing (WES) of the patient-parent trio was conducted (**Figure 1 II-1, I-1, I-2**). WES of the family members yielded more than 14.1 Gb of data at 92× the average depth with an overall coverage

TABLE 1 | *IGF2* genetic defects and clinical features of patients with Silver-Russell syndrome.

Phenotype	Present Case	Begemann et al., 2015				Yamoto et al., 2017	Deguo Liu et al., 2017	Walid Abi Habib et al., 2018		Cathryn Poulton et al., 2018
		Patient 1	Patient 2	Patient 3	Patient 4			Case 1	Case 2	
Country of Origin	China	Germany				Japan	China			Australian Aboriginal
Genetic Defect	NM_000612.4: c.157+5G > A	NM_001127\ 598.2:c. 191G > A	NM_00112 7598.2: c.191G > A	NM_00112 7598.2: c.191G > A	NM_00112 7598.2: c.191G > A	NM_000612.5: c.110_117delin sAGGTAA	NM_000612: c.101G > A	NM_0006 12.5:c.78C > G	NM_000612.5: c.158_159dup	NM_000612.5: c.157+3A > C
Source of Variation	Paternal allele, *De novo*	Paternal	Paternal	Paternal	Paternal	Paternal allele, *De novo*	Paternal allele, *De novo*	*De novo*	*De novo*	Paternal allele, *De novo*
Gender	M	M	F	M	F	M	M	M	F	F
Height of Father	178	178	178	182	150.5 (patient1)	173				
Height of Mother	163	163	163	163	147	167				
Gestational-age	40	43	36	36	35	31	37	32	29	32
Small for Gestational Age	+	+	+	+	+	+	+	+	+	+
Head Circumference at Birth	27					NA	33	27/–2.13	23.5/–2.3	28
Prominent Forehead(1–3-years-old)	+	+	+	–	+	+	+	+	+	+
Body Asymmetry	–	–	–	–	–	–	+	–	–	
Feeding Difficulties and/or Low BMI	+	+	+	–	+	+	+	+	+	+
Postnatal Growth Failure	+	+	+	+	+	+	+	+	+	+
Relative Macrocephaly	+	+	+	+	+	+	+	+	+	+
Hypoplastic Placenta	+					+				
Oligohydramnios	+		+			NA				
Triangular Face	+	+	+	+	+	+	+	+	+	
Micrognathia	+	+	+	–	+	+	+			+
Cleft Palate	–					+				
Low-set Ears	+	+	+	+	+	+	+			
Hearing Impairment	–					Borderline hearing impairment				
Low Muscle Mass	+									
Clinodactyly	+		+			+				+
Ectrodactyly	–					+				
Polydactyly	–					+				
Syndactyly	–					+				
Shoulder Dimples	–									+
Bone Age Delay	+	+	+	+	NA					
Low-intelligence	–	+	+	–	–					
Hypospadia	–			+	–	+				
Abnormal Scrotum	–					+				
Cryptorchidism	–			+		+				
Microphallus	–					+				
Heart defect	–	+	+		+	+	+			+
Brain	–									Posterior periventricular white matter loss and periventricular leukomalacia
Motor Delay	+	+	+	–	+		–			+
Speech Delay	+	+	+	–	–	–				
Other Abnormity	NA									
GH Response	+	+	+	+	+	NA	+	+	+	+

greater than 97.65%. A total of 675 variants with minor allele frequencies of <0.01 in any of the study-associated databases (dbSNP, Exome Aggregation Consortium, 1000 Genomes Project, HapMap project, and in-house database) were selected for further analysis. Variant analysis was performed based on an autosomal recessive inheritance model, including compound heterozygous and homozygous variants, or based on an autosomal dominate inheritance model, including heterozygous variants. In addition, *CDKN1C* and *IGF2* gene variants were considered in the analysis. No *CDKN1C* variant was found, whereas a *de novo IGF2* gene variant, NM_000612.4: c.157+5G > A, was identified. Because *IGF2* is a paternally expressed gene, *IGF2* gene defects on the paternal chromosome could induce disease. A heterozygous SNP rs3213225 (NM_000612.4:c.157+61C > T) adjacent to c.157+5G > A was additionally located in the same read. This SNP was paternal in origin, and the mother did not harbor this variant. Finally, the presence of the variant was verified by Sanger sequencing.

IN VITRO FUNCTIONAL ANALYSIS

To confirm the function of the newly identified variant, we conducted *in vitro* functional analysis with RT-PCR and Sanger sequencing. Total mRNA of the proband and a healthy control was extracted from blood, and cDNA was synthesized from the total mRNA by performing reverse transcription with a modified oligo(dT) primer. PCR was conducted with a pair of primers binding exons 1 and 3 (**Figure 2A**). The wild type mRNA produced the expected PCR product of 700 bp. However, the mRNA of the proband yielded a shorter electrophoretic band of 600 bp, indicating a truncated mRNA (**Figure 2B**). Sanger sequencing of the RT-PCR products from the healthy control and the patient indicated that the NM_000612.4: c.157+5G > A variant led to the skipping of exon 2 in the *IGF2* gene (**Figure 2C**). This exon skipping resulted in a loss of the coding sequence for exon 2 and, more importantly, the loss of the AUG initiation codon.

DISCUSSION

The estimated incidence of SRS is 1:30,000 to 1:100,000. Nevertheless, the incidence may be underestimated because of limitations in molecular diagnosis techniques (Wakeling et al., 2017). The major molecular etiology of SRS is based on loss of methylation on chromosome 11p15 and maternal uniparental disomy of chromosome 7, which account for 30–60% and 5–10% of molecular diagnoses in SRS patients, respectively (Wakeling et al., 2017). The molecular etiology of the remaining undiagnosed patients warrants further investigation. A maternal *CDKN1C* gain-of-function variant has been reported as a rare cause of SRS (Eggermann et al., 2014). Recently, five reports have identified *IGF2* loss-of-function variants as new contributors to the molecular etiology of SRS, on the basis of studies including individuals of German, Japanese, Chinese, and Australian Aboriginal descent (**Table 1**) (Begemann et al., 2015; Liu et al., 2017; Yamoto et al., 2017; Abi Habib et al., 2018; Poulton et al., 2018). In our SRS patient from China, we found a novel *IGF2* splicing variant, NM_000612.4: c.157+5G > A, which is located on the paternal allele. According to *in*

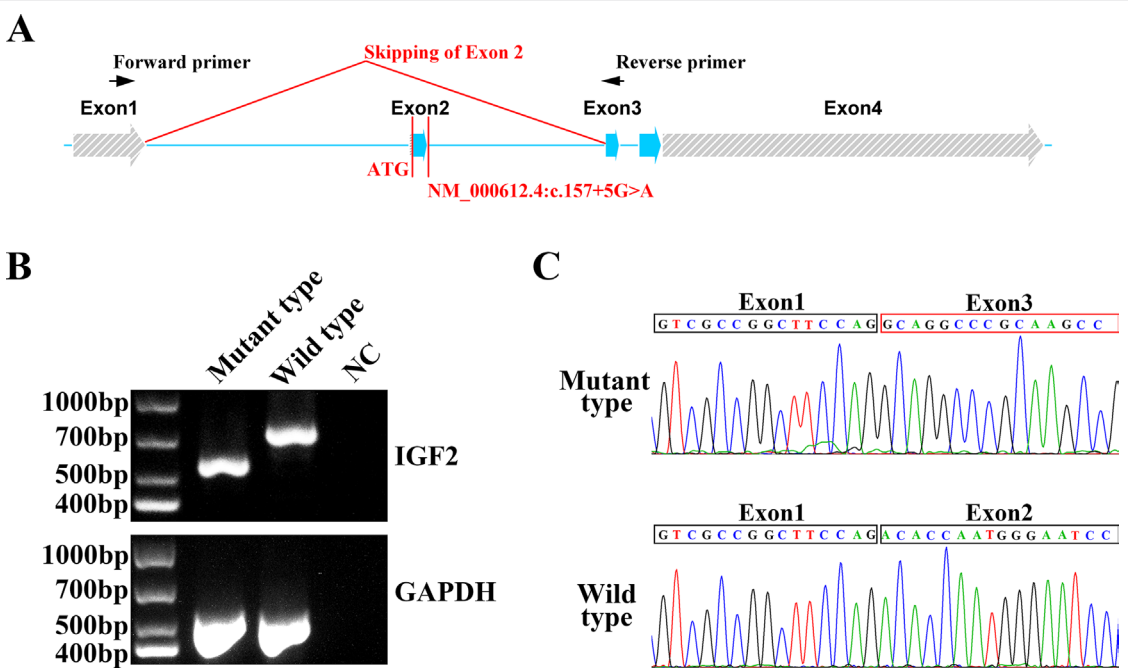


FIGURE 2 | *In vitro* functional analysis of *IGF2* splicing variant. **(A)** The *IGF2* gene was consisted with four exons. The NM_000612.4: c.157+5G>A variant was in intron 2. The AUG initiation codon of *IGF2* was in exon 2. The RT-PCR primers were designed for exon 1 and exon 3. **(B)** Agarose gel electrophoresis of the RT-PCR products from the proband and the healthy control. The PCR of the mutant type mRNA from the proband produced a truncated electrophoretic band of about 600 bp. GAPDH was used as an internal control. **(C)** Sanger sequencing of mutant type and wild type PCR products. Exon 2 skipping was clearly observed in mutant type mRNA, while the normal mRNA sequence was observed in the wild type.

vitro functional analysis by RT-PCR, the variant results in skipping of exon 2, which contains the *IGF2* initiation codon. The variant is absent from control databases including gnomAD (http://gnomad.broadinstitute.org/), ExAC (http://exac.broadinstitute.org/), and the 1000 Genomes project (http://www.internationalgenome.org/). According to the ACMG guidelines, the NM_000612.4: c.157+5G > A variant is described as PVS1 (initiation codon or single or multi-exon deletion), PS2 (*de novo*), and PM2 (absent from controls), and therefore is classified as pathogenic (Richards et al., 2015). The SNP rs3213225 (NM_000612.4:c.157+61C > T) was located in the deep intron region, with a relatively high minor allele frequency (0.449) in 1,000 genomes. The SNP is in cis with the the c.157+5G > A *de novo* mutation, and 56 nt away from it. We consider that rs3213225 has no obvious effects on splicing, and it is very unlikely to be related to phenotype in this case. While it is very unlikely that the common cis polymorphism rs3213225 has any impact on the splicing defect caused by the *de novo* mutation, we cannot positively exclude that possibility. The six previously identified variants include missense, frameshift, stop-gain, and splicing variants (**Table 1**). No major variation pattern appears to exist for *IGF2*.

SRS, first described by Silver et al. and Russell (Silver et al., 1953; Russell, 1954), is mainly characterized by small size for gestational age, postnatal short stature, body asymmetry, relative macrocephaly, a prominent forehead, and feeding difficulties. A patient with more than four of the major characteristic features would be clinically diagnosed with SRS (Azzi et al., 2015). Other phenotypic characteristics often observed in SRS patients include a downturned mouth, clinodactyly of the fifth finger, shoulder dimples, syndactyly of the 2/3 toes, low muscle mass, a prominent heel, autism, and diagnosed cognitive disabilities (Wakeling et al., 2017). However, the phenotypes of *IGF2* gene variants associated with SRS may be different. On the basis on the cases linked to *IGF2* variants (**Table 1**), we conclude that most of these patients do not have body asymmetry (nine in ten patients), a major classification condition according to the NH-CSS for SRS clinical diagnosis. This difference may be a primary condition for diagnosis of SRS caused by epigenetic changes or the presence of an *IGF2* variant. This finding, as well as the case summary, may be useful for the molecular diagnosis of SRS.

In addition, low muscle mass (one in ten patients) was observed in our patient. This finding has not previously been reported in patients with SRS caused by *IGF2* variant (**Table 1**). Hypoplastic placenta (two in ten patients), oligohydramnios (two in ten patients), bone age delay (four in ten patients), low intelligence (two in ten patients), and speech delay (three in ten patients) were uncommon phenotypes in patients with SRS (**Table 1**). Apart from small size for gestational age, postnatal short stature, body symmetry, relative macrocephaly, it can be concluded that low muscle mass, as described in this report, in addition to the previously described phenotypes, are the second characteristics of SRS caused by *IGF2* variants.

A different variant reported in an Australian Aboriginal family, NM_000612.5:c.157+3A > C, also causes skipping of exon 2 (Poulton et al., 2018). Despite the same *in vitro* functional results, the phenotypes of the two patients were not fully consistent. Hypoplastic placenta, oligohydramnios, low muscle mass, bone age delay, and speech delay were observed in only our patient. Shoulder dimples, heart defects, posterior periventricular white matter loss, and periventricular leukomalacia were found only in the Australian Aboriginal patient. These results indicate the clinical heterogeneity of the *IGF2* variant causing SRS. In addition, the phenotypes of SRS caused by *IGF2* variants may differ according to ethnicity. This viewpoint has previously been described by Poulton et al.(Poulton et al., 2018). To date, all nine patients treated with growth hormone (GH) presented positive effects (**Table 1**), thus indicating that this therapy is an effective treatment for *IGF2*-associated SRS.

To date, four patients from a multigenerational family and five patients with *de novo IGF2* gene variants have been reported in only five reports worldwide (Begemann et al., 2015; Liu et al., 2017; Yamoto et al., 2017; Abi Habib et al., 2018; Poulton et al., 2018). Herein, we present the second reported case of SRS caused by an *IGF2* splicing variant, in a Chinese individual, and identified a novel pathogenic *IGF2* splicing variant. We suggest that an *IGF2* gene variant should be considered in the molecular diagnosis process, especially when body asymmetry is not observed in the clinical diagnosis of an SRS patient. In addition, we identified low muscle mass in the patient, a previously unreported phenotype, thus expanding the phenotypic spectrum of *IGF2*-associated SRS. In a comparison in two different ethnic groups yielding the same molecular result of skipping of exon 2, we observed several different phenotypes. These findings indicate the heterogeneity and ethnic differences of SRS caused by *IGF2* variants. However, owing to the limited number of reported cases, further investigation is necessary to verify the phenotypic spectrum, typical characteristics, and clinical therapy in *IGF2*-variant-associated SRS. Collectively, we hope that our findings will facilitate the clinical and molecular diagnosis of SRS.

ETHICS STATEMENT

This study was approved by the Medicine Ethics Committee of Shenjing Hospital of China Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

C-LX and JW conceived and designed the experiments. YL, CL, and C-XL helped in patient workup and recruitment of the patients and their family members. HL, Z-TZ, and S-WY performed the experiment. YM, WL, L-YK, and BL helped in genetic analysis. H-KJ, JL-L, and JW wrote the paper.

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Conflict of Interest: Author YM, WL, and LY-K were employed by company Basecare Medical Device Co., Ltd., Suzhou.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Genetics

CASE REPORT
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A *De Novo* Mutation in *DYRK1A* Causes Syndromic Intellectual Disability: A Chinese Case Report

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Autosomal dominant mental retardation-7 (MRD7) is a rare anomaly, characterized by severe intellectual disability, feeding difficulties, behavior abnormalities, and distinctive facial features, including microcephaly, deep-set eyes, large simple ears, and a pointed or bulbous nasal tip. Some studies show that the disorder has a close correlation with variants in *DYRK1A*. Herein we described a Chinese girl presenting typical clinical features diagnosed at 4 years old. Whole-exome sequencing of the familial genomic DNA identified a novel mutation c.930C > A (p.Tyr310*) in exon 7 of *DYRK1A* in the proband. The nonsense mutation was predicted to render the truncation of the protein. Our results suggested that the *de novo* heterozygous mutation in *DYRK1A* was responsible for the MRD7 in this Chinese family, which both extended the knowledge of mutation spectrum in MRD7 patients and highlighted the clinical application of exome sequencing.

Keywords: *DYRK1A*, intellectual disability, microcephaly, nonsense mutation, whole-exome sequencing

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BACKGROUND

DYRK1A encodes dual specificity tyrosine-phosphorylation-regulated kinase 1A, which contains a nuclear targeting signal sequence, a protein kinase domain, a leucine zipper motif, and a highly conserved 13-consecutive-histidine repeat. This protein catalyzes the phosphorylation of serine and threonine residues on exogenous substrates, as well as phosphorylation of its own kinase domain. The protein is ubiquitously expressed in fetal and adult tissues, with a high expression in the brain (Song et al., 1996; Galceran et al., 2003; Martinez de Lagran et al., 2012). DYRK1A-related intellectual disability syndrome is characterized by mild to severe range of intellectual disability including impaired speech development, microcephaly, and autism spectrum disorder including anxious and/or stereotypic behavior problems. Also, affected individuals often have other symptoms such as typical facial gestalt, feeding problems, seizures, hypertonia, gait disturbances, and skeletal system abnormalities. Rarely, endocrine problems and dental, ophthalmologic, and/or cardiac anomalies are reported (Bronicki et al., 2015; Ji et al., 2015; Ruaud et al., 2015; van Bon et al., 2016). To date, there are more than 2,000 genes associated with intellectual disability (Human Phenotype Ontology Database: http://compbio.charite.de/hpoweb/showterm?id=HP:0000118#id=HP:0001249). Deciphering

Abbreviations: MRD7, Autosomal Dominant Mental Retardation-7 tal Retardation-7; DYRK, Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase; DYRK1A, Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1A; SNP, Single-Nucleotide Polymorphism; CNV, Copy Number Variation; MRI, Magnetic Resonance Imaging; WES, Whole Exome Sequencing.

developmental disorders study group and studies showed that *DYRK1A*-related intellectual disability syndrome accounted for 0.1–0.5% of individuals with intellectual disability and/or autism (Courcet et al., 2012; O'Roak et al., 2012; van Bon et al., 2016).

As the technologies develop, DYRK1A-related intellectual disability syndrome was detected by cytogenetic analysis and Fluorescence in situ hybridization (FISH) to investigate karyotype abnormalities, chromosomal microarray analysis (CMA) or array-CGH to find copy number variations (CNV), and direct Polymerase Chain Reaction (PCR)/panel/clinical exome sequencing/exome sequencing to explore single nucleotide variants and small insertions/deletions (Moller et al., 2008; Yamamoto et al., 2011; Courcet et al., 2012; Valetto et al., 2012; Redin et al., 2014; Bronicki et al., 2015; Ji et al., 2015; Rump et al., 2016; van Bon et al., 2016; Weisfeld-Adams et al., 2016; Evers et al., 2017).

In this study, we identify a novel nonsense mutation in *DYRK1A* by whole-exome sequencing (WES) in a small Chinese family with the severe phenotype of the syndrome. We also review the previously published cases originated from the ClinVar database and Medline search to further summarize variations in *DYRK1A* identified in MRD7, which provide convenience for clinical application.

MATERIALS AND METHODS

Patient Samples

The patient with clinical features was highly consistent with MRD7. Parents took her to Nanjing Maternity and Child Health Care Hospital for genetic counseling when she was 4 years old. Written informed consent form was obtained from the legal guardians of the patient for the molecular genetic analysis and the publication of this case report. Our study was approved by the Ethics Committee of the hospital. Peripheral blood of the family was collected in EDTA anticoagulant tubes, and genomic DNA was isolated from 400 μl peripheral blood using the Automated Nucleic Acid Extractor (Concert Bioscience, Xiamen, China) according to the manufacturer's protocols.

Chromosomal Microarray Analysis

Human cyto12 single-nucleotide polymorphism (SNP) array beadchip with 300,000 probes (Illumina, San Diego, CA, USA) (Leung et al., 2011) was used for whole genome scan. SNP array was carried out according to the manufacturer's instructions. For SNP array, CNV analysis was carried out using KaryoStudio V1.4.3.0 (Hu et al., 2015).

Whole-Exome Sequencing

DNA of the trio was sent for whole exome capture using the SureSelect Human All Exon V6 (Agilent Technologies, USA). According to the manufacturer's recommendations, the resulting libraries was sequenced on Illumina HiSeq 2000. Over 85 Mbs of mappable sequences were generated, resulting in a depth of coverage>30X for more than 97.4% of RefSeq-coding exons. Reads were aligned to Human genome GRCh37/hg19 with the Burrows-Wheeler Aligner36 (BWA.0.6.2) (Li and Durbin, 2010). The Genome Analysis Toolkit 2.6-4 (McKenna et al., 2010) was used for genotyping and indel discovery and single-nucleotide variants, as well as for indel realignment and base quality score recalibration. Candidate events were inspected using the Integrative Genomics Viewer (IGV), while coverage was evaluated with the GATK Depth of Coverage tool by rejecting bases with base quality of <30 and reads with mapping quality of <20.

Sanger Sequencing

PCR primers were designed to amplify exon 7 of *DYRK1A*. The mutation of the proband was sequenced from a 230 bp DNA fragment amplified using primer pair 5'-TGTTGAAGTTAATCAATGGAACCCT-3' and 5'-ACCCGA GGGACCACATA TCA-3'. Sanger sequencing was performed using the ABI 3730xl DNA automated sequencer (Applied Biosystems, Foster City, CA, USA).

Case Presentation

The girl was the firstborn child of healthy nonconsanguineous Chinese parents from Anhui Province. She was cesarean born with a length of 47 cm (−2 SD), weight of 3180 g (−1 SD), and a head circumference of 31 cm (<−2 SD). From birth onward, she had feeding problems and febrile seizures, regularly. The electroencephalogram (EEG) was normal or slightly abnormal during the first year of life. She was able to sit unsupported around the age of 10 months and walk independently around the age of 23 months, and non-fluent motoric movements and hypoactivity were noted. She was described as having a broad-based clumsy tread, and exhibited a mild tremor. She started to pronounce syllables around 2 years old and used simple words around the age of 3 years. Nowadays, she could not speak with full sentences and presented with significantly lower IQ (67).

At 4 years old, the girl had a slender posture with a length of 90 cm (−1.5 SD) and presented with significantly smaller head size of 38.5 cm (<−2 SD) compared to the same age children. Typical facial dysmorphisms included deep-set eyes, pointed nasal tip, large ears, a downturned mouth, and micrognathia (**Figure 1A**). A cerebral magnetic resonance imaging (MRI) showed mild widened lateral ventricles, enlarged pericerebral spaces, high palate, a thin corpus callosum, and delayed myelination but without structural congenital anomalies (**Figure 1B**).

Chromosomal abnormalities and submicroscopic chromosomal imbalances at the whole genome level of the girl by CMA did not reveal any anomaly (data not shown). Using WES, a heterozygous nonsense variant (chr21:38865324C > A; c.930C > A; p.Tyr310*) in the coding region of exon 7 of the *DYRK1A* gene (NM_130436.2) was identified in the proband. Nevertheless, none of the mutation at this site was found in her parents (**Figures 2A, B**). Sanger sequencing confirmed this conclusion (**Figure 2C**).

The nonsense variant in the amino acid residue tyrosine 310, a perfectly conserved amino acid in *DYRK1A* and *DYRK1A* vertebrate orthologues, was a heterozygous truncation mutation and interfered with the protein kinase activity. This variant was absent from the Genome Aggregation Database browsers (gnomAD, http://gnomad.broadinstitute.org/) and the Exome Aggregation Consortium databases (ExAC, http://exac.broadinstitute.org/) or dbSNP (http://www.ncbi.nlm.nih.

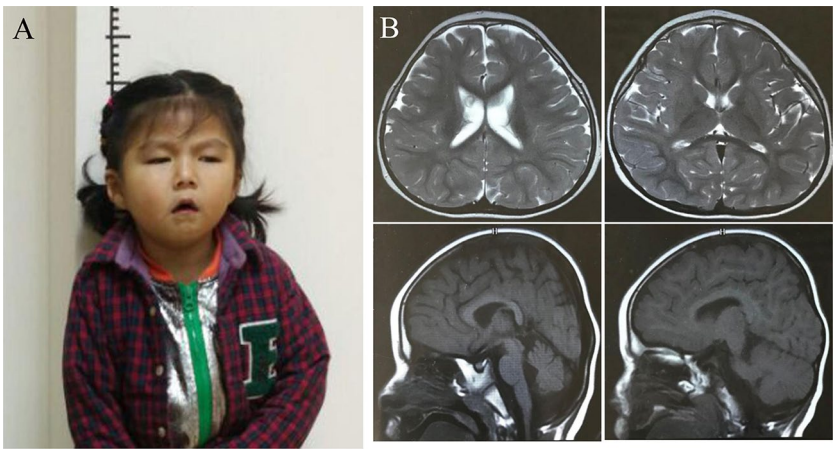


FIGURE 1 | Clinical and imaging features of the proband **(A)** The picture illustrates the facial dysmorphic features consisting of microcephaly, micrognathia, deep-set eyes, large ears, pointed nasal tip, and a downturned mouth. Written informed consent for publication of medical data and identifiable images was obtained from the parents of the patient. **(B)**. Brain MR images showed mild prominence of lateral ventricles, enlarged pericerebral spaces, high palate, delayed myelination, and a thin corpus callosum when the girl was at 4 years of life.

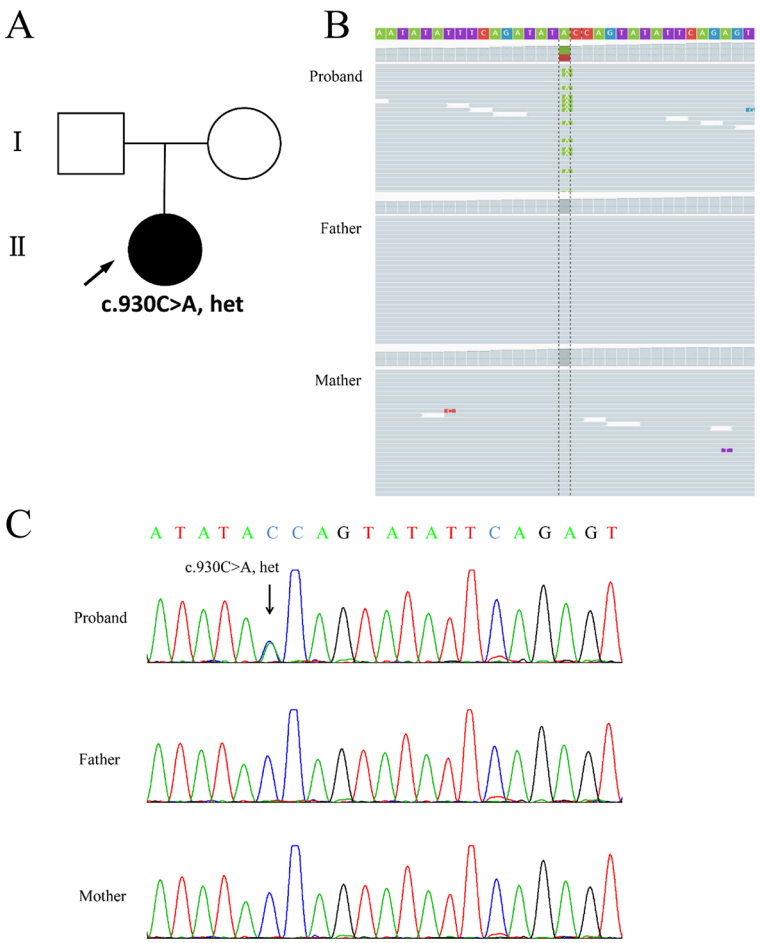


FIGURE 2 | Genetic findings from the family. **(A)** Pedigree of the family with segregation of the identified *DYRK1A* mutation. The square represents male, and circles represent female. Filled symbol indicates the affected individual. **(B)** Visualization of mutation in the *DYRK1A* gene is shown on the Integrative Genomics Viewer. Variation c.930C > A was heterozygous in the proband. **(C)** The variation of c.930C > A is a nonsense mutation (p.Tyr310*) identified in the proband. The parents were tested and did not carry the mutation. Black arrows indicate the point mutation.

gov/snp), which excluded that it represents a polymorphism. Meanwhile, the loss-of-function intolerance (pLI) for *DYRK1A* is 1.00, which is based on the ExAC sequencing data, suggesting strong intolerance to functional mutations. Taken together, we hypothesize that the nonsense mutation causes the severe clinical phenotype by ACMG recommendations for standards and guidelines for the interpretation of sequence variants (Richards et al., 2015). A heterozygous variant in PKHD1L1 (NM_177531.6: c.9352G>T, Glu3118*); a heterozygous variant in PRODH2 (NM_021232.1: c.457C>T, Arg153*) and a heterozygous variant in SDK2 (NM_001144952.2: c.1865delT, Leu622Argfs*29) was also identified, which were ruled out because the genes were recessive inheritance and only one variant of each gene was identified in the patient. Moreover, the phenotype of the patient was not consistent with the genes (**Supplementary Table S1**).

DISCUSSION

In this study, we described a Chinese girl characterized by severe intellectual disability, delayed motor development, seizures, microcephaly, and special facial features for the first time. Using WES, we identified a *de novo* heterozygous truncated mutation (c. 930C > A, p.Tyr310*) in *DYRK1A*, which was responsible for autosomal dominant mental retardation-7 (MRD7). This mutation was predicted to cause premature truncation of the DYRK1A protein and presumably impairment of kinase activity, thereby contributing to phenotype in the patient. The highly conserved dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK) family includes the members of *DYRK1B*, *DYRK2*, *DYRK3*, and *DYRK4*. As a member of the DYRK subfamily, *DYRK1A* contains a kinase domain, located centrally in the protein, spanning from amino acid residue 158 to residue 479. Meanwhile, *DYRK1A* comprises two nuclear localization signals at the N-terminus (NLS) (Soundararajan et al., 2013), a DYRK homology (DH)-box, a PEST domain, a speckle-targeting signal (STS), a histidine repeat, and a region rich in serines and threonines at the C-terminus (Song et al., 1996). During the early embryonic development in Drosophila, *DYRK1A* is expressed in neuroepithelial progenitor cells defined the transition step from proliferations to neurogenic divisions (Hammerle et al., 2002). Remarkably, mice with heterozygous mutation of *DYRK1A* showed a significant body size reduction

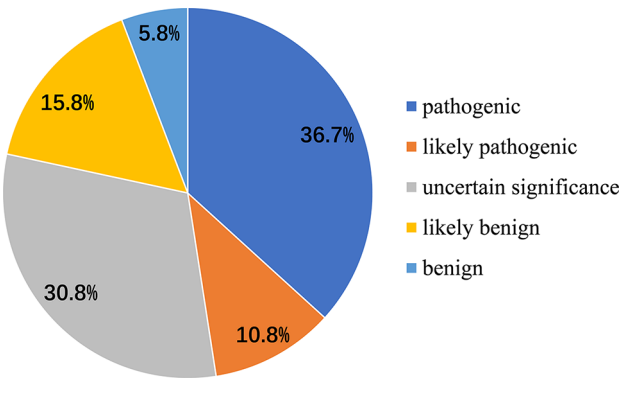


FIGURE 3 | Classification of mutations in *DYRK1A* associated with MRD7 Pathogenic variants were seen in 36.7%, likely pathogenic variants were 10.8%, benign variants were found in 5.8%, and likely benign variants and uncertain significance variants were 37/120 (30.8%) and 19/120 (15.8%), respectively.

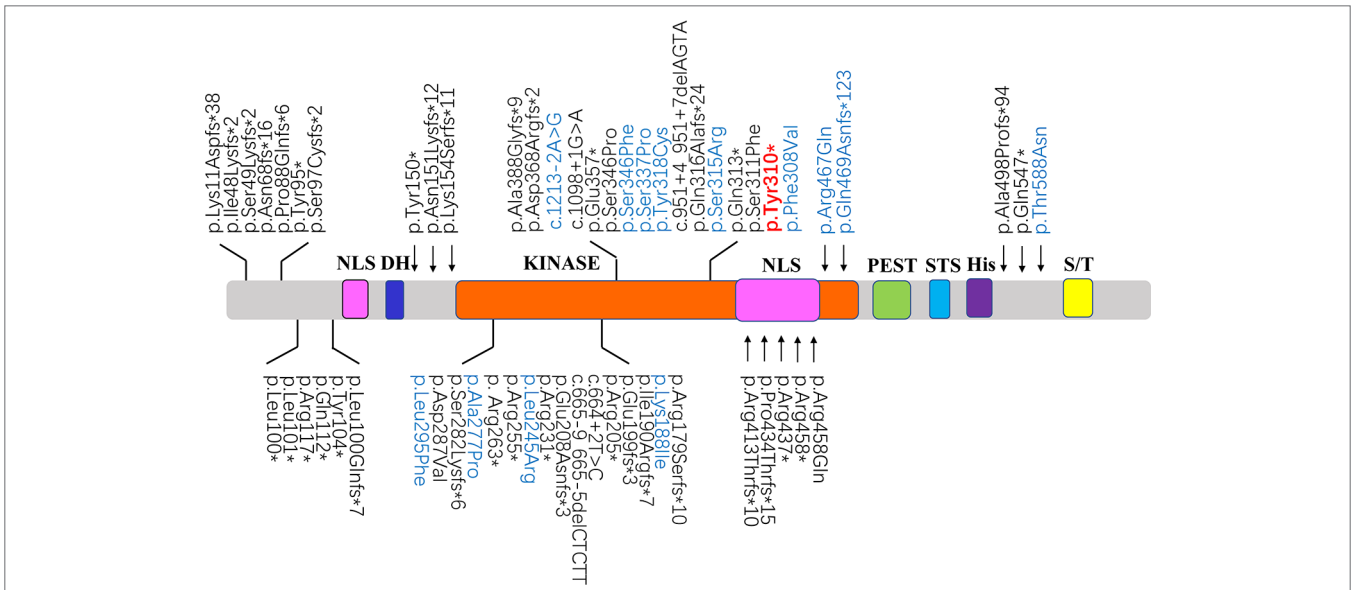


FIGURE 4 | Schematic diagrams showing structure of DYRK1A. The recently identified pathogenic variants and likely pathogenic variants were shown in black and blue, respectively. The position of the mutation identified in this study are shown in red. NLS, nuclear localization signal; DH, DYRK homology domain; KINASE, protein kinase domain; PEST, domain enriched in proline, glutamic acid, serine, and threonine residues; STS, the speckle-targeting signal; His, histidine repeats; S/T, serine and threonine-rich region.

and a decreased size of the brain than those wild type mice, and mice with one functional copy of the *DYRK1A* gene also revealed motor defects, altered behaviors, and intrauterine growth restriction (Fotaki et al., 2002; Fotaki t al., 2004; Martinez de Lagran et al., 2007). Tejedor et al. reviewed the role of *DYRK1A* gene in neurogenesis and characterized the protein as a regulator of multiple neurodevelopmental procedures, itemizing many possible interacting proteins and/or substrates (Tejedor and Hammerle, 2011).

In human, the *DYRK1A* gene located in 21q22.2 on chromosome 21 maps to the critical region of Down syndrome (DS) (Guimera et al., 1996). In 2008, Moller et al. firstly described two unrelated patients with truncation of *DYRK1A* lead to microcephaly, developmental delay, feeding difficulty, and epilepsy, probably through restrain neural differentiation (Moller et al., 2008). Courcet et al. identified a 69 kb deletion and a frameshift mutation (c.290_291delCT; p.Ser97Cysfs*98) in *DYRK1A*. While microcephaly, language delay, and seizures were considered as fixed features, *DYRK1A* mutation was found in 1/70 patients (1.4%). Hence, the authors suggested that *DYRK1A* analysis could be mostly considered when patients present with the phenotype (Courcet et al., 2012).

The strikingly similar features of previously reported individuals with MRD7 were intrauterine growth retardation (IUGR), microcephaly, severe intellectual disability, brain abnormalities (MRI), global developmental delay, speech and motor delay, seizures, behavioral issues, feeding difficulties, broad-based gait, and dysmorphic facies (Bronicki et al., 2015; Luco et al., 2016; Widowati et al., 2018). In this report, the patient with the *de novo* heterozygous mutation of *DYRK1A* (c.930C > A, p.Tyr310*) displayed typically clinical features, which are closely resembling the syndrome.

We also analyzed 120 disease-associated variants obtained from the ClinVar database and the formerly reported Medline search (**Supplementary Table S2**). These reported variants were all absent from the parental genomes, thereby proving their *de novo* occurrence. Uncertain significance variants and likely benign variants were found in 37/120 (30.8%) and 19/120 (15.8%). Benign variants were observed in 7/120 (5.8%). Remarkable, 36.7% was recognized as pathogenic variants (44/120) likely pathogenic variants were seen in 13/120 (10.8%) (**Figure 3**). Among the pathogenic and likely pathogenic variants(**Figure 4**), the disruptive mutations included nonsense, frameshift, and splice site variants, which affected residues widespread within the protein, all predicted premature stop codons leading to a potential loss of function.

Technological improvement in high-throughput sequencing has satisfied clinical requirements and thus hastened the identification of novel pathological variants in *DYRK1A* gene (Yamamoto et al., 2011; Ji et al., 2015; Ruaud et al., 2015; van Bon et al., 2016). As compared to conventional sanger sequencing, WES provides an effective alternative method that aids genetic diagnosis especially in cases with overlapping features among neurogenesis. Additionally, the clinical utility of WES would be promoted significantly within the near future as the cost is reduced.

Concluding Remarks

In conclusion, we identified a novel nonsense mutation in *DYRK1A* in a Chinese family, expanding the mutation spectrum of MRD7. Meanwhile, we provide a review of the formerly reported cases to summarize variations in *DYRK1A* gene, which can provide convenience for clinical application. And also, we emphasize the value of WES for genetic diagnosis in rare diseases.

DATA AVAILABILITY STATEMENT

All datasets for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The study was reviewed and approved by the Ethics Committee of Nanjing Maternity and Child Health Care Hospital in China. The parents of the patient provided written informed consent for publication of medical data and for molecular genetic analysis of the related gene.

AUTHOR CONTRIBUTIONS

FQ and BS performed the experiments, interpreted the data, and wrote the manuscript. CW, YW, and RZ collected clinical information and provided genetics counseling. GL provided patient samples and determined the phenotype based on the clinical criteria. LM performed experiments and analyzed the data. PH and ZX designed the study and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01194/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Newborn Screening for Spinal Muscular Atrophy in China Using DNA Mass Spectrometry

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Background: Spinal muscular atrophy (SMA) is the most common neurodegenerative disorder and the leading genetic cause of infant mortality. Early detection of SMA through newborn screening (NBS) is essential to selecting pre-symptomatic treatment and ensuring optimal outcome, as well as, prompting the urgent need for effective screening methods. This study aimed to determine the feasibility of applying an Agena iPLEX SMA assay in NBS for SMA in China.

Methods: We developed an Agena iPLEX SMA assay based on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and evaluated the performance of this assay through assessment of 167 previously-genotyped samples. Then we conducted a pilot study to apply this assay for SMA NBS. The *SMN1* and *SMN2* copy number of screen-positive patients were determined by multiplex ligation-dependent probe amplification analysis.

Results: The sensitivity and specificity of the Agena iPLEX SMA assay were both 100%. Three patients with homozygous *SMN1* deletion were successfully identified and conformed by multiplex ligation-dependent probe amplification analysis. Two patients had two *SMN2* copies, which was correlated with severe SMA type I phenotype; both of them exhibited neurogenic lesion and with decreased muscle power. Another patient with four *SMN2* copies, whose genotype correlated with milder SMA type III or IV phenotype, had normal growth and development without clinical symptoms.

Conclusions: The Agena iPLEX SMA assay is an effective and reliable approach for population-based SMA NBS. The first large-scale pilot study using this assay in the Mainland of China showed that large-scale implementation of population-based NBS for SMA is feasible.

Keywords: spinal muscular atrophy, newborn screening, Agena iPLEX assay, MassARRAY genotyping, *SMN1*, *SMN2*

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder and the leading genetic cause of infant mortality, with an incidence around 1 in 10,000 live births (Verhaart et al., 2017a; Verhaart et al., 2017b). About 95% of the SMA-affected patients are caused by the homozygous deletion of the survival of motor neuron 1 (*SMN1*) gene, resulting in deficiency of SMN protein (Lefebvre et al., 1995; Lefebvre et al., 1997). The *SMN1* gene is located in chromosome 5q13 containing *SMN2*. *SMN1,* and *SMN2* are highly homologous differing by only five nucleotides (Lefebvre et al., 1995; Cartegni and Krainer, 2002). The number of *SMN2* copies correlates with the severity of phenotypes. To be more specific, larger number of *SMN2* copies are associated with milder phenotypes (Mailman et al., 2002; Calucho et al., 2018).

Based on the age of onset and clinical severity, SMA is classified into five subtypes (0–IV) (Verhaart et al., 2017b). 60% of SMA-affected patients have severe SMA type I, and these patients usually progress to respiratory failure and even die within the first 2 years of life if they are left untreated (Kolb et al., 2017; Verhaart et al., 2017b). Early medical intervention on preventing motor neuron loss could generate maximal benefits to SMA-affected patients (Mendell et al., 2017). However, most patients are currently diagnosed with significant delay (Lin et al., 2015). Therefore, early detection of SMA through newborn screening (NBS) prior to the onset of neurodegeneration is essential to providing pre-symptomatic treatment and ensuring optimal outcome (Phan et al., 2015).

Given the serious clinical phenotype and high frequency of SMA, especially novel therapies drastically altering the course of disease and prolonging survival. Currently, SMA has been included into the recommended universal screening panel in the United States, and some states have lunched the SMA NBS program (Kanungo et al., 2018; Fabie et al., 2019). Furthermore, nationwide NBS for SMA are now being evaluated in other countries (Saffari et al., 2019). Thus, including SMA into NBS program has been highlighted recently, and NBS methods for SMA are needed more than ever.

Unlike conventional NBS practices, SMA does not have a specific biochemical analyte, thus DNA testing is undoubtedly the best approach. Several methods have been developed to detect the *SMN1* genotype in dried blood spot (DBS) samples, including real-time PCR (RT-PCR) (Pyatt and Prior, 2006; Taylor et al., 2015), competitive oligonucleotide priming PCR (Kato et al., 2015; Ar Rochmah et al., 2017), liquid microbead arrays (Pyatt et al., 2007), high-resolution melting analysis (Dobrowolski et al., 2012), and droplet digital PCR (ddPCR) (Vidal-Folch et al., 2018). However, standard methods for SMA NBS are still lacking. An ideal NBS assay must be cost-efficient, with high throughout, and easy to perform and automate. The Agena iPLEX assay is a MassARRAY genotyping platform based on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Calvo et al., 2010). It is one such platform that has successfully been utilized for identification of thousands of gene variations (Gabriel et al., 2009). The MassARRAY assay consists of an initial locus-specific PCR reaction, followed by single base extension using dideoxynucleotide terminators of a variant-specific oligonucleotide primer which anneals immediately upstream of the target site (Jurinke et al., 2004). Multiplexing application of the MassARRAY system, allowing for simultaneous assessment of multiple single-nucleotide polymorphisms (SNPs)/variants, is a cost-efficient way to augment high-throughput genotyping output. Herein, we described the development of an Agena iPLEX SMA assay to detect the homozygous *SMN1* deletion, which is applicable to SMA NBS. The performance of this assay was systemically studied and further evaluated by applying it to screen 29,364 newborns.

MATERIALS AND METHODS

Subjects and Samples Preparation

To evaluate the sensitivity and specificity of the Agena iPLEX SMA assay design of this study, 167 newborns in September 2017, including one SMA case and 166 normal controls, were enrolled. The copy numbers of *SMN1* and *SMN2* genes of these 167 newborns were genotyped by using multiplex ligation-dependent probe amplification analysis (MLPA) method, which is the clinical golden-standard SMA approach. Pilot NBS was then performed to validate the application possibility of our design. The target screening sample size was calculated based on the incidence of 1:17,181 in Taiwan of China. Using PASS software (package 11.0), with permissible error of 0.03% and a two-sided 95% confidence interval, the necessary sample size needed to achieve statistical significance was 19,046 (**Supplementary File 1**). In order to increase the screening reliability, a total of 29,364 newborns from six hospitals were recruited for pilot NBS between March 2018 and June 2018. All newborns with expanded NBS results within the reference range. The participating hospitals include Children's Hospital, Zhejiang University School of Medicine (n = 14,686), Quanzhou Maternity and Children's Hospital (n = 2917), Huaihua Maternal and Child Health Care Hospital (n = 2905), Jining Maternal and Child Health Family Service Center (n = 2951), Yancheng Maternity and Child Health Care Hospital (n = 2965), and Anhui Women and Child Health Care Hospital (n = 2940). Blood samples were collected by heel stick and spotted on Whatman 903 filter paper. DBS samples of 167 and 29,364 newborns were sent to Hangzhou Genuine Clinical Laboratory (Hangzhou, Zhejiang, China) for SMA testing after the center had completed NBS. Genomic DNA was extracted from the DBS samples using a Qiagen Blood DNA mini kit (Qiagen, Hilden, Germany), and then preserved at −20°C refrigerator after measuring the concentration. DNA quality and quantity were confirmed using a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The concentration of DNA extracted from DBS was 12.513 ± 5.838 ng/μl. The project was approved by the Ethical Committee of Children's Hospital, Zhejiang University School of Medicine. Written informed consents were obtained from parents of all the infants.

Design of the Agena iPLEX SMA Assay

Due to the high similarity of *SMN1* and *SMN2* genes, two well-known positions (c.840 and c.1155) were used as targets for designing PCR and single-base extension (SBE) primers (**Figure 1**). The iPLEX assay consists of a target-specific PCR reaction, followed by SBE using molecular weight-modified dideoxynucleotide terminators of an extension primer which anneals immediately upstream of the polymorphic site of interest. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, the distinct mass of the extended primer identifies the SNP allele. The nucleotide of c.840 position is C and T in *SMN1* and *SMN2* genes, respectively. The nucleotide of c.1155 position is G and A in *SMN1* and *SMN2* genes, respectively. PCR and SBE primers (patent applications in progress) were designed for these two variants of *SMN1* and *SMN2* genes by using MassARRAY Assay Design 3.1 software (Agena, San Diego, CA) with 80 ≤ amplicon length (bp) ≤120 and 4,300 ≤ Mass Range (Da) ≤ 9,400. One PCR primer pair was designed for amplifying exon 7 of these two genes, and another was for exon 8. Beside these two variants of *SMN1* and *SMN2* genes, we also designed other polymorphic markers for sample identification (data not shown).

MassARRAY-Based Genotyping

In the multiplex PCR reaction, DNA extracted from DBS was used to amplify an approximately 100 bp region targeting the two *SMN1/SMN2* variants of interest, including the c.840C/T and c.1155G/A. The 5 μl reaction containing 1 × PCR buffer (Agena), 2 mM $MgCl_2$, 100 nM each amplification primer, 500 μM dNTPs, 25 ng DNA, and 1 U PCR enzyme (Agena). PCR conditions were 94°C for 4 min, followed by 45 cycles of 94°C for 20 s, 60°C for 30 s, 72°C for 60 s and a final incubation at 72°C for 3 min. Following the PCR, unincorporated dNTPs were inactivated by the addition of shrimp alkaline phosphatase (SAP) to the PCR reaction product [7 μl reaction containing 0.24× SAP buffer (Agena) and 0.07 U/μl SAP (Agena)]. Following SAP treatment, SBE onto the mutation site using the extension primers and assay-specific iPLEX terminator nucleotide mixes were performed (9 μl reaction containing 0.222× iPLEX buffer (Agena), 9 mM each iPLEX terminator nucleotide (Agena), 0.5–1 μM each extension primer (primer adjustment according to the primer mass based on regression method) and 1× iPLEX Pro DNA polymerase (Agena)]. PCR conditions were 94°C for 30 s, followed by 40 cycles of [94°C for 5 s, then 5 cycles of (58°C for 5 s, 80°C for 5 s)], and a final incubation at 72°C for 3 min. The mass spectrum from time-resolved spectra was retrieved by using a MassARRAY mass spectrometer (Agena), and each spectrum was then analyzed using SpectroTYPER software (Agena) to perform the genotype calling.

MLPA Analysis

The *SMN1* and *SMN2* copy number were determined using MLPA [SALSA MLPA probemix P060 SMA (MRC-Holland, Amsterdam, Netherlands)]. The MLPA assay was performed according to the manufacture's instruction. The PCR products were detected by ABL 3500XL capillary electrophoresis (Applied Biosystems, Foster City, CA, USA). The data were analyzed using the Coffalyzer software (version 3.5) to determine potential CNV (copy number variations) of exons. In brief, the dosage quotient values of 0, 0.4–0.65, 0.8–1.2, 1.3–1.65, and 1.75–2.15 indicates homozygous deletion, heterozygous deletion, normal copy number, heterozygous duplication, and homozygous duplication, respectively (**Supplementary files 2** and **3**).

RESULTS

Validation Study

The Agena iPLEX SMA assay was validated in a double-blind testing of 167 previously-genotyped DBS samples with known *SMN1* and *SMN2* copy numbers. There was a clear distinction between positive samples with homozygous *SMN1* deletion and normal individuals (**Figure 2**). The only case of SMA-affected patient was accurately identified from 167 samples of different genotypes, and the analytical results showed 100% concordance. Thus, the sensitivity and specificity of this screening assay were both 100%.

NBS for SMA

A total of 29,364 individuals were screened, and three newborns with SMA were identified. All these positive patients were confirmed to have homozygous deletions of *SMN1* exons 7 and 8 by MLPA, yielding an incidence of 1:9788 (**Figures 3** and **Figure 4**). Among which two patients (patients 1 and 2) had

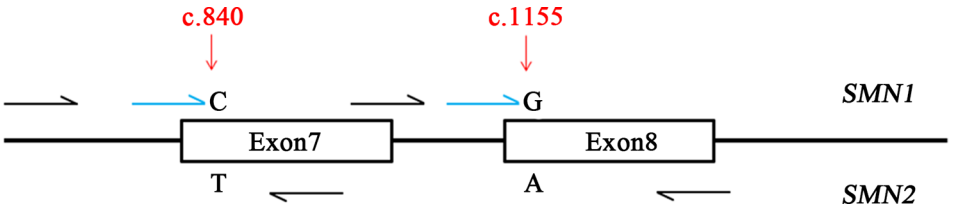


FIGURE 1 | Design of the Agena iPLEX spinal muscular atrophy (SMA) assay. The iPLEX assay consists of a target-specific PCR reaction, followed by single-base extension using molecular weight-modified dideoxynucleotide terminators of an extension primer which anneals immediately upstream of the polymorphic site of interest two well-known positions of *SMN1* and *SMN2* genes (c.840 and c.1155) were used as targets for designing PCR and single-base extension primers. Black and blue colors indicate multiplex PCR and extension primers, respectively.

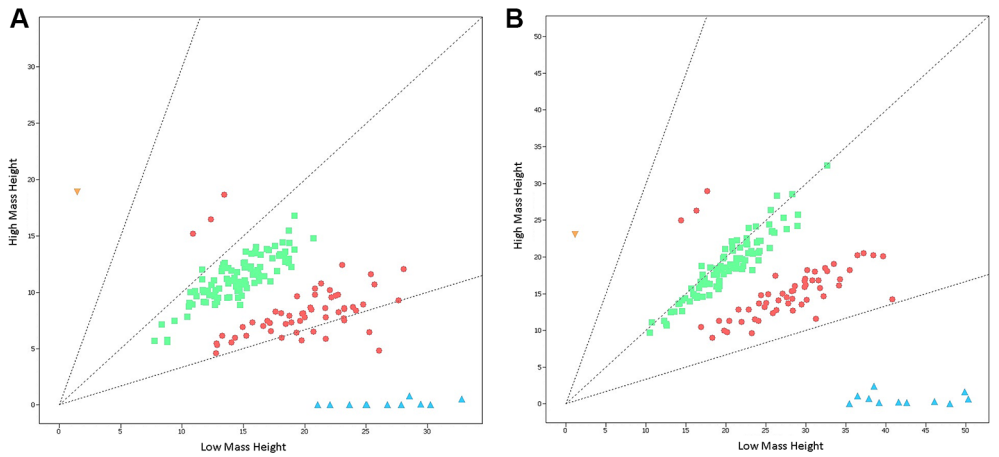


FIGURE 2 | Cluster plots of 167 previously-genotyped samples detected by the Agena iPLEX SMA assay. **(A)** Cluster plots of samples at the nucleotide of c.840 in exon 7 of *SMN1* and *SMN2* genes. **(B)** Cluster plots of samples at the nucleotide of c.1155 in exon 8 of *SMN1* and *SMN2* genes. Low mass height represents the signal strength of *SMN1*, while high mass height represents the signal strength of *SMN2*. The yellow triangle (*SMN1:SMN2* = 0:2) indicate SMA-affected positive sample with homozygous *SMN1* deletion. The red circles (*SMN1:SMN2* = 2:1 or 1:2), the green squares (*SMN1:SMN2* = 2:2), and the blue triangles (*SMN1:SMN2* = 2:0) indicate normal non-homozygous deletion samples.

two *SMN2* copies, which was correlated with severe SMA type I phenotype. Both patients exhibited neurogenic lesion and showed decreased muscle power. While another patient (patient 3) had four *SMN2* copies, with genotype correlated with milder SMA type III or IV phenotype; the patient had normal growth and development without symptoms during the latest follow-up visit (**Table 1**).

DISCUSSION

The Agena iPLEX assay is a robust platform for the detection of many genetic variations, which has been approved for clinical diagnosis. In this study, through taking full advantage of the homologous sequences of *SMN1* and *SMN2*, we translated deletions to mutations and successfully established an Agena iPLEX SMA assay to detect homozygous *SMN1* deletion. The sensitivity and specificity of the Agena iPLEX SMA assay were both 100%. Then, we conducted a pilot study to apply this assay in SMA NBS. Three patients having SMA with homozygous deletions of *SMN1* exons 7 and 8 were successfully identified and diagnosed by MLPA. The sensitivity of this assay applied in NBS was 95%, because this assay detected only 95% of homozygous *SMN1* deletion mutations.

Although there are several technologies available for SMA NBS, whereas each method has its limitations. For example, ddPCR is relatively costly and the instrument is scarce; liquid microbead array is rarely used in less developed regions; RT-PCR requires normalization or standard curves; competitive oligonucleotide priming PCR involves multiple steps of post-PCR manipulations leading to labor intensive; and high-resolution melting analysis requires an experienced researcher to optimize the reaction conditions and analyze the data. Therefore, we explored the use of Agena iPLEX SMA assay for the identification of homozygous *SMN1* deletion. This assay is automated with easier operation, and it is capable to analyzing larger batches of samples daily. Meanwhile, the results are easy to interpreted and even implemented in small/medium-sized NBS laboratories. Moreover, many quality reference materials such as gender and SNPs can be built in for sample traceability. Furthermore, the Agena iPLEX SMA assay has great adaptability with DBS specimens, and previous studies have demonstrated the successful use of Agena iPLEX assay for Fabry NBS (Lu et al., 2018). Last but not least, the Agena iPLEX SMA assay has the advantage of flexibility in that more diseases can be incorporated into the current assay, such as integrated severe combined immunodeficiency, the first DNA-based NBS condition that was detected by measuring the T-cell receptor excision circles, for prospective molecular screening. Thus, the Agena iPLEX SMA assay has great potential to be widely applied in NBS for SMA.

NBS is tasked with differentiating affected patients from healthy individuals, however, some carriers may be identified in actual work. The identification of carriers is problematic because it increases unnecessary stress to the parents. The approach in this study was designed to exclusively detect only homozygous *SMN1* deletion and the unwanted detection of SMA carriers can be effectively avoided. Of note is that a small proportion (5% of total) of *SMN1* intragenic variants cannot be detected through the current Agena iPLEX SMA assay, which needs to be clarified during the process of informed consent.

There were several pilot studies on NBS of SMA and the feasibility of DNA-based NBS for SMA has been demonstrated. For instance, Jennifer et al. used a multiplex TaqMan RT-PCR to screen 3826 newborns in New York. In this research, one neonate with SMA was successfully detected and the study was accepted by these families (Kraszewski et al., 2018). Chien et al. (2017) used a RT-PCR combined with ddPCR as second-tier testing screened 120,267 newborns in Taiwan of China, 15 newborns

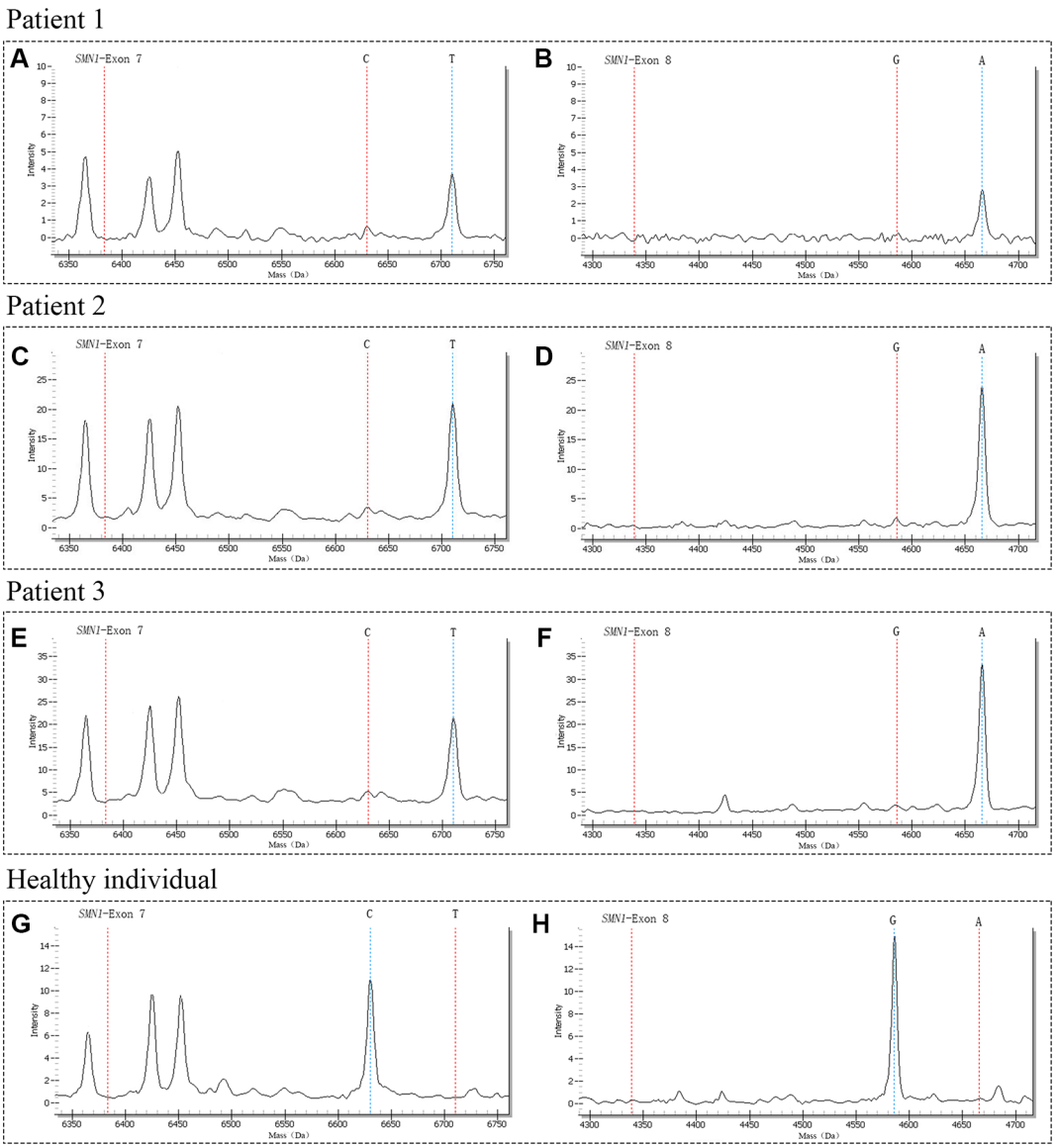


FIGURE 3 | The mass spectra of SMA-affected positive patients and healthy individual. Patient 1 had only one peak at 6710.3 Da **(A)** and 4665.9 Da **(B)** respectively, indicating the homozygous deletions of *SMN1* exons 7 and 8; Patient 2 had only one peak at 6710.3 Da **(C)** and 4665.9 Da **(D)** respectively, indicating the homozygous deletions of *SMN1* exons 7 and 8; Patient 3 had only one peak at 6710.3 Da **(E)** and 4665.9 Da **(F)** respectively, indicating the homozygous deletions of *SMN1* exons 7 and 8; The control of healthy individual had only one peak at 6630.3 Da **(G)** and 4586.0 Da **(H)** respectively, indicating with normal copy number of *SMN1*. The red vertical dotted lines on the left indicate the mass of unextension primer, the red and blue vertical dotted lines on the right indicate the mass of primer after extension.

were tested positive for primary screening, and 7 patients were finally diagnosed as SMA. Another study conducted by Thomas et al. using liquid microbead arrays screened 40,103 DBS samples, four positive patients with SMA were successfully identified (Prior et al., 2010). The current study revealed that Agena iPLEX SMA assay is applicable for SMA NBS; and the frequency of SMA in our population is 1:9788, which is similar to 1:10,026 in Ohio and higher than 1:17,181 in Taiwan of China (Prior et al., 2010; Chien et al., 2017). Therefore, Agena iPLEX SMA assay has high specificity and sensitivity, and at high throughput, is an effective and reliable approach for population-based SMA NBS.

This is the first large-scale study of NBS for SMA in the Mainland of China, two patients with severe SMA type I were detected. However, because the first U.S. Food and Drug Administration approved drug for SMA therapy, Spinraza, which was not available in China, both patients in this series were only eligible for adjuvant therapy such as nutritional and respiratory support (Finkel et al., 2017; Ottesen, 2017; Gidaro and Servais,

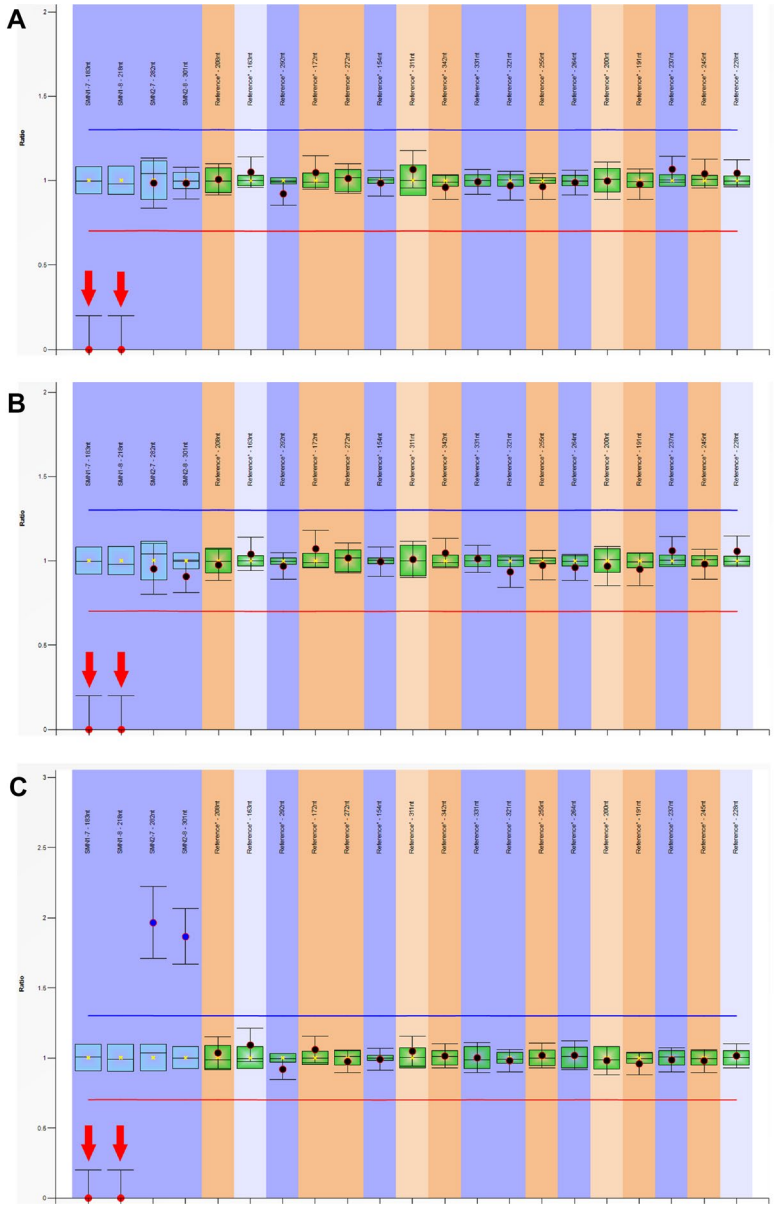


FIGURE 4 | Multiplex ligation-dependent probe amplification (MLPA) analysis of *SMN1* and *SMN2* genes. The dosage quotient (DQ) values of 0, 0.4–0.65, 0.8–1.2, 1.3–1.65, and 1.75–2.15 indicates homozygous deletion, heterozygous deletion, normal copy number, heterozygous duplication, and homozygous duplication, respectively. The homozygous deletions of *SMN1* exons 7 and 8 were detected in three patients, **(A)** patient 1 (*SMN1:SMN2* = 0:2); **(B)** patient 2 (*SMN1:SMN2* = 0:2); **(C)** patient 3 (*SMN1:SMN2* = 0:4).

TABLE 1 | Newborn screening results for spinal muscular atrophy.

Patients no.	Gender	Province/city	Age of onset	MassARRAY-based genotype	*SMN1:SMN2* copies by MLPA	Clinical features (Classification)	Evolution
1	Female	Zhejiang	<3 months	Homozygous deletions of *SMN1* exons 7 and 8	0:2	Exhibited neurogenic lesion and with decreased muscle power (I)	Died at 5 months old
2	Male	Zhejiang	<3 months	Homozygous deletions of *SMN1* exons 7 and 8	0:2	Exhibited neurogenic lesion and with decreased muscle power (I)	Severe
3	Male	Hefei	Not found	Homozygous deletions of *SMN1* exons 7 and 8	0:4	Normal (III or IV)	Well with normal growth and development

MLPA, multiplex ligation-dependent probe amplification.

2019). Consequently, the treatment of these two patients were not effective, one patient died and the other is still under poor prognosis. But more importantly, the detection of SMA is of great significance for the genetic counseling and reproductive guidance of these families, especially due to the limitation that carrier screening has not been widely carried out in China. China has long-term faced many challenges in the screening and treatment of SMA, including low level of public awareness of the disease, lack of genetic testing channels, and effective treatment drugs, as well as lack of standardized long-term follow-up and management (Ke et al., 2019). These challenges have seriously hindered the development of diagnosis and treatment of SMA in China, and have brought many difficulties to SMA-affected patients and their families. Fortunately, Spinraza was introduced in China in April 2019, indicating that the incurable dilemma of SMA should be resolved. Simultaneously with the launch of Spinraza, The Diagnosis and Treatment Center for SMA was founded in China and will provide better medical services for Chinese SMA-affected patients. In addition, the Chinese government is currently paying increasing attention to SMA and has formulated a series of policies which benefit patients and their families. The current study demonstrated the feasibility of SMA NBS, which will undoubtedly advance the progress of SMA in China.

In summary, we developed a simple and automated high-throughput SMA assay for SMA NBS. The assay is rapid (7 h from DNA preparation to data report), inexpensive (the running cost is approximately $3/sample), and with high specificity and sensitivity. Thus, the assay could be recommended as an efficient tool for SMA NBS. The first large-scale pilot study using this assay in the Mainland of China showed that large-scale implementation of population-based NBS for SMA is feasible. We believe this work could advance NBS for SMA.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The project was approved by the Ethical Committee of Children's Hospital, Zhejiang University School of Medicine (reference number: 2018-IRB-077).

AUTHOR CONTRIBUTIONS

YL performed experimental work, paper writing and drafting; C-HL designed the study and participated in paper editing; XY carried out the mutation analysis and paper editing; LZ carried out the genetic tests, mutation analysis and paper editing; CH participated in manuscript preparation; JY, YuS, CY, XC, HH, QM, XS, YaS and ZH followed the patients and collected the clinical data; XH was mentors who designed and guided the research study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01255/full#supplementary-material

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Conflict of Interest: CL is currently an employee of Feng Chi Biotech Corp. LZ is currently an employee of Genuine Diagnostics Company. CH is currently an employee of Zhejiang Biosan Biochemical Technologies Corp.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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A Novel Homozygous Nonsense Mutation p.Cys366* in the WNT10B Gene Underlying Split-Hand/Split Foot Malformation in a Consanguineous Pakistani Family

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Split hand/split foot malformation (SHFM) or ectrodactyly is characterized by a deep median cleft of the hand or foot, hypoplasia or aplasia of the metacarpals, metatarsals, and phalanges. It is a clinically and genetically heterogeneous group of limb malformations. This study aimed to identify the pathogenic variant in a consanguineous Pakistani family with autosomal recessive SHFM. Peripheral blood samples were obtained, DNA was extracted, *WNT10B* coding and noncoding regions were PCR amplified and Sanger sequencing was performed using workflow suggested by Thermo Fisher Scientific. A novel homozygous nonsense variant (c.1098C>A; p.Cys366*) was identified in the *WNT10B* gene in the index patients, which probably explains SHFM type 6 in this family in comparison with similar data from the literature.

Keywords: Pakistani family, SHFM, autosomal recessive mode, gene variant, non-sense mutation

INTRODUCTION

Split-hand/split-foot malformation (SHFM; OMIM:225300) or ectrodactyly is a rare limb developmental disorder. It is characterized by a deep median cleft of the hand or foot corresponding to the central rays of the autopod (1, 2). Phalangeal aplasia and hypoplasia of metacarpals and metatarsals are also signature features of SHFM. It has an incidence rate of ∼1 in 90,000 live births (3, 4). Both individuals and families with SHFM can have a highly variable presentation ranging from minor clefting at the central ray to severe, lobster claw-like deformity (5, 6). It can exist as an isolated entity or as a complex syndrome. Few patients have been reported exhibiting craniofacial defects, ectodermal dysplasia or intellectual disability (5–7). Typically, SHFM is

inherited as an autosomal dominant trait with incomplete penetrance; however, several cases have been reported with an autosomal recessive or X-linked inheritance pattern (7–9). To date, eight types of non-syndromic SHFM were described and mapped to different chromosomes. Four autosomal dominant types have been mapped to chromosomes 2q31 (SHFM-5; MIM 606708), 3q27 (SHFM-4; MIM 605289), 7q21 (SHFM-1; MIM 183600), 10q24 (SHFM-3; MIM 246560), and the X-linked form to Xq26.3 (SHFM-2; MIM 313350) (10–15). The autosomal recessive SHFM-6 (MIM 225300) was mapped to chromosome 12q13.12 harboring the wingless-type MMTV integration site family member 10 (*WNT10B*, MIM 601906) (8, 9). Mutations in several genes have been associated with SHFM including *TP63*, *DLX5*, *DLX6*, *ZAK*, *FGFR1*, *EPS15L1*, and *WNT10B;* however, the clinical features are often indistinguishable (16–18). In the zebrafish, the expression of *TP63*, *DLX5*, *DLX6*, *FGFR1*, and *WNT10B* was shown in the fin's apical ectodermal ridge (AER) cells (16). A similar pattern was also observed in the mouse limbs (19).

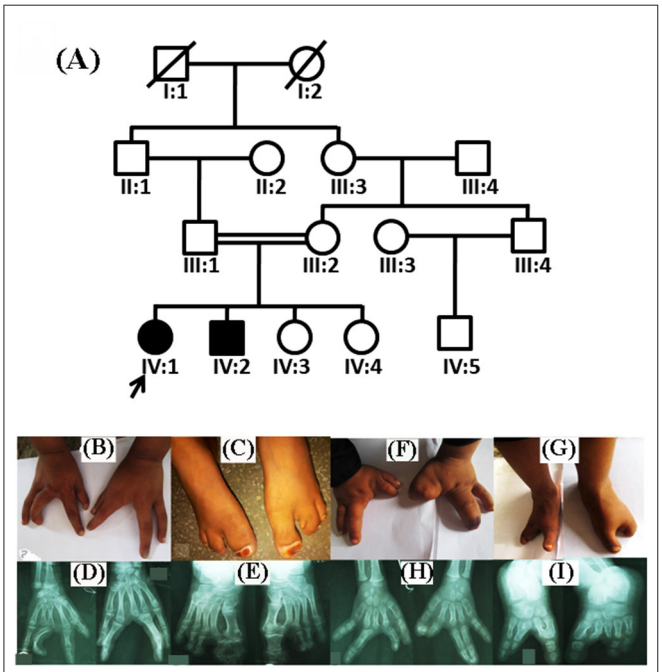


FIGURE 1 | Pedigree analysis and clinical features of split hand/foot malformation (SHFM) observed in the family. **(A)** A consanguineous pedigree showing two affected members (IV:1 and IV:2) in the fourth generation. Affected individuals in the pedigree are shown with shaded symbols and unaffected with open symbols. **(B,C)** 14 years old affected individual (IV:1), showing fusion of proximal phalanx with a median cleft in both hands, and clinodactyly in the left hand. Feet showing complex pre axial syndactyly. **(D,E)** Radiographic examination of the affected individual (IV:1) revealed median cleft in both hands, aplasia, with complex syndactyly, while feet showed complex preaxail syndactyly of the toes and 1st and 2nd metatarsals. **(F,G)** Affected individual (IV:2) having aplasia in both hands and feet, missing digits and complex syndactyly, feet exhibiting typical median cleft phenotypes. **(H,I)** Radiographs of the same affected individual revealed missing digits in both hands and feet, complex preaxial and postaxial syndactyly.

In this study, we described a consanguineous family of Pakistani origin showing SHFM with recessive inheritance. Sanger sequencing of *WNT10B* was used to possibly detect a novel homozygous nonsense mutation that co-segregating with SHFM phenotype within the studied family. We provided a brief phenotype comparison of this family to other cases described in the literature.

CASE PRESENTATION

Methods

Ethics and Consent Approval

The study design and protocol were approved by the Institutional Review Board (IRB) at Quaid-i-Azam University Islamabad Pakistan, the Ethical Review Committee (ERC) of Peking Union Medical College (Beijing, China), and China Medical University (Shenyang, China). For minors, written informed consent was signed by their parents.

Patients and DNA Sample Collection

The two index patients (IV:1, IV:2) of Pakistani origin were children from a consanguineous marriage (**Figure 1A**). Blood samples were collected from individuals III:1, III:2, IV:1, IV:2, and IV:3. The QIAquick DNA extraction kit (QIAamp, Qiagen, Valencia, CA, USA) was used for genomic DNA extraction. The DNA quantity and quality were assessed using nanodrop-2000 spectrophotometer (Thermo Scientific, Schaumburg, IL, USA).

Sanger Sequencing of *WNT10B*

Genomic sequence of *WNT10B* gene, including exons, introns, 5′ untranslated region (UTR) and 3′ UTR, was retrieved from the University of California Santa Cruz genome database browser (UCSC; http://genome.ucsc.edu/). Oligonucleotide primer pairs of the five exons in WNT10B were designed with Gene Runner software (version 5.0.69 Beta; Hastings Software, Inc., Hastings, NY) (**Table 1**). For all family members, all coding and noncoding regions of the *WNT10B* was PCR-amplified in 20 μL reaction volume with 10 pMol of each primer pair and sequenced by Sanger's method after purification. Standard sequencing protocol was followed using BigDye® Terminator v3.1 and Cycle Sequencing Kit (Applied Biosystems, USA). BioEdit tool was applied to analyze and detect mutation in sequenced data using NCBI GeneBank accession number [NG_023347.1] as a reference for alignment. In addition, 200 ethnically-matched

TABLE 1 | Primers for *WNT10B* PCR amplification.

Primers	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product length (bp)
WNT10B-1	ggagagggtgtgtgagagag	tggctctctatgcgtctctg	598
WNT10B-2	ctgaacccgcatcaagtctc	gtcggtgtttctatggcctg	187
WNT10B-3	caggccatagaaacaccgac	ctagggtaggagagcaggga	583
WNT10B-4	ttacctccaccatcacaccc	gcctctcaaactctaaccagg	471
WNT10B-5	ctccatttgtccctccctgt	ttccagggaccaagagtgac	669

control samples were sequenced to assess the allele frequency of the novel variant.

RESULTS

Clinical and Radiological Examinations

Both patients (IV:1 and IV:2) had a physical examination and X-rays performed by an orthopedic surgeon. Patients during the time of recruitment for genetic analysis were 14 (IV:1) and 12 (IV:2) years of age. Both patients of the family exhibited SHFM phenotype with involvement of hands and feet segregated in an autosomal recessive manner. Parents of the affected patients were normal and healthy. The patient IV:1 had complex syndactyly, clinodactyly, dysplastic hands and feet (**Figures 1B–E**). In contrast, the patient IV:2 had syndactyly in the left hand and both feet, aplasia of the radial ray of hand and hallux valgus deformity in the big toe. The distal phalanx of the middle finger was also missing. Photos of the hands and feet are shown in **Figures 1F–I**. No other dysmorphic features were observed. Both patients had an average intellect and were attending regular school classes with satisfactory performance.

Radiological Examinations

During the radiological evaluation, the patient IV:1 showed an absence of the first metacarpal and multiple phalanges in both hands. Both feet had deep midline cleft and syndactyly **(Figures 1B–E)**. Radiographic examination of Patient IV:2 showed the absence of multiple metatarsals and phalanges in both feet (**Figures 1F–I**). Detailed clinical information of both patients were compared to previously reported SHFM cases as seen in **Table 2**.

Mutation Confirmation

Analysis of Sanger sequencing shown in **Figure 2A** identified a homozygous nonsense variant (c.1098C>A; p.Cys366*) in exon 5 of the *WNT10B* gene in both affected individuals (IV:1 and IV:2) of the family. Their parents were heterozygous for the same variant (**Figure 2B**). This variant leads to premature termination of the 389 amino acid protein in the main WNT domain (**Figure 2C**). Other vertebrate species indicated in **Figure 1D** share similar variant and an overall gene sequence.

In-silico Analysis

The novelty and pathogenicity of the identified variant were predicted using different online *in silico* analysis tools including Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant From Tolerant (SIFT, http://sift.jcvi.org/), Protein Variation Effect Analyzer (PROVEAN, http://provean.jcvi.org/), Mutation Taster (http://www.mutationtaster.org/), Varsome (https://varsome.com/), and Combined Annotation Dependent Depletion (CADD, https://cadd.gs.washington.edu/). Finally, for the interpretation of variants, American College of Medical Genetics and Genomics (ACMG) 2015 guidelines were used (20) (**Table 3**). The variant (c.1098C>A; p.Cys366*) was

TABLE 2 | Clinical features and WNT10B variations detected in the present family and those families reported previously.

DNA variation	Protein variation	Exon location	Major clinical phenotype	Ethnicity
c.994C>T	p.Arg332Trp	5	Syndactyly, Postaxial syndactyly 3rd and 4th fingers with almost fused nail beds, clinodactyly in finger 5, polydactyly type 1.	Turkish (8)
c.986C>G	p.Thr329Arg	5	Pre-axial syndactyly of 1st and 2nd toe, post-axial syndactyly of 3rd and 4th toe, absence of 2nd toe, hypoplasia in 2nd digit, Agenesis of the distal ray at meta-carpophalangeal joint level, fixed flexion contracture of both 4th and 5th digit at the proximal interphalangeal joint level, pre-axial polydactyly type 1.	Pakistani (9)
c.1165_1168delAAGT c.300_306dupAGGGCGG	p.Lys388Glufs*36, p.Leu103Argfs*53	5 3	polydactyly, pre- axial syndactyly, campodactyly, dysplastic hands and cleft feet, hallux valgus deformity of big toe and rudimentary bud of lesser toes.	Pakistani (24)
c.460C>G	p.Gln154*	4	Pre-axial syndactyly of index finger, Mesoaxial type of syndactyly, cleft hand with the absence of a middle finger, Aplasia of the middle finger, missing central toes, missing of great thumb, hallux valgus deformities of big toe.	Pakistani (25)
c.300_306dupAGGGCGG	p.Leu103Argfs*53	3	Syndactyly of the 1st and 2nd toe, hypoplasia of 1st metacarpal, complex fusion of 3rd and 4th finger, bilateral hypoplasia and fusion of the 3rd and 4th finger, claw toe deformity.	Pakistani (25)
c.676C>T c.338-1G>C	p.Arg226* –	4 3	Syndactyly, polydactyly, flexion contracture of the left index finger, dysplasia of the distal/middle phalanges of the right index finger, and dysplasia of the postaxial toes of both feet.	Saudi (12)
c.695_697delACA	p.Asn232del	4	Asymmetric longitudinal deficiency of the central rays of hands, absence of the middle and distal phalanges of the 3rd right and left fingers, delayed ossification of all carpal bones, asymmetric longitudinal deficiency of central rays of feet, fusion of the right 1st and 2nd metatarsals, absence of the left 2nd, 3rd, and 4th metatarsals and their corresponding phalanges, absence of the right 3rd metatarsal and 2nd, 3rd, and 4th proximal, middle, and distal phalanges.	Indian (26)
c.1098C>A	p.Cys366*	5	Proximal phalanx with median cleft in both hands, clinodactyly in left hand. Feet showing complex preaxial syndactyly, median cleft in both hands, aplasia, with complex syndactyly, complex pre-axial syndactyly of the toes and 1st and 2nd metatarsal, complex preaxial and post axial syndactyly in hand and feet.	This study

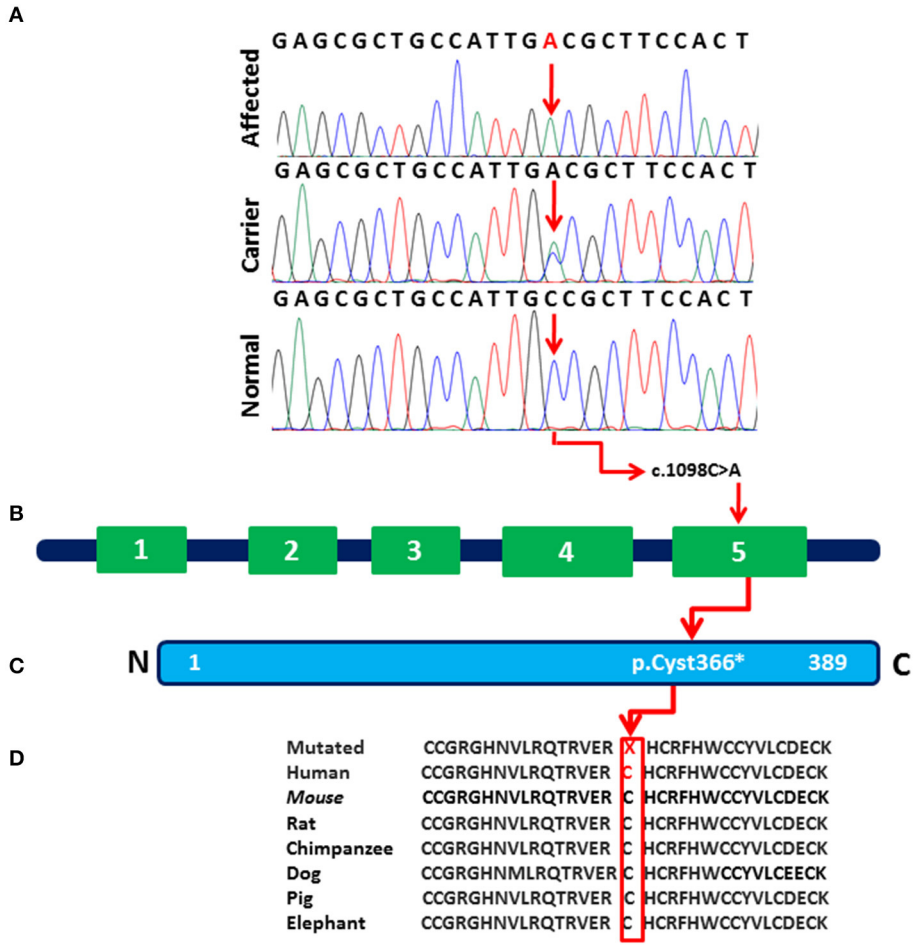


FIGURE 2 | (A) Sequence chromatogram of the *WNT10B* gene showing a homozygous nonsense mutation (c.1098C>A; p.Cys366*) in affected individuals (IV:1 and IV:2), a heterozygous variant in (III:1, III:2, IV:3) and control reference in 200 ethnically-matched normal individuals. **(B)** The exon/intron structure of the *WNT10B* gene along with reported mutation causing SHFM phenotypes. Variant (c.1098C>A) marked with black color represents our index patients. **(C)** Variant c.1098C>A (p.Cys366*) detected in the present study affect the last region (amino acids 367–389) of the *WNT10B* domain. **(D)** The amino acids mutated in the affected individuals are conserved in different species.

not observed in gnomAD (https://gnomad.broadinstitute.org/), 1000 Genomes (http://www.1000genomes.org/), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), and in the Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/) and was not present in 200 ethnically-matched control individuals.

DISCUSSION

In this study, we identified a novel homozygous nonsense variant c.1098C>A in *WNT10B* in a consanguineous family having SHFM. The nonsense variant (c.1098C>A; p.Cys366*) results in a Cysteine amino acid substitution to stop codon at a position 366 which results in a shorter protein formation (21). A number of families has been reported previously from Pakistan having different mutations in the *WNT10B* leading to SHFM phenotypes (22–24). Khan et al. (9), Aziz et al. (24), and Ullah et al. (25), studied several Pakistani consanguineous SHFM families using linkage analysis followed by direct sequencing and identified pathogenic variants in the *WNT10B* gene (9, 24, 25). The Clinical features of affected members in these families exhibited SHFM phenotype, which is inherited in the form of autosomal recessive pattern. The SHFM features observed in our patients show similarities to those reported previously (8, 9, 24, 26). Detailed clinical comparisons of our patients with that reported earlier are presented in **Table 2**.

During organogenesis, Wnt signaling plays a significant role in proximal-distal outgrowth as well as dorso-ventral patterning of limb formations (27). Wnt signaling is essential in cartilage, bone, muscle and joint development (28). Other WNT genes such as *WNT3*, *WNT4*, *WNT6*, *WNT7A*, *WNT7B*, *WNT9B*, *WNT10A*, and *WNT16* as well as *WNT10B* show higher expression throughout the limb bud ectoderm in all phases of mouse limb formation with the exception of the apical ectodermal ridge where Wnt10b expression is only seen at embryonic day 11.5 (E11.5) (29, 30).

A few individuals were described carrying *WNT10B* variants exhibiting developmental tooth alterations, low bone mass or obesity but causality was not established (25, 26, 31, 32). Such phenotypes were not observed in our patients. Until now, 20 different types of (missense, nonsense, splice site, and frameshift) mutation have been identified in the *WNT10B* gene causing different human disorders including obesity, dental anomalies, and SHFM **(Table 4)** (HGMD: http://www.hgmd.org). Among the 20 HGMD reported mutations, only 12 mutations showed associations with SHFM anomalies. In line with previous reports, the *WNT10B* mutation (p.Cys366*) identified in this study is predicted to affect the development of the hands and feet resulting in SHFM type 6. Our findings support the vital function of *WNT10B* in the human skeleton development.

TABLE 3 | Molecular information of homozygous variant on chromosome 12 of *WNT10B* family.

Family ID	Family
Affected individuals	Male,1Female
Transcript ID	NM_003394.4
Gene name	*WNT10B*
Chromosomal location	12q13.12
MIM number	601906
Chromosome position	Chr12:49359950
Nucleotide change	c. 1098C>A
Protein change	p.Cys366*
1000G_ALL	–
ExAC_Freq	–
LRT	0.000/Damaging
gnomAD	–
SIFT Score_pred	–
Polyphen2 Score_pred	–
Mutation Taster Score_pred	–
FATHMM_MKL	0.986/damaging
CADD Score	38/damaging
PROVEAN Score_pred	–
DANN	0.991/damaging
ACMG classification	Pathogenic
Variant status	Novel
Segregation with phenotype	Yes

CONCLUSION

We have reported a novel sequence variant (c.1098C>A; p.Cys366*) in the *WNT10B* gene in a consanguineous Pakistani family presenting with SHFM type 6. This study further extended the spectrum of mutations in the *WNT10B* gene which might be helpful in proper molecular diagnosis and genetic counseling.

WEB RESOURCES

1000 Genomes—http://www.1000genomes.org/;
Exome Variant Server—http://evs.gs.washington.edu/EVS/;
GnomAD—https://gnomad.broadinstitute.org;
dbSNP—http://www.ncbi.nlm.nih.gov/SNP/;
OMIM—http://www.omim.org/;
HGMD—http://www.biobase-international.com/products/hgmd

TABLE 4 | Mutations identified in *WNT10B* gene and associated HGMD disorders identified in different ethnic groups.

Transcript ID	Gene name	DNA variation	Protein variation	Mutation location	Mutation type	HGMD* reported phenotype	Ethnicity of reported families
NM_003394.4	*WNT10B*	c.1098C>A	p.Cys366*	Exon 5	Nonsense	Split Hand/Foot Malformation (Present study)	Pakistan
NM_003394.4	*WNT10B*	c.265G>A	p.Asp89Asn	Exon 3	Missense	Dental Anomalies, isolated	Thailand
NM_003394.4	*WNT10B*	c.475G>C	p.Ala159Pro	Exon 4	Missense	Dental anomalies, isolated	Thailand
NM_003394.4	*WNT10B*	c.994C>T	p. Arg332Trp	Exon 5	Missense	Split hand/foot malformation	Turkey
NM_003394.4	*WNT10B*	c.569C>G	p.Pro190Arg	Exon 4	Missense	Oligodontia	Chinese
NM_003394.4	*WNT10B*	c.632G>A	p.Pro211Gln	Exon 4	Missense	Oligodontia	Chinese
NM_003394.4	*WNT10B*	c.661C>T	p.Arg221Trp	Exon 4	Missense	Split hand/foot malformation	United State
NM_003394.4	*WNT10B*	c.767G>A	p.Cys256Tyr	Exon 5	Missense	Obesity	European
NM_003394.4	*WNT10B*	c.786G>A	p.Trp262*	Exon 5	Nonsense	Oligodontia	Chinese
NM_003394.4	*WNT10B*	c.849C>A	p.Ile283Ile	Exon 5	Missense	Oligodontia	Chinese
NM_003394.4	*WNT10B*	c.851T>G	p.Phe284Cys	Exon 5	Missense	Oligodontia	Chinese
NM_003394.4	*WNT10B*	c.986C>G	p.Thr329Arg	Exon 5	Missense	Split hand/foot malformation	Pakistan
NM_003394.4	*WNT10B*	c.986C>A	p.Thr329Lys	Exon 5	Missense	Split hand/foot malformation	United State
NM_003394.4	*WNT10B*	c.1052G>A	p.Arg351His	Exon 5	Missense	Split hand/foot malformation	Thailand
NM_003394.4	*WNT10B*	c.1087C>T	p.Arg363Cys	Exon 5	Missense	Split hand/foot malformation	Thailand
NM_003394.4	*WNT10B*	c.695_697delACA	p.Asn232del	Exon 4	Frameshift	Split hand/foot malformation	Indian
NM_003394.4	*WNT10B*	c.458_461dupAGCA	p.D115Afs*47	Exon 4	Frameshift	Split hand/foot malformation	Switzerland
NM_003394.4	*WNT10B*	c.293_299dupAGGGCGG	–	Exon 3	Frameshift	Split hand/foot malformation	Pakistan
NM_003394.4	*WNT10B*	c.300_306dupAGGGCGG	p.Leu103Argfs*53	Exon 3	Frameshift	Split hand/foot malformation	Pakistan
NM_003394.4	*WNT10B*	c.338-1G>C	–	Intron 3	Splice site	Split hand/foot malformation	Saudi Arabia
NM_003394.4	*WNT10B*	c.1165_1168delAAGT	p.Lys338Glufs*36	Exon 5	Frameshift	Split hand/foot malformation	Pakistan

HGMD, Human genome mutation database.*

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study design and protocol were approved by the Institutional Review Board (IRB) at Quaid-i-Azam University Islamabad Pakistan, the Ethical Review Committee (ERC) of Peking Union Medical College (Beijing, China), and China Medical University (Shenyang, China). For minors, written informed consent was signed by their parents. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AK and RW participated in the design of the study, performed PCR, gene sequencing, and manuscript writing. AK, MU, MAA, and SH studied family, collected blood samples, and extracted DNA. MAn, WA, and MAl participated in manuscript preparation and XZ collected funds and supervised the study progress. All authors read and approved the final manuscript.

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A 7-Year Report of Spectrum of Inborn Errors of Metabolism on Full-Term and Premature Infants in a Chinese Neonatal Intensive Care Unit

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Inborn errors of metabolism (IEMs) have great repercussions in neonatal intensive care units (NICUs). However, the integrative analysis of the incidence for full-term and premature neonates of IEMs in NICUs have not been reported. In this study, we aimed to estimate the incidence of IEMs in the NICU population so as to better evaluate the impact of IEMs on Chinese NICUs. A total of 42,257 newborns (proportion of premature as 36.7%) enrolled to the largest Chinese NICU center for a sequential 7 years screen, and 66 were diagnosed with IEMs. The prevalence of IEMs in total, full-term, and premature infants was 1:640, 1:446, and 1:2,584, respectively. In spectrum of our NICU, diseases that cause endogenous intoxication like methylmalonic acidemia accounted for 93.9% (62/66), and this ratio was higher in full-term infants with 98.3% (59/60), while the most prevalent disease in premature newborn was hyperphenylalaninemia (50%, 3/6), respectively. The genetic analysis of 49 cases revealed 62 potentially pathogenic mutations in 10 well-documented pathogenic genes of IEMs, among which 21 were novel. In conclusion, differences in incidence and spectrum of full-term and premature births we obtained in NICU will provide diagnostic guidelines and therapeutic clues of neonatal IEMs for pediatricians.

Keywords: newborn screening, neonatal intensive care unit, inborn errors of metabolism, incidence of inborn errors of metabolism, spectrum of genes and mutations

INTRODUCTION

Inborn errors of metabolism (IEM) are a phenotypically and genetically heterogeneous group of disorders caused by a defective enzyme, cofactor, or transporter in a metabolic pathway, leading to metabolic malfunctioning and/or the accumulation of toxic intermediate/terminal metabolites. This group of disorders involve in metabolism of various substances, such as amino acid, organic acidemias, fatty acid, and so on. As new concepts and techniques become available for identifying biochemical phenotypes, the number of such disorders has been constantly increasing up to more

than 1,000 to date (Mak et al., 2013). Though individually rare, the cumulative incidence is about 1:800 in live births (Pampols, 2010). IEMs can present at any age from newborns to the elders. The clinical spectrum of IEM is diversified, either multi-systemic or with single-organ involvement, which can lead to death or severe disabilities if in time intervention were not introduced.

Newborn screening (NBS) for IEMs can prevent death and disability through early diagnosis and pre symptomatic treatment, recognizing as a huge success in field of public health of the last 50 years (Wilcken and Wiley, 2015). With the development of multiple testing and applications of molecular testing, especially the tandem mass spectrometry (MS/MS) technology, NBS has been rapidly improved and widely spread worldwide (Ombrone et al., 2016). In China mainland, MS/MS screening for IEMs using dry blood spot (DBS) launched in 2004 (Han et al., 2008). At present, the strategy has been administrated unbiasedly for normal newborns in some developed regions, whereas subjected exclusively for infants with high-risk inclination known as selective screening in other areas. Conclusions drawn from different studies in this field are controversial due to different selection criteria and target groups. The incidence of IEMs on normal newborns in different cities and regions of China was from 1/1,683 to 1/8,304 (Guo et al., 2018). While in some studies on selective screening of high-risk children with IEMs, the incidence was as high as 1:16 (on 4,981 cases) (Han et al., 2008), 1:51 (on 6,210 cases) (Sun et al., 2011), or 1:53 (on 16,075 cases) (Jiang et al., 2015).

As about 25% of IEMs can have manifestations in the neonatal period, newborns requiring admission to the neonatal intensive care unit (NICU) due to severe symptoms or low survival ability should actively consider the effects of IEMs by general practitioners (Jouvet et al., 2007; Couce et al., 2011; El-Hattab, 2015). Despite few studies from 31 cases in Spain (Couce et al., 2011) and 724 cases in China (Tu et al., 2012) reporting the distribution of IEMs in the corresponding NICU, the evaluation of impacts for IEM in NICU are still largely unveiled. In this study, we aim to estimate the incidence and characteristics of IEMs in full-term and premature neonates in a NICU in mainland China based on the screening results of 42,257 newborns in 7 years. We discovered that the total incidence of IEMs in a Chinese NICU was 1:640; while that in full-term neonates was 1:446 and that in premature ones was 1:2,584. Among the nine kinds of IEMs diagnosed in our study, the most common disease in full-term or all NICU neonates was methylmalonic acidemia (MMA). We found 62 potentially pathogenic mutations (including 21 novel ones) in 10 well-documented pathogenic genes of diagnostic cases of IEMs, and the most prevalent defective gene was *MMUT*. Our results enriched the understanding of the characteristics and genetics of neonatal IEMs and provided related diagnostic and therapeutic clues for neonatal pediatricians.

MATERIALS AND METHODS

Study Participants

All the newborn patients admitted to the NICU of the affiliated BaYi Children's Hospital of Seventh Medical Center of PLA General Hospital between January 1, 2010, and December 31, 2016 were enrolled to conduct biochemical analysis using MS/MS and gas chromatography–mass spectrometry (GC-MS) to screen IEMs. The hospital is a medical center for neonates in North China, attending seriously ill newborns (aged between several hours and 30 days) with low survival ability. A total of 42,257 newborns admitted to the NICU, including 26,750 full-term infants (GA ≥ 37 weeks) and 15,507 premature infants (GA <37 weeks), were enrolled for the study (**Supplementary Table 1**). Informed consent was obtained from the patient's guardians for experimentation. All experiments were conducted in adherence to standard biosecurity and institutional safety procedures. 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.

Tandem Mass Spectrometry Analysis

Dried blood filter papers were collected from neonates fed more than 48 h, and transferred to metabolic screening laboratory in BaYi Children's Hospital on the same day. Blood amino acids and acylcarnitines were analyzed with MS/MS using in butyl-derivatized specimens (Shigematsu et al., 2002). An API 3200 (Applied Biosystems, Foster City, CA, USA) was used for MS/MS.

Gas Chromatography–Mass Spectrometry Analysis

Urine samples of the infants with primary positive result in MS/MS analysis were taken to test the metabolic profiles using GC-MS. Urine metabolome analysis with urease-pretreatment was conducted as reported previously (Kuhara, 2002). A "TRACE GC ULTRA-ISQ" instrument (Thermo Fisher Scientific, San Jose, CA, USA) was used for the analysis.

Biochemical Diagnoses

Biochemical diagnoses of IEMs were based on the results of MS/MS and/or GC-MS indicating obvious specific abnormal metabolites, in accordance with clinical data by physicians who specializing in IEMs.

Genetic Analysis

Genomic DNA was extracted from peripheral blood leukocytes obtained from the patients using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The lists of known inheritable genetic disease–related genes in the panels for captured and targeted next-generation sequencing were described previously (Yang et al., 2013; Chen et al., 2017). The amplified DNA was specifically enriched using a biotinylated capture probe (MyGenostics, MD, USA). Sanger sequencing was used to confirm the mutations. The polymerase chain reaction product was purified using solid phase reversible immobilization beads (Beckman Coulter, Inc.) according to the manufacturer's protocol (He et al., 2010; Wu et al., 2011). The enrichment libraries were sequenced using an Illumina HiSeq 2000

sequencer. Short read mapping and alignment were performed using the Burrows Wheeler Aligner software.

Clinical Data Analysis

The medical records of full-term MMA patients with definite genetic defect were analyzed, and the rates of each symptom were calculated to assess the difference between *MMUT* and *MMACHC* defects in NICU.

Statistical Analysis

All analyses were done using SPSS software, version 19.0. The differences between rates of each symptoms were tested using χ^2 or Fisher exact tests, if appropriate. A P value less than 0.05 indicated a statistically significant difference.

Availability of Data and Materials

The clinical data of the patient is available in the record room of the Seventh Medical Center of PLA General Hospital. The sequencing and mass spectrometry data is available in the laboratory of BaYi Children's Hospital. Basic information of subjects and the genetic analysis and clinical symptom data of the patients are presented in the additional supporting files.

RESULTS

Incidence and Composition of Inborn Errors of Metabolism in a Chinese Neonatal Intensive Care Unit

Among all 42,257 neonates admitted to the NICU, 66 patients were biochemically diagnosed with IEMs. This included 60 IEM patients of 26,750 full-term neonates and 6 IEM patients with IEMs of 15,507 premature neonates. The total prevalence in the NICU was 1:640 (66/42, 257), and the prevalence of full-term and premature cases was 1:446 (60/26, 750) and 1:2,584 (6/15, 507), respectively. Further, 60 full-term cases of IEM [(34 male and 26 female; median age 5 days (0–30 days), median gestation 39.21 weeks (37.00–42.00 weeks), and median birth weight 3,000 g (1,600–4,400 g)] included 46 cases of MMA (26 cases of isolated MMA and 20 cases of combined MMA/homocystinuria), 4 cases of propionic acidemia (PA), 3 cases of urea cycle disorders (UCD), 3 cases of maple syrup urine disease (MSUD), 2 cases of tyrosinemia (Tyr), 1 case of isovaleric acidemia (IVA), and 1 case of very long-chain acyl-CoA dehydrogenase deficiency (VLCADD). Also, six premature cases of IEM [five male and one female; median gestation 33.07 weeks (26.86–36.71 weeks), and median birth weight 2015 g (1,000–3,200 g)] included three cases of phenylketonuria (PKU) and one case each of MMA combined with Hcy, UCD, and glutaric aciduria type II (GAII) (see **Table 1**).

A Genotypic Spectrum of Cases Biochemically Diagnosed With Inborn Errors of Metabolism in a Chinese Neonatal Intensive Care Unit

Of 66 patients biochemically diagnosed with IEMs, 49 were sent for gene analysis, and 62 potentially pathogenic mutations in 10 genes were detected (**Table 2**). Twenty-one (33.9%) of these mutations were found unreported in this study (**Table 2**). Further, the eight types of IEMs associated with these mutations were completely consistent with the biochemical diagnosis. The distribution of mutations in different genes in 49 cases are shown in **Figure 1**. The most prevalently mutated gene was *MMUT* related to the occurrence of isolated MMA.

In 38 MMA cases who underwent genetic analysis, 43 potentially pathogenic mutations were found in three genes (including *MMUT*, *MMACHC*, and *HCFC1*) (**Table 2**). Among 27 *MMUT* mutations, the most common mutation was c.323G > A (p.R108H) in exon 2 which detected in four patients, and most of these mutations (74.1%, 20/27) were identified only in isolated cases (**Supplementary Table 2**).

Fifteen *MMACHC* mutations were identified in 17 cases, and the most common mutation was c.609 G > A (p.W203*), which occurred in 38.2% of all disease alleles in exon 4. The exon 4 of *MMACHC* in this study encompasses the largest number of mutations (66.7%, 10/15) which including two unreported ones (**Table 2**).

TABLE 1 | Incidence of inborn errors of metabolism in neonatal intensive care unit population and case numbers of full-term and premature.

Disease		Incidence in NICU		Case numbers		Proportion (%)	
				Full-term	Premature		
MMA	Isolated	1:899	1:1,625	26	0	71.2	39.4
	With Hcy		1:2,012	20	1		31.8
PA		1:10,564		4	0	6.1	
UCD		1:10,564		3	1	6.1	
MSUD		1:14,086		3	0	4.5	
PKU		1:14,086		0	3	4.5	
Tyr		1:21,128		2	0	3.0	
IVA		1:42,257		1	0	1.5	
VLCADD		1:42,257		1	0	1.5	
GAII		1:42,257		0	1	1.5	
Total		1:640		60	6	100	

TABLE 2 | Gene mutation spectrum of 49 cases of inborn errors of metabolism in Chinese neonatal intensive care unit.

Gene (cases)	Exons	Variant	Type of mutation	Reference PMID/ ClinVar ID※	Allele frequency % (n)	IEMs presentation
***MMUT* (20)**	2	c.323G > A (p.R108H)	Missense	11528502	10.0 (4)	Isolated methylmalonic acidurias
	3	c.729_730insTT (p.D244Lfs*39)	Frame shift	16281286	7.5 (3)	
	5	c.914T > C (p.L305S)	Missense	16281286	5.0 (2)	
	5	c.944dupT (p.Y316Lfs*11)	Frame shift	25863090	5.0 (2)	
	6	c.1106G > A (p.R369H)	Missense	9285782	5.0 (2)	
	6	c.1280G > A (p.G427D)	Missense	16281286	5.0 (2)	
	11	c.1874A > C (p.D625A)	Missense	30712249	5.0 (2)	
	2	c.91C > T (p.R31*)	Nonsense	16435223	2.5 (1)	
	2	c.322C > T (p.R108C)	Missense	16281286	2.5 (1)	
	3	c.424A > G (p.T142A)	Missense	19806564	2.5 (1)	
	3	c.683G > A (p.R228Q)	Missense	9554742	2.5 (1)	
	4	c.755dupA (p.H252Qfs*6)	Frame shift	23430940	2.5 (1)	
	6	c.1107dupT (p.T370Yfs*22)	Frame shift	30098236	2.5 (1)	
	IVS9	c.1677-1G > A	Splicing	16281286	2.5 (1)	
	10	c.1679G > A (p.C560Y)	Missense	16435223	2.5 (1)	
	12	c.2080C > T (p.R694W)	Missense	7912889	2.5 (1)	
	13	c.2179C > T (p.R727*)	Nonsense	16281286	2.5 (1)	
	3	c.428A > G (p.H143R)	Missense	Unreported (17113806)	2.5 (1)	
	3	c.470T > A (p.V157D)	Missense	Unreported (30712249)	2.5 (1)	
	4	c.861C > G (p.Y287*)	Nonsense	Unreported	2.5 (1)	
	4	c.877C > T (p.Q293*)	Nonsense	Unreported	2.5 (1)	
	6	c.1153_1154delTT (p.L385Afs*6)	Inframe deletion	Unreported	2.5 (1)	
	IVS9	c.1677-2A > G	Splicing	Unreported	2.5 (1)	
	10	c.1759T > C (p.Y587H)	Missense	Unreported (15643616, 22614770)	2.5 (1)	
	10	c.1787delA (p.E596Gfs*2)	Frame shift	Unreported	2.5 (1)	
	IVS11	c.1956+1del G	Splicing	Unreported	2.5 (1)	
	13	c.2194G > C (p.A732P)	Missense	Unreported	2.5 (1)	
***MMACHC* (17)**	4	c.609G > A (p.W203*)	Nonsense	16311595	38.2 (13)	Combined methylmalonic aciduria and homocystinuria, cblC type
	2	c.217C > T (p.R73*)	Nonsense	16311595	8.8 (3)	
	4	c.658_660delAAG (p.K220del)	Inframe deletion	16311595	8.8 (3)	
	2	c.271 dupA (p.R91Kfs*14)	Frame shift	16311595	5.9 (2)	
	4	c.567dupT (p.I190Yfs*13)	Frame shift	19370762	5.9 (2)	
	1	c.80A > G (p.Q27R)	Missense	16311595	2.9 (1)	
	3	c.315C > G (p.Y105X)	Nonsense	20631720	2.9 (1)	
	3	c.331C > T (p.R111*)	Nonsense	16311595	2.9 (1)	
	3	c.398_399delAA (p.Q133Rfs*5)	Frame shift	16311595	2.9 (1)	
	4	c.445_446del TG (p.C149Hfs*32)	Frame shift	26287336	2.9 (1)	
	4	c.616C > T (p.R206W)	Missense	16311595	2.9 (1)	
	4	c.666C > A (p.Y222*)	Nonsense	16311595	2.9 (1)	
	4	c.615C > A (p.Y205*)	Nonsense	558292※	2.9 (1)	
	4	c.658A > C (p.K220Q)	Missense	Unreported	5.9 (2)	
	4	c.511delG (p.V171Cfs*39)	Frame shift	Unreported	2.9 (1)	
***HCFC1* (1)**	18	c.4475C > G (p.P1492R)	Missense	373423※	100.0 (1)	Combined methylmalonic aciduria and homocystinuria, cblX type
***PCCA* (2)**	2	c.130_131insAT (p.C44Yfs*3)	Frame shift	Unreported	25.0 (1)	Propionic acidemia
	2	c.131G > T (p.C44F)	Missense	Unreported	25.0 (1)	
	19	c.1746+3G > C	Splicing	Unreported	25.0 (1)	
	—	exon 7-9 deletion	Large deletion	Unreported	25.0 (1)	
***OTC* (2)**	2	c.214G > T (p.E72*)	Nonsense	Unreported	50.0 (1)	Urea cycle disorder (ornithine transcarbamylase deficiency)
	10	c.1016T > G (p.V339G)	Missense	25932215	50.0 (1)	
***ASL* (1)**	8	c.544C > T (p.R182*)	Nonsense	17326097	50.0 (1)	Urea cycle disorder (argininosuccinic aciduria)
	10	c.706C > T (p.R236W)	Missense	17326097	50.0 (1)	
***BCKDHA* (1)**	1	c.108+4A > G	Splicing	Unreported	50.0 (1)	Maple syrup urine disease
	2	c.117dupC (p.R40Qfs*11)	Frame shift	8037208	50.0 (1)	

(Continued)

TABLE 2 | Continued

Gene (cases)	Exons	Variant	Type of mutation	Reference PMID/ ClinVar ID※	Allele frequency % (n)	IEMs presentation
***PAH* (3)**	6	c.611A > G (p.Y204C)	Missense	23430918	16.7 (1)	Phenylketonuria
	6	c.688G > A (p.V230I)	Missense	8268925	16.7 (1)	
	7	c.728G > A (p.R243Q)	Missense	2071149	33.3 (2)	
	7	c.764T > C (p.L255S)	Missense	2014802	16.7 (1)	
	11	c.1199G > A (p.R400K)	Missense	16256386	16.7 (1)	
***FAH* (1)**	6	c.494C > T (p.S165F)	Missense	Unreported	50.0 (1)	Tyrosinemia type 1
	9	c.782 C > T (p.P261L)	Missense	9633815	50.0 (1)	
***IVD* (1)**	1	c.134T > G (p.L45R)	Missense	Unreported (2063866)	50.0 (1)	Isovaleric acidemia
	12	Exon 12 deletion	Large deletion	Unreported	50.0 (1)	

Under the column "Reference," the PMID in () references for a different amino acid change in previously reported positions; ※ means the variant was unreported in PubMed while has been annotated in ClinVar.

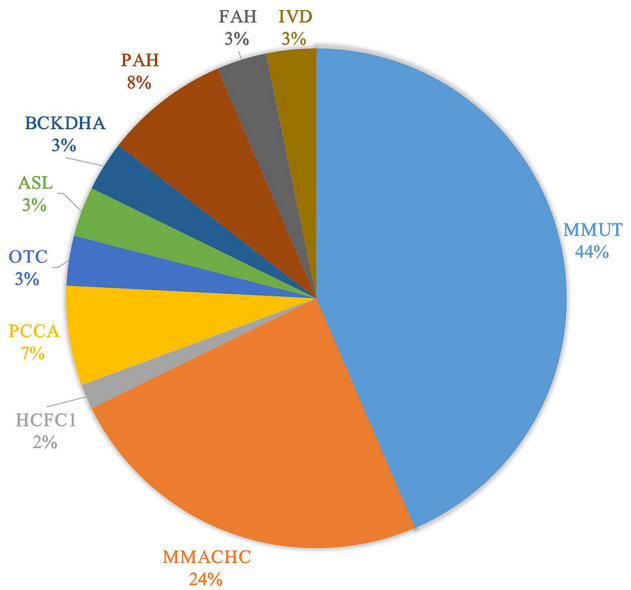


FIGURE 1 | The genotypic spectrum in 49 cases of inborn errors of metabolism in Chinese neonatal intensive care unit.

Clinical Characteristics of Neonates With Methylmalonic Acidemia in the Neonatal Intensive Care Unit

There were 20 *MMUT* and 17 *MMACHC* defects in 38 MMA neonates (80.8%, 38/47) that performed genetic analysis. The differences in clinical characteristics of the above two in the NICU were analyzed, and the only case of premature birth with *MMACHC* defect was excluded because multiple clinical symptoms caused by premature birth are easily confused with MMA.

When admitted to the NICU, 11 cases among the 16 full-term patients with *MMACHC* mutations showed clinical symptoms suspected of IEMs. Further, 4 of 11 were early onset within 0–7 days after birth. According to the results of vitamin B12 loading test, all patients with *MMACHC* mutations were diagnosed with vitamin B12 responsiveness. However, four deaths still occurred during an acute metabolic crisis in the NICU (**Supplementary Table 3**). The common symptoms of 16 *MMACHC* cases in the NICU included respiratory distress/pneumonia (15, 93.8%), poor response or milk refusal (11, 68.8%), metabolic acidosis (9, 56.2%), anemia (9, 56.2%) and encephalopathy (9, 56.2%) (**Table 3**).

All 20 cases with *MMUT* mutations presented metabolic symptoms suspected of IEMs upon admission to the NICU, and 90.0% (18/20) developed these symptoms within 0–7 days after birth. Sixteen cases of 20 showed non-responsiveness to vitamin B12. Among non-responsiveness cases, 14 died during an acute metabolic crisis in the NICU; while four patients who diagnosed with vitamin B12 responsiveness were discharged with a better health condition. The common symptoms of 20 *MMUT* cases in the neonatal period included metabolic acidosis (20, 100%), poor response or milk refusal (19, 95.0%), electrolyte disturbances (17, 85.0%), respiratory distress/pneumonia (16, 80.0%), coagulant function abnormality (12, 60.0%), and jaundice/liver failure (11, 55.0%) (**Table 3**).

DISCUSSION

In this study, we reported the incidence of IEMs in the NICU population including full-term and preterm neonates for 7 years in north China mainland. The disease spectrum of IEMs in a Chinese NICU was obtained based on the data of 42,257 newborns. The prevalence of IEMs in our NICU as 1:640, while the data reported were higher (1–2%) based on much fewer cases (Couce et al., 2011; Tu et al., 2012). This difference might have originated from the distinct standards of enrollment to the NICU. The reported high incidence of IEMs comes from survey of elder infants or children with a higher risk of metabolic disorders, while the present study focused on a population of neonates within 1 month of admission to the NICU. High proportion of premature neonates (36.7%) and more accurate top limit time of admission are the hallmarks of our survey. The incidence of full-term neonates was 1:446 in this study, which was approximately six times more than 1:2,584 in premature neonates and 7–10 times more than 1:3,065 to 1:4,300 in

TABLE 3 | Manifestations of full-term methylmalonic acidemia cases with *MMUT* (20) and *MMACHC* (16) defects in neonatal intensive care unit.

Manifestations	*MMUT* % (n=20)	*MMACHC* % (n=16)	*p* value
Responsiveness to VitB12	**20.0 (4)**	**100 (16)**	**0.0000**
Early onset in 0-7 days	**90.0 (18)**	**25.0 (4)**	**0.0001**
Metabolic acidosis	**100.0 (20)**	**56.2 (9)**	**0.0014**
Electrolyte disturbances	**85.0 (17)**	**31.2 (5)**	**0.0017**
Neonatal death	**70.0 (14)**	**25.0 (4)**	**0.0176**
Coagulant function abnormality	**60.0 (12)**	**25.0 (4)**	**0.0485**
Glucose metabolism dysfunction	**45.0 (9)**	**12.5 (2)**	**0.0671**
Poor response or milk refusal	**95.0 (19)**	**68.8 (11)**	**0.0689**
Hyperammonemia	**20.0 (4)**	**0 (0)**	**0.1131**
Jaundice/liver failure	**55.0 (11)**	**31.2 (5)**	**0.1914**
Anemia	**35.0 (7)**	**56.2 (9)**	**0.3128**
Progressive encephalopathy	**35.0 (7)**	**56.2 (9)**	**0.3128**
Respiratory distress/pneumonia	**80.0 (16)**	**93.8 (15)**	**0.3549**
Skin lesions	**10.0 (2)**	**25.0 (4)**	**0.3738**
Respiratory failure	**15.0 (3)**	**6.2 (1)**	**0.6129**
Congenital heart disease	**15.0 (3)**	**12.5 (2)**	**1.0000**
Infection/sepsis	**15.0 (3)**	**18.8 (3)**	**1.0000**
Mature low birth weight	**15.0 (3)**	**18.8 (3)**	**1.0000**
Myocardial damage	**30.0 (6)**	**25.0 (4)**	**1.0000**
Renal injury	**10.0 (2)**	**6.2 (1)**	**1.0000**
Seizures	**20.0 (4)**	**18.8 (3)**	**1.0000**

normal newborns (Frazier et al., 2006; Shi et al., 2012; Lim et al., 2014; Therrell et al., 2015; Wilcken and Wiley, 2015). In addition, full-term neonates accounted for 90.9% (60/66) of all IEM cases in a Chinese NICU population with the ratio of full-term and premature infants as 1.72:1. Despite several IEMs are known to be associated with premature birth (Carrillo-Carrasco et al., 2012) (Vianey-Saban et al., 2016) (Guibaud et al., 2017), no direct association between IEMs and premature birth can be concluded when reviewing the clinical history of all six cases of premature in our study.

In the spectrum of diseases that we obtained, a total of 53 cases in four kinds of organic aciduria (MMA, PA, IVA, and GAII) accounted for 80.3%, 12 cases in 5 kinds of amino acidic disorders accounted for 18.2%, and 1 case of fatty acid oxidation disorders accounted for the remaining 1.5%. Further, 93.9% (62/66) of these cases belong to the group that caused endogenous intoxication (including MMA, PA, UCD, MSUD, TyrI, IVA, GAII) which can display early onset in neonatal period (Saudubray et al., 2002) and this ratio of endogenous intoxication group was more in full-term infants with 98.3% (59/60), while the dominating type of IEM among preterm patients was PKU (50%, 3/6), a typical kind of late-onset IEMs. However, the most common IEM among full-term neonates was MMA (including isolated type and combined type with Hcy), which was also the most common IEM in all NICU cases, accounting for 71.2% (isolated type as 39.4%, combined type with Hcy as 31.8%) in disease spectrum. PKU and MMA ranked the top 2 of all kinds of IEMs diagnosed in the Chinese population, the proportion of MMA are between 10.2 and 36.6%. (Han et al., 2008; Shi et al., 2012; Guo et al., 2018). Intriguingly, the proportion of MMA was dramatically higher in our NICU. Meanwhile, the incidence of MMA in NBS was reported to be 1:3,920 in Shandong, 1:6,032 in Henan, 1:26,000 in Shanghai/Beijing, and 1:46,532 in Zhejiang, respectively (Hong et al., 2017; Zhou et al., 2018). Strikingly, this ratio in our NICU was 1:899.

The higher incidence of IEMs in sick full-term as opposed to premature newborns doesn't indicate that the full term neonates are more prone to develop IEMs, rather than IEMs were not a frequent cause of premature births, at least in this cohort. Our result likely reflects the fact that maternal and placental metabolism protects infants with IEMs until birth and they present soon after delivery and exposure to stress and feeds. The higher incidence of IEMs in the NICU cohort compared to normal/NBS cohorts reflects the disease burden of early onset intoxication disorders that typically present with severe metabolic ketoacidosis and hyperammonemia in the first hours/days of life. And the detection of more PKU cases in the premature cohort doesn't mean that PKU caused the prematurity, as it is a more common IEM.

Late onset IEMs like PKU, VLCADD often do not have clinical manifestations in the neonatal period. We reviewed these cases of late onset IEMs found in our study, they admitted to NICU because of pneumonia, infection, and other common causes, without obvious symptoms suspected IEMs. These cases have been diagnosis of IEMs by MS/MS screening much earlier before symptoms appear and were immediately subjected to treatment, indicating the advantages of MS/MS screening in early intervention of IEMs.

Detailed analysis of hotspot mutant genes was also conducted in this integrative study. The *MMACHC* mutations were reported to account for the majority of Chinese MMA cases (Guo et al., 2018; Liu et al., 2018); while in the spectrum of IEM genes in NICU we obtained, *MMUT* mutations were more common. This might because of worsened clinical manifestations of *MMUT* defect in the neonatal period. Moreover, many neonatal deaths displaying this defect had not experienced diagnosis, leading to loss of the statistic reports from

surviving cases of elder children. In previous reports, the two most common mutations of *MMUT* were c.729_730insTT and c.323G > A among Chinese patients with different ages of onset ranging from newborn to 8 years (Han et al., 2015). However, in our study based on neonatal patients, the proportion of c.323G > A was slightly higher than c.729_730insTT (**Table 2**). A total of 20 mutations accounting for half of the disease alleles were identified only in one allele, and a large proportion of new mutations (37.0%) and compound heterozygotes (100%) were found, demonstrating the highly pleomorphic nature and genetic heterogeneity of *MMUT* gene as reported. Parallelly, among 15 different *MMACHC* mutations identified in 17 cases, the most common mutation was c.609 G > A (p.W203*) in exon 4, which occurred in 38.2% of all disease alleles. Strikingly, we found that the mutations of *MMACHC* in a Chinese NICU were concentrated at the C-terminal of exon 4 (9 in 15). Furthermore, a hotspot mutation in c.658 (p.220) was found in highly conserved regions of homologous sequences on exon 4 with a frequency of 14.7%.

The limited number of patients included in this study, the high frequency of compound heterozygotes, and the lack of enzymatic studies all rendering the assessment the precise relationship between gene mutations and clinical manifestations difficult. An *MMACHC* mutation [c.609G > A (p.W203*)], which was reported to be the most common hot site associated with early onset (Yu et al., 2015), was found in the homozygous state in two cases (cases 21 and 22 in **Supplementary Table 3**) of the study. Both cases had severe neonatal symptoms such as roaring production of lactic acid. One died of metabolic crisis, and the other was discharged from the NICU in a good condition after active treatment in NICU. In addition, an unreported missense mutation of *MMACHC* as c.658A > C (p.K220Q) was homozygous in case 23 (**Supplementary Table 2**). Case 23 was admitted due to poor appetite, dyspnea, and fever at 28 days of age. The clinical symptoms of this case were mild without typical lactic acid, intoxication, and metabolic disorders of electrolytes and blood sugar; also, vitamin B12 treatment was found to be effective in this case. Two mutations in c.658 (located at p.220), including c.658A > C, c.658_660delAAG, accounted for 14.7% in all disease alleles of *MMACHC*, and c.658_660delAAG was reported to be common in the Chinese population and associated with early onset (Zhou et al., 2018). We speculated that the c.658 is a mutation hotspot in Chinese neonates.

Synergistically, the correlation between clinical features and *MMUT* and *MMACHC* defects in full-term neonates were calculated in this study. The number of patients with early onset in PN0–PN7 days, metabolic acidosis, electrolyte disturbances, coagulant function abnormality, and neonatal death were significantly larger in *MMUT* defect than in *MMACHC* defect, while the quantity of cases responded to vitamin B12 treatment with significantly improved biochemical indicators was much smaller in *MMUT* defect than in *MMACHC* defect (**Table 3**). The mortality of *MMUT* cases (70.0%) was much higher than the total mortality of IEMs in the NICU (53.0%, 35 deaths in 66 cases were all attributed to the endogenous toxic type of IEMs). On the other side, the mortality of *MMACHC* defects was only 25.0%. The dampened survival rate in these cases of IEMs was mostly due to the reluctance of the parents for accepting the fit-time treatments after informing the result of biochemical diagnosis. Moreover, 21.2% (14/66) of diagnosed IEM cases had a history of IEMs or neonatal death in siblings, but their parents had no prenatal counseling or genetic testing. Therefore, it is necessary to improve parents' cognition of IEMs and the rate of genetic diagnosis of probands.

To sum up, we screened 42,257 NICU newborns by mass spectrometry and diagnosed 66 cases of IEMs. We found in Chinese NICU the incidence of IEMs was 1:640, and the incidence in full-term neonates (1:446) was significantly higher than that in premature ones (1:2,584). The most common IEM in total and full-term infants was MMA (in particular the isolated type), while in premature infants was PKU. The higher incidence and the overwhelming proportion (> 90%) of endogenous intoxication type of IEMs in disease spectrum we obtained reflects the disease burden of these disorders in NICU. We found 62 potentially pathogenic mutations in 10 genes of IEMs in genetic analysis, 33.9% (21) of them were novel. The obtained spectra of mutation genes indicated that the most prevalent defect gene was *MMUT*. Our results enriched the clinical characteristics and genetic data of neonatal IEMs and provided diagnostic clues and therapeutic insights for neonatal pediatricians.

DATA AVAILABILITY STATEMENT

The clinical data of the patient is available in the record room of the Seventh Medical Center of PLA General Hospital. The sequencing and mass spectrometry data is available in the laboratory of BaYi Children's Hospital. Basic information of subjects and the genetic analysis and clinical symptom data of the patients are presented in the additional supporting files. The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Medical ethics committee of PLA general hospital" with written informed consent from all the patients' guardians for experimentation. All procedures followed were in accordance with the ethical standards of "Medical ethics committee of PLA general hospital" and with the Helsinki Declaration.

AUTHOR CONTRIBUTIONS

WZ and YY performed and interpreted the results of DBS and urine screening of IEMs, analyzed the patient's data, and wrote the manuscript. WP and YC analyzed the genetic data and the structural of the protein. JC and YM performed the medical assessment, provided the clinical data, assisted with the data

analysis, and created the figures. LY, XW, and YL involved in sample collection, implementation of DBS and urine screening, and drafting the manuscript. YW and ZF provided guidance in patient management, participated in the manuscript preparation and revision. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01302/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Diagnosis and Management of Beckwith-Wiedemann Syndrome

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Beckwith-Wiedemann syndrome (BWS) is a human genomic imprinting disorder that presents with a wide spectrum of clinical features including overgrowth, abdominal wall defects, macroglossia, neonatal hypoglycemia, and predisposition to embryonal tumors. It is associated with genetic and epigenetic changes on the chromosome 11p15 region, which includes two imprinting control regions. Here we review strategies for diagnosing and managing BWS and delineate commonly used genetic tests to establish a molecular diagnosis of BWS. Recommended first-line testing assesses DNA methylation and copy number variation of the BWS region. Tissue mosaicism can occur in patients with BWS, posing a challenge for genetic testing, and a negative test result does not exclude a diagnosis of BWS. Further testing should analyze additional tissue samples or employ techniques with higher diagnostic yield. Identifying the BWS molecular subtype is valuable for coordinating patient care because of the (epi)genotype-phenotype correlations, including different risks and types of embryonal tumors.

Keywords: Beckwith-Wiedemann syndrome, methylation, diagnostic testing, mosaicism, cancer predisposition, tumor screening

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INTRODUCTION

Beckwith-Wiedemann syndrome (BWS) is a human imprinting disorder that leads to overgrowth. It is associated with genetic and epigenetic changes on the chromosome 11p15 region (1), which includes imprinted genes that regulate fetal and postnatal growth. BWS is often diagnosed neonatally or in early childhood and has a broad clinical spectrum of features that vary in severity. These features include macroglossia, abdominal wall defects, lateralized overgrowth, enlarged abdominal organs, and an increased risk for developing embryonal tumors during early childhood (2). BWS is now considered a spectrum (BWSp) ranging from classic BWS to isolated lateralized overgrowth (2, 3). BWS has an estimated prevalence of 1 affected child in 10,340 live births (4), with an increased risk associated with assisted reproductive technologies (ART) of around 1 in 1,100 (5–7).

To determine if molecular testing should be pursued and to establish a clinical diagnosis of BWS, a clinical scoring system is used (2). In this system, cardinal features include macroglossia, omphalocele, lateralized overgrowth, bilateral Wilms tumor, hyperinsulinism, and specific pathological findings such as adrenal cytomegaly or placental mesenchymal dysplasia. Each cardinal feature is given 2 points. Suggestive features include birthweight greater than two standard deviations above the mean, facial nevus simplex, polyhydramnios or placentomegaly, ear creases or pits, transient hypoglycemia, embryonal tumors, nephromegaly or hepatomegaly, and umbilical hernia or diastasis recti. Each suggestive feature is given 1 point. Patients with a clinical score $\geq$2 merit genetic testing for BWS. Patients with a score $\geq$4 based on cardinal and suggestive features

satisfy a clinical diagnosis of classical BWS, even without molecular confirmation. Patients with a score <2 do not meet criteria for genetic testing. If a patient with a score ≥2 has negative genetic testing, alternative diagnoses and/or referral to a BWS expert should be considered. Genetic testing is also recommended for patients with a family history of BWS and a known heritable pathogenic 11p15 anomaly, which occurs in about 10–15% of patients (1).

BWS is a mosaic disorder and, as such, may warrant genetic testing on multiple tissues because a patient may have cells of different genetic or epigenetic compositions in their body (8). In the case of BWS, a patient may have some cells that carry the epigenetic/genetic change and some cells that do not. Mosaicism itself can also vary in severity within a patient as different tissues can have different ratios of affected to unaffected cells. Thus, molecular confirmation is not always possible due to tissue mosaicism. More details about testing are below under Molecular Genetic Testing for BWS.

MOLECULAR MECHANISMS OF BWS

BWS involves molecular aberrations within a cluster of imprinted genes on the chromosome 11p15.5-11p15.4 region, as depicted in **Figure 1**. There are two functionally independent domains, the telomeric and the centromeric, each with its own imprinting control region (1). The telomeric domain includes the long non-coding RNA (lncRNA) *H19* and insulin-like growth factor 2 (*IGF2*). *H19* is a maternally expressed lncRNA in the embryo and placenta, but it is silenced in most tissues after birth except in cardiac and skeletal muscle (9, 10). It may have a role in both tumor formation and suppression (11). *IGF2* is a growth factor that is paternally expressed in the fetus and placenta, and biallelically expressed in the liver after birth (12). The telomeric domain is controlled by the *H19/IGF2* intergenic differentially methylated region (*H19/IGF2*:IG DMR), also known as imprinting control region 1 (IC1) that is located 2 kilobases upstream of *H19* (1). Shared enhancers of *H19* and *IGF2* and a CTCF binding factor-dependent insulator located between the two genes control imprinting of this locus (13, 14). In mouse, CTCF binds to the imprinting control region (ICR) on the maternal allele to produce an insulator that results in expression of *H19* and silencing of *Igf2* (15). On the paternal allele, methylation of the ICR prevents CTCF binding, which leads to *Igf2* expression and silencing of the *H19* promoter. Similar mechanisms of regulation occur in humans, but the IC1 region is much larger in humans (16).

The centromeric domain includes *KCNQ1*, the regulatory lncRNA *KCNQ1OT1*, and *CDKN1C*, a cell cycle inhibitor. *KCNQ1* encodes a voltage-gated potassium channel, and it is initially maternally expressed during early embryogenesis but becomes biallelically expressed during fetal development (17). *KNCQ1OT1* is a paternally expressed lncRNA transcribed antisense to *KCNQ1* (18). *CDKN1C* encodes a G1 cyclin-dependent kinase inhibitor, and it negatively regulates cell growth and proliferation. It is expressed in the embryo and placenta as well as postnatally in the body (19). The centromeric domain is controlled by the *KCNQ1OT1* transcription start site differentially methylated region (*KCNQ1OT1*:TSS DMR), also known as imprinting control region 2 (IC2) that is located on the 5′ end of *KCNQ1OT1* and includes the promoter region for *KCNQ1* (20). In mice, the maternal allele is methylated so that *Kcnq1ot1* is not expressed and *Kcnq1* and *Cdkn1c* are expressed. On the paternal allele, the *Kcnq1ot1* promoter is not methylated so the lncRNA is expressed and *Kcnq1* and *Cdkn1c* are silenced (21). Regulation of this ICR seems to be similar in mice and humans (16).

About 80% of patients with BWS have a known molecular defect in the 11p15 region, most commonly due to aberrant DNA methylation (1). Normally, the paternal allele is methylated at IC1 and the maternal allele is methylated at IC2 (**Figure 1A**). These methylation marks are established in the germline and must be maintained throughout the reprogramming that occurs post-fertilization in the zygote (22). Gain of methylation at IC1 on the maternal allele (IC1 GOM) is found in about 5–10% of patients. This leads to downregulation of *H19* and expression of *IGF2* on the maternal allele (**Figure 1B**) (23). Loss of methylation at IC2 on the maternal allele (IC2 LOM) is found in about 50% of patients (24). This leads to derepression of *KCNQ1OT1* and downregulation of *CDKN1C* on the maternal allele (**Figure 1C**) (23, 25). Paternal uniparental isodisomy (pUPD) occurs when a patient has two copies of the paternal chromosome 11p15 and none of the maternal, and this occurs in about 20% of patients (24). The extent of the disomy can range from segmental to genome wide, but with regards to the 11p15 region, pUPD leads to overexpression of *IGF2* and decreased *CDKN1C* expression (**Figure 1D**) (26). pUPD can be caused by errors in meiosis I or meiosis II in the gametes, or more frequently in BWS by postzygotic errors in mitotic recombination during early embryogenesis (27, 28). *CDKN1C* mutations on the maternal allele occur in about 5% of sporadic cases and 40% of familial BWS (**Figure 1E**) (24). If the mutation is maternally inherited, there is a 50% chance recurrence risk with variable expressivity (29). Chromosomal abnormalities including duplications, deletions, and translocations of the 11p15 region are found in <5% of patients (24). These alterations are usually inherited in an autosomal dominant manner, and the recurrence risk depends on the sex of the parent carrying the affected allele (1).

There is a higher frequency of multiple gestations in patients with BWS compared to the general population (30). The majority of cases are monozygotic female twins with discordant features where one twin is more affected by BWS than the other (30), but there is great variability in the degree of phenotypic discordance (31). It is hypothesized that an epigenetic event causing BWS occurs prior to, and perhaps triggers the twinning process (30, 32, 33). The affected cells diffuse among the embryos in a multiple pregnancy, resulting in a mosaic distribution. Cohen et al. (31) present this theory of "diffused mosaicism" where the timing of the embryologic twinning relative to the timing of the epigenetic event likely influences the degree of BWS affectedness and degree of mosaicism.

Assisted reproductive technologies (ART), such as *in vitro* fertilization (IVF) and intracytoplasmic sperm inject (ICSI)

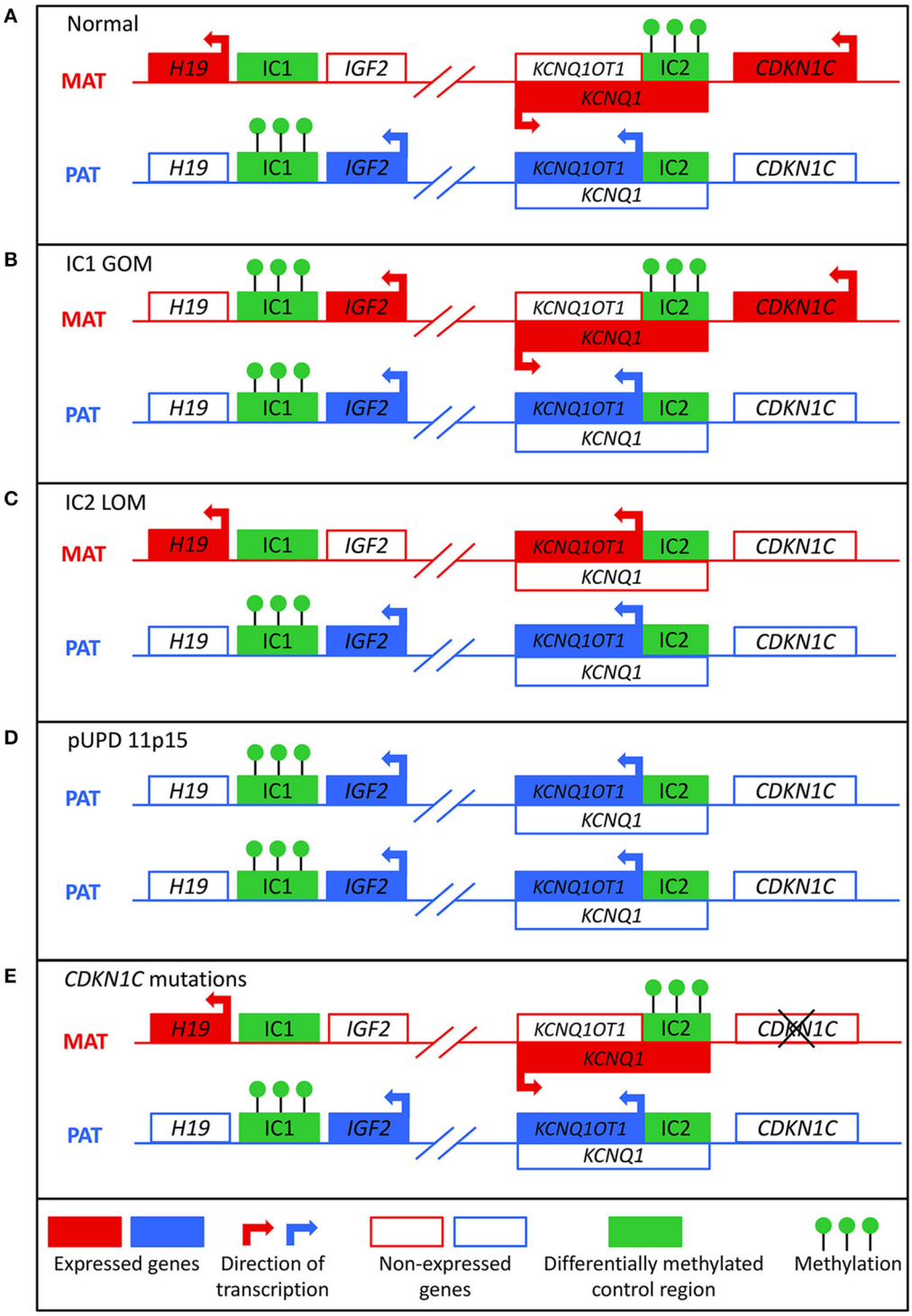


FIGURE 1 | Molecular mechanisms that can lead to Beckwith-Wiedemann syndrome. **(A)** In normal cells, the paternal allele is methylated at imprinting control region 1 (IC1) and the maternal allele is methylated at imprinting control region 2 (IC2). **(B)** In IC1 gain of methylation (IC1 GOM), both the maternal and paternal alleles are methylated at IC1, which leads to downregulation of *H19* and overexpression of *IGF2*. **(C)** In IC2 loss of methylation (IC2 LOM), the maternal allele loses methylation at IC2, which leads to expression of *KCNQ1OT1* and downregulation of *CDKN1C*. **(D)** In paternal uniparental disomy of 11p15 (pUPD), there are two copies of the paternal chromosome, which leads to overexpression of *IGF2* and decreased expression of *CDKN1C*. **(E)** Mutations of the maternal *CDKN1C* gene can result in loss of function.

may impact the establishment and/or the maintenance of DNA methylation at imprinted loci (7, 34). There is a 10-fold increased risk of BWS with ART and an absolute risk of about 1 in 1,100 (5). More than 90% of children with BWS conceived by ART have IC2 LOM (5). Further research is needed to illuminate the relationship between ART and imprinting defects.

MOLECULAR GENETIC TESTING FOR BWS

Overview of Genetic Testing Strategies

Mosaicism can pose a significant challenge to genetic testing in BWS because different tissues may have different proportions of affected BWS cells (8). First-line diagnostic testing is usually performed on DNA derived from blood-leukocytes (2). Other samples such as buccal swabs, skin fibroblasts, or cells of mesenchymal origins including surgical resections and/or excisions of hyperplastic tissues, can improve the detection of mosaicism (3, 35, 36). A negative result does not exclude a diagnosis of BWS and may be the result of low-level mosaicism that is below the level of detection, a rare balanced chromosomal rearrangement, or another currently unrecognized cause (2). In up to 20% of patients with a BWS phenotype, a molecular diagnosis remains unknown (1). This may be due to tissue mosaicism, as testing multiple tissues improves diagnostic yield (3). Patients without a confirmed molecular diagnosis should be evaluated for clinical features suggestive of different diagnosis and appropriate additional testing should be considered (2). If other features are not present and the clinical score is ≥4, the

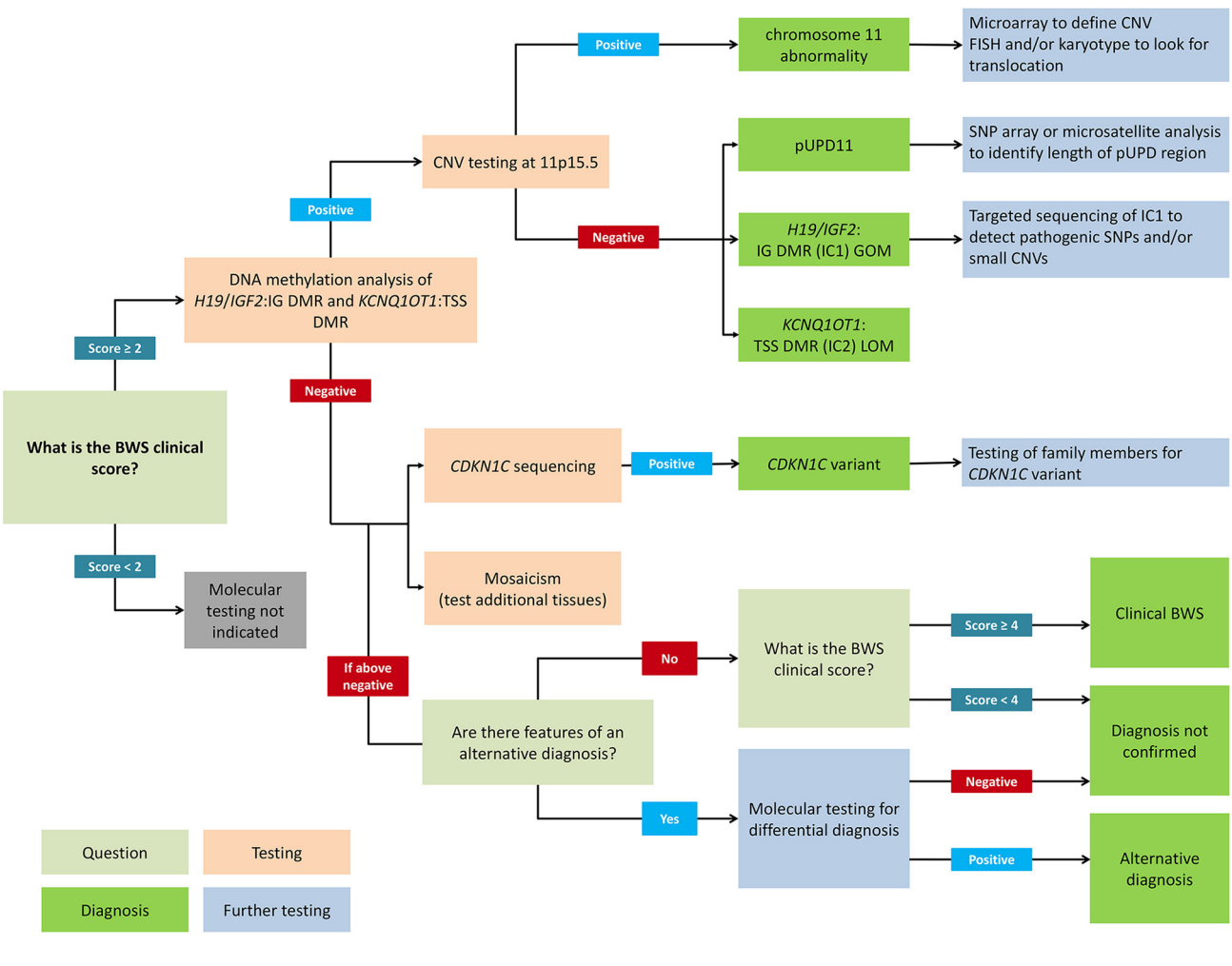


FIGURE 2 | Flowchart for molecular diagnosis of Beckwith-Wiedemann syndrome. Patients with BWS clinical score ≥2 should receive genetic testing while patients with clinical score <2 do not meet the criteria for testing. Recommended first-line testing (highlighted in orange) should analyze methylation at *H19/IGF2*:IG DMR (IC1) and *KCNQ1OT1*:TSS DMR (IC2) and copy number variation (CNV). These tests can yield positive molecular diagnoses of chromosome 11 abnormalities, paternal uniparental disomy of chromosome 11 (pUPD), IC1 gain of methylation (IC1 GOM), and IC2 loss of methylation (IC2 LOM) (highlighted in dark green). Further testing (highlighted in blue) can determine chromosomal abnormalities more precisely. If DNA methylation testing is negative, *CDKN1C* sequencing is recommended, or additional tests for rare chromosomal translocations. Negative test results can also be due to tissue mosaicism, and additional tissue samples can be tested. Differential diagnoses should also be considered, but patients with clinical score ≥4 can have a clinical diagnosis of BWS even without molecular confirmation. Adapted from Brioude et al. (2).

patient may have classical BWS without molecular confirmation (2). Patients with a score <4 and with isolated lateralized overgrowth may still be part of the BWSp (3).

Figure 2 presents a flowchart for how to approach a molecular diagnosis for BWS. First-line testing procedures should determine the IC1 and IC2 methylation level and the differentially methylated region (DMR) copy number (2). Abnormal methylation is present in IC1 GOM, IC2 LOM, pUPD11 (which shows both IC1 GOM and IC2 LOM), and in copy number variations (CNVs) (35). Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is currently the most common diagnostic test because it simultaneously detects percent methylation and DMR copy number status (37, 38). However, other methylation-specific techniques are more sensitive to low-level mosaicism, and multiple tissues should be tested in patients with low-level mosaicism (36, 39, 40). To confirm pUPD, chromosomal microarray analysis (CMA) such as a single nucleotide polymorphism (SNP) based microarray analysis can detect low-level mosaicism and determine the length of the region of pUPD, which may impact care (27, 41, 42). Genome-wide pUPD (GWpUPD), or mosaic paternal isodisomy, may affect up to 10% of patients with pUPD of chromosome 11p15 and involves additional clinical features and elevated tumor risk (26, 43). *CDKN1C* mutations are detected through gene sequencing, and detection of a pathogenic variant allows for cascade testing of family members to clarify recurrence risk (29, 44).

If a CNV is detected, chromosome microarray is recommended to determine the size and nature of the deletion or duplication (41, 45). Karyotyping or fluorescence *in situ* hybridization (FISH) can also be used to identify chromosomal translocations depending on the nature of the breakpoints (46). In patients with IC1 GOM, up to 20% may have small CNVs in the DMR, which are associated with a high risk of recurrence (47, 48). These can sometimes be detected using MS-MLPA but require targeted IC1 sequencing, especially if there is a family history of BWS (49, 50). Deletions in IC2 are rare (45), and there is currently no specific recommendation to analyze CNVs in patients with IC2 LOM (2). About one third of IC2 LOM patients have been reported to have a multilocus imprinting disturbance (MLID), but the clinical significance is uncertain so testing is usually not indicated (51).

For BWS patients who are part of a multiple pregnancy, knowing the zygosity and chorionicity is important for appropriate diagnosis. For dizygotic dichorionic gestations, no evaluation is indicated for the unaffected twin but for monozygotic monochorionic and dichorionic gestations, the twin should receive a clinical examination by a geneticist (31). To accurately diagnose discordant monozygotic twins, buccal swab is the preferred source of DNA because DNA from blood cells or saliva may show aberrant methylation in an unaffected twin due to shared circulation during fetal development (52).

Diagnostic Tests for Aberrant Methylation

The most widespread diagnostic test is methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) because it can detect both DMR methylation and copy number variation (37, 38). However, it cannot determine the size or content of CNVs, and other tests such as CMA or FISH analyses are more suitable for this (37). MS-MLPA is a polymerase chain reaction (PCR)-based method and uses multiple test probes in the 11p15 region and in other loci across the genome (37, 38). Some of the probes for the 11p15 region are methylation specific and contain the Hha I restriction enzyme site within a CpG island. After the probes and DNA incubate together, the sample is divided into two aliquots, one for traditional MLPA and the other for methylation-specific (MS) analysis. The first aliquot for traditional MLPA uses the ligation of two half-probes to detect CNVs. The second aliquot for MS analysis includes the addition of Hha I restriction enzyme, which specifically targets unmethylated sequences for degradation so only methylated samples are amplified. The relative amounts of the target DNA sequence are quantified by fluorescently-labeled primers, which are used to calculate the methylation index (37, 38). MS-MLPA can also identify the parent of origin of small genomic duplications and deletions (38), but further testing such as a CMA or FISH is recommended. It is important to have a wide cohort of controls and to match analyses with controls from the same tissue (36). Using a single experiment, MS-MLPA can detect CNV and methylation status within the 11p15 region with high specificity and reliability (45). Furthermore, this test can be performed efficiently and at low costs with a small quantity of DNA, making it an ideal first-line diagnostic test (37, 38).

To more precisely study low-level mosaicism, other methylation-sensitive PCR methods are needed. In quantitative methylation-sensitive PCR, genomic DNA is treated with sodium bisulfite then amplified with quantitative TaqMan PCR (39, 40). The bisulfite treatment deaminates unmethylated cytosines to uracil while methylated cytosines are preserved. The TaqMan probes are labeled with different fluorophores to discriminate between methylated and unmethylated DNA, and the methylation index is calculated based on the fluorescence intensity from each allele. Using allele-specific methylated multiplex real-time quantitative PCR (ASMM RTQ-PCR), Azzi et al. (40) identified a range of normal methylation smaller than that of MS-MLPA, meaning the assay is more sensitive and is able to detect minute changes in methylation. Another method is methylation-specific high-resolution melting (MS-HRM) that uses differences in melting profile of methylated and unmethylated DNA to detect methylation (53). Following bisulfite treatment and PCR amplification, a fluorescent intercalating dye is added to the DNA, and the change in fluorescence is monitored as the DNA melts. The unmethylated allele will begin melting at a lower temperature followed by the methylated allele. MS-HRM has similar results and limitations as MS-MLPA and other methylation-specific PCR techniques (53).

Diagnostic Tests for Uniparental Disomy and Chromosomal Abnormalities

Chromosomal microarray analysis (CMA), FISH, and karyotyping can detect copy number variations, chromosomal abnormalities, and can confirm uniparental isodisomy (46). CMA is the most common microarray technology used to

identify deletions and duplications, but it provides limited information on the structural rearrangements. Either FISH or karyotyping is usually performed with CMA to confirm results and to identify translocations and insertions (46).

There are two major microarray analyses: comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) analysis. Both microarrays detect submicroscopic changes that are too small to be detected through conventional karyotyping by comparing hybridization intensities between the patient sample and normal reference DNA (54). Through this approach, labeled patient DNA and labeled reference DNA compete to hybridize to normal metaphase-arrested human DNA. An equal distribution of the hybridized patient and reference DNA is indicative of a healthy sample, whereas an imbalanced ratio is indicative of chromosomal aberrations in the patient DNA (54). CGH can identify deletions or duplications of a few kilobases in size whereas SNP probes can identify variations at a single site in DNA when present (55). A major limitation of CGH is its inability to detect balanced chromosomal rearrangements, or UPD and low-level mosaicism (46, 54).

SNP microarrays can simultaneously detect gain/loss CNVs and copy-neutral loss of heterozygosity, as well as the parent of origin of CNVs to detect uniparental disomy (27, 45, 56). MS-MLPA and methylation-sensitive PCR methods can detect pUPD indirectly when both IC1 and IC2 have abnormal methylation, but SNP microarrays can quantitatively determine the size of UPD based on the extent of the SNPs affected (26, 36, 41). SNP microarrays can detect uniparental disomy when both alleles in the patient come from a single parent. SNP microarrays are the most sensitive method for UPD and the associated mosaicism (41), and can distinguish low-level mosaicism (1–5%) from normal samples using B-allele frequencies (BAF) (27, 42). They are also able to distinguish UPD from chromosomal abnormalities more precisely than karyotyping or FISH (42). Furthermore, a genome-wide SNP array can be utilized to distinguish mosaicism from chimerism, which occurs when there are two different cell lines with two complete sets of DNA within the body (27). Microsatellite arrays, which analyze highly polymorphic short tandem repeats (STRs) in the DNA, are similar to SNP microarrays in that both can detect UPD, but SNP microarrays are more sensitive (41).

While microarrays identify changes at specific regions of the genome, karyotyping identifies larger chromosome differences. Karyotyping detects structural changes >3–10 Mb in size and it is well-suited for complex rearrangements involving multiple chromosomes (46).

Fluorescence *in situ* hybridization (FISH) can detect structural changes of genes with higher resolution than karyotyping (57). FISH is a hybridization technique that uses fluorescent probes to bind to specific DNA sequences with high specificity in order to detect the presence or absence of these stretches of DNA (58). Prior knowledge of the abnormal region is required to design FISH probes, and only a few probes can be used at a time (58). While karyotyping and FISH can also distinguish chromosomal abnormalities from mosaic UPD, they cannot determine the size of a disomy nor the size of a chromosomal deletion or duplication (42). After identifying the chromosomal abnormalities in the patient, testing should be extended to other family members as appropriate (2).

Diagnostic Tests for *CDKN1C* Mutations

To detect *CDKN1C* mutations or other gene mutations in the 11p15 region, genetic sequencing is performed. Briefly, PCR is performed to amplify the region of interest, then Sanger sequencing is used to query the sequence for candidate mutations.

Prenatal Testing

BWS can be diagnosed molecularly in some prenatal cases, but due to mosaicism, a negative test result cannot exclude a diagnosis (23) and postnatal testing should also be performed to confirm results (59). Genetic counseling should include discussion of the benefits and limitations of each test offered. Testing is usually indicated by abnormal ultrasound, including omphalocele, macroglossia, or enlarged abdominal organs in the fetus. Placental mesenchymal dysplasia, polyhydramnios, or increased alpha-fetoprotein (AFP) in the second trimester can also occur (59). Positive family history mainly arises from *CDKN1C* mutations or chromosomal abnormalities such as deletions/duplications. Both native and cultured amniocytes can be used for testing, but cultured cells may show features that do not correlate with the true biological status of the fetus or placenta (59). If testing is undertaken, methylation testing and *CDKN1C* sequencing is recommended. Testing on chorionic villus sampling (CVS) can be limiting because of confined placental mosaicism, which might not reflect the (epi)genetic status of the fetus (60) and thereby CVS testing that is negative or showing low-level changes would require an amniocentesis and/or postnatal testing for further evaluation. Maternal contamination is also a possibility, so parallel microsatellite analysis of maternal and fetal short tandem repeats (STRs) is strongly advised (59). While a positive methylation or chromosomal test result confirms a diagnosis of BWS, a negative result cannot exclude a diagnosis. Due to the complexity and heterogeneity of BWS, tissue mosaicism and other molecular alterations could escape detection. Postnatal testing should be performed to confirm any results (59).

MANAGEMENT AND CARE OF PATIENTS WITH BWS

Determining the molecular subtype of BWS is important because there are correlations between clinical phenotype and (epi)genotype (61–63). **Figure 3** depicts facial photographs of patients with different molecular subtypes of BWS. IC1 GOM patients tend to have large birth weights, enlarged abdominal organs, and high incidence of tumors (28%) especially Wilms tumors, while IC2 LOM patients typically present with omphalocele, macroglossia, and nevus simplex, and have the lowest incidence of tumors (2.6%). UPD patients tend to have lateralized overgrowth and hyperinsulinism and an intermediate tumor incidence (16%). It is unknown if there is a correlation between the severity of the phenotype and the

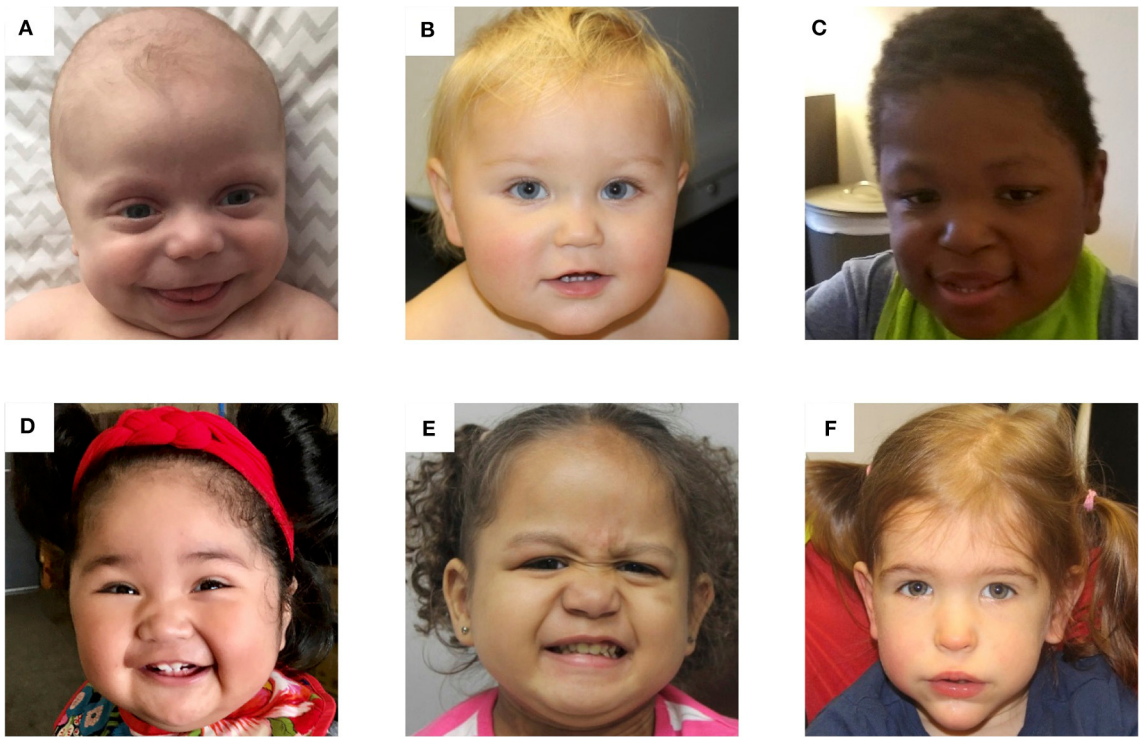


FIGURE 3 | Photos of six patients with BWS due to **(A)** IC2 loss of methylation (IC2 LOM), **(B)** IC1 gain of methylation (IC1 GOM), **(C)** chromosomal rearrangements (deletions, duplications), **(D)** paternal uniparental isodisomy 11 (pUPD), **(E)** genome-wide paternal uniparental isodisomy (GWpUPD), and **(F)** *CDKN1C* mutation. Written consent was obtained from the parents of every participant to publish these identifying images.

level of mosaicism or chromosomal isodisomy (61). Patients with *CDKN1C* mutations have similar features as those with IC2 LOM including omphalocele and nevus simplex, and they have an estimated tumor incidence of 6.9%, although limited data exists (62).

Prenatal Care

For patients with a prenatal diagnosis, the management of individual congenital anomalies should follow standard protocols. BWS is associated with increased risk of polyhydramnios, gestational hypertension, pre-eclampsia, and preterm births (64–66), so appropriate arrangements for delivery and neonatal care should be made. Post-delivery complications may include neonatal hypoglycemia, respiratory obstruction due to macroglossia, and surgical repair of omphalocele (2).

Hypoglycemia and Hyperinsulinism

Hypoglycemia occurs in about 30–60% of patients, and BWS-related neonatal hypoglycemia is due to excess insulin (62, 67, 68). It is generally transient and resolves within a few days, but in some cases persistent hyperinsulinism (HI) occurs, and therefore neonates with suspected BWSp should be screened for hypoglycemia before discharge (2). HI may require medical treatment such as diazoxide or somatostatin analogs such as octreotide and lanreotide, or in some cases subtotal pancreatectomy in HI that persists despite maximal medical therapies (68). Congenital HI is a rare condition seen most commonly in pUPD patients, and a diagnosis should be made with an endocrinologist who is familiar with this condition. HI in BWS can occur with or without mutations in the beta cell potassium channel genes (*ABCC8* and *KCNJ11*), which are also on chromosome 11p near the BWS region (68).

Macroglossia

Macroglossia is seen in about 90% of patients with BWS (61, 62), and about 40% of children undergo a tongue reduction surgery (69). Need for surgery and timing of surgery depends on the clinical status of the patient. The most common indications for surgery include respiratory problems, obstructive sleep apnea, feeding difficulties, persistent drooling, problems with speech and articulation, and orthodontic problems (70, 71). An airway evaluation and polysomnography can provide further assessment for obstructive sleep apnea (72). If there are respiratory problems, surgery might need to be performed earlier in the neonatal period. Studies show that patients who receive surgery before 2–3 years tend to have good outcomes with favorable results including cosmetic improvement, adequate tongue mobility, and no substantial effect on taste (69, 71).

Abdominal Wall Defects

For omphalocele and other abdominal wall defects, no specific recommendations have been given with regards to patients with

BWS. The management of these features should follow standard protocols and local practices (2).

Growth and Lateralized Overgrowth

Overgrowth in BWS occurs in about 43–65% of patients (61, 62), and lateralized overgrowth can occur when one side of the body is larger than the other. Postnatal growth is generally in the upper percentiles but slows down in late childhood (73). Lateralized overgrowth is the most frequent feature in pUPD patients (63), and the management will depend on the affected limbs. Leg length discrepancy (LLD) may require shoe lifts or surgical correction in some cases (74).

Tumor Screening Protocols

BWS is a cancer predisposition syndrome with an overall tumor risk of about 8%, but each molecular subgroup is associated with a different tumor incidence and types of tumors (75, 76). The most common types of tumors are Wilms tumor (52%) and hepatoblastoma (14%), followed by neuroblastoma (10%), rhabdomyosarcoma (5%), and adrenal carcinoma (3%) (62). Cancer risk is the highest during the first 2 years of life and declines afterwards, and there is currently no evidence of an increased tumor risk in adults with BWS (2). IC1 GOM is associated with the highest incidence of tumor (28%), followed by pUPD (16%), *CDKN1C* mutation (6.9%), and the lowest with IC2 LOM (2.6%) (61, 62, 75). Patients with IC1 GOM are predisposed to Wilms tumor, which accounts for 95% of tumors in this group (62, 75), while patients with IC2 LOM are more likely to develop hepatoblastoma (77), and patients with *CKDN1C* mutations are predisposed to neuroblastoma (62, 75). Wilms tumor and hepatoblastoma occur with similar frequencies in patients with pUPD, and patients with GWpUPD seem to develop similar types of tumor as those with segmental pUPD but with an increased incidence of hepatic and/or adrenal tumors extending into young adulthood (78, 79). For patients with a clinical diagnosis of BWSp or negative molecular testing, there needs to be further research to understand their cancer risk (80).

Tumor screening protocols are recommended for earlier detection of tumors, reducing morbidity, and increasing patient survival. Guidelines developed by the American Association for Cancer Research Childhood Cancer Predisposition Workshop (AACR-CCPW) include full abdominal ultrasound (USS) every 3 months from diagnosis until the 4th birthday, renal ultrasound every 3 months from age 4–7 years, and AFP screening every 3 months until the 4th birthday for all patients with BWSp (81). Patients with *CDKN1C* mutations should also receive neuroblastoma screening, which includes urine VMA/HVA and chest X-ray every 3 months until the 6th birthday then every 6 months from age 6–10 years, in addition to abdominal imaging and AFP screening (82). In contrast Brioude et al. (2), an international consensus group consisting primarily of European experts, recommends abdominal ultrasounds every 3 months from diagnosis until the 7th birthday for the BWSp subgroups that are at the highest risk of cancer including IC1 GOM, pUPD, *CDKN1C* mutation, and other genomic rearrangements of the region and clinical BWS (2).

To screen for hepatoblastomas, measurements of serial serum alpha-fetoprotein (AFP) could lead to earlier detection than abdominal ultrasounds. However, interpreting AFP levels can be difficult during infancy and early childhood due to variable concentrations and wide range of normal values (83–85). The consensus group stated that AFP measurements should not be offered routinely because of the low incidence of hepatoblastoma in BWSp and the difficulties in interpretation (2).

AACR-CCPW identifies a 1% tumor risk threshold and therefore recommends abdominal USS and AFP screening for all subtypes of BWSp (81). While patients with IC2 LOM have an overall lower risk of tumor development, they have an increased risk of hepatoblastoma (77), which has significantly lower event-free survival rates compared to Wilms tumor or other embryonal tumors. Patients with localized and lower stage hepatoblastoma can achieve high survival rates between 80 and 100%, but patients with late stage tumors face a poorer prognosis (86, 87). Serum AFP levels to screen for hepatoblastoma should be interpreted in the context of the clinical picture, and patients with BWS tend to have higher AFP levels in early childhood compared to normal pediatric values (83, 84, 88). AFP levels are expected to decline over time and can be tracked with normograms (89), and large rises in AFP levels should be further investigated by repeat testing and additional imaging (81). AFP screening can be used to distinguish hepatoblastoma from infantile hepatic hemangioma, a benign vascular neoplasm (90, 91). Monitoring serial serum AFP levels can allow for early detection of hepatoblastoma, even before detection by abdominal imaging, which can lead to better patient outcomes (92, 93). Recently Mussa et al. (94) have developed a less invasive method for measuring AFP levels using dried blood spots that is as accurate as traditional venipuncture.

Children with BWS and Wilms tumor tend to have higher incidence of recurrence (95) and potential co-occurrence of progressive non-malignant renal diseases and bilateral Wilms tumor (96, 97). Multifocal or diffuse nephrogenic rests in one or both kidneys (nephroblastomatosis) are not easily distinguishable from Wilms tumor and may require MRI for diagnosis (97). Partial nephrectomy and nephron-sparing strategies for Wilms tumors are recommended if possible (96, 98). The presence of nephro-urological anomalies in patients with BWSp is 28–61% (99), including cortical and medullary cysts in about 10% of patients and higher incidence of hypercalciuria and nephrolithiasis (100). For adults with BWS, a detailed clinical review and renal ultrasound should be performed at 16 years to develop specific recommendations for surveillance for ongoing problems (2). There is no apparent association between BWSp and predisposition to common adult-onset carcinomas (101), but further research on adults with BWS is needed.

Cardiac Features

Cardiac defects occur in up to 13–20% of patients with BWS, and there is higher incidence of congenital heart disease compared to the general pediatric population (61, 102, 103). Minor anatomical defects should be monitored by echocardiogram until spontaneous resolution, but more severe defects might require surgical correction (2). Although rare, patients with IC2 CNVs and/or genomic rearrangements of the region may

be predisposed to long QT syndrome and require follow-up throughout adulthood (104, 105).

Cognitive and Neurological Features

Patients with BWS usually have normal cognitive development, and a broader differential diagnosis should be considered in patients with an overgrowth disorder and learning disability without a 11p15 anomaly (2, 106). However, developmental delay can be associated with prematurity, severe hypoglycemia, unbalanced chromosomal rearrangements, and GWpUPD (78).

Psychological Well-Being

The diagnosis of BWSp can affect patients and families psychologically and socially. Parents may be unprepared for the diagnosis because in many cases there is no relevant family history (2). The increased tumor risk and surveillance protocol can cause increased anxiety, but a survey of parents with children with BWS revealed that tumor screening decreases worry and is not burdensome (107). In children with macroglossia, some parents are worried that a large protruding tongue and persistent drooling may affect peer interactions and increase emotional difficulties (70, 108). Healthcare professionals should be aware of these psychosocial issues and refer families to specialists including genetic counselors, social workers, and psychologists, or offer support groups as appropriate (2).

DISCUSSION

BWSp is a complex multisystem disorder that can result from a variety of molecular changes in the 11p15 region. A range of different genetic diagnostic tests are used to detect aberrant methylation and chromosomal abnormalities, and the presented genetic testing strategies can guide clinicians when establishing a molecular diagnosis for BWSp. However, tissue mosaicism continues to pose a challenge to genetic testing, and a negative test result cannot exclude a diagnosis of BWSp. Even with negative molecular testing, a BWS clinical score $\geq$4 based on cardinal and suggestive features satisfies a clinical diagnosis of BWS. Nevertheless, identifying a molecular diagnosis is important in coordinating the care and management of patients with BWSp and testing multiple tissues if possible can improve molecular diagnosis.

AUTHOR CONTRIBUTIONS

KW performed the literature search and drafted the manuscript and some of the figures. JK performed a literature search and drafted the section on some of the testing techniques and made one of the figures. KD helped conceptualize the project and edited the manuscript. JMK conceptualized, organized, and edited the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Risk of Necrotizing Enterocolitis Associated With the Single Nucleotide Polymorphisms *VEGF* C-2578A, *IL-18* C-607A, and *IL-4 Receptor α-Chain* A-1902G: A Validation Study in a Prospective Multicenter Cohort

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The etiology of necrotizing enterocolitis (NEC) is multifactorial and an underlying genetic predisposition to NEC is increasingly being recognized. A growing number of studies identified single nucleotide polymorphisms (SNPs) of selected genes with potential biological relevance in the development of NEC. However, few of these genetic studies have been replicated in validation cohorts. We aimed to confirm in a cohort of 358 preterm newborns (gestational age <30 weeks, 26 cases of NEC ≥ Bell stage II) the association with NEC of three candidate SNPs: the vascular endothelium growth factor (*VEGF*) C-2578A polymorphism (rs699947), the interleukin *(IL)-18* C-607A polymorphism (rs1946518), and the IL-4 receptor α-chain *(IL-4Rα)* A-1902G polymorphism (rs1801275). We observed that allele and genotype frequencies of the three SNPs did not significantly differ between the infants with and without NEC. In contrast, the minor G-allele of the *IL-4Rα* A-1902G polymorphism was significantly less frequent in the group of 51 infants with the combined outcome NEC or death before 34 weeks postmenstrual age than in the infants without the outcome (0.206 vs. 0.331, $P = 0.01$). In addition, a significant negative association of the G-allele with the combined outcome NEC or death was found using the dominant (adjusted odds ratio, aOR: 0.44, 95% CI 0.21–0.92), recessive (aOR 0.15, 95% CI 0.03–0.74), and additive (aOR 0.46, 95% CI 0.26–0.80) genetic models. In conclusion our study provides further evidence that a genetic variant of the *IL-4Rα* gene may contribute to NEC.

Keywords: VEGF, IL-18, IL-4 receptor α-chain, polymorphisms, necrotizing enterocolitis, preterm

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INTRODUCTION

Necrotizing enterocolitis (NEC) is the leading cause of morbidity and mortality from gastrointestinal disease in very and extremely preterm infants (1, 2). As extensively discussed in several exhaustive reviews, the etiology of NEC is multifactorial and largely related to immaturity of the gastrointestinal tract (1–9). Besides low gestational age (GA), a complex interplay of other factors, such as type of feeding, gut dysbiosis, the high susceptibility of intestinal mucosal surface to inflammatory processes, or intestinal hypoperfusion may contribute to the pathogenesis of NEC (1–9). Nevertheless, NEC affects a minority of (very) preterm infants and clinical risk factors provide a limited explanation of the inter-individual variability in NEC susceptibility (3, 10). In the last years, an underlying genetic predisposition to NEC is increasingly being recognized (3, 10, 11).

As recently reviewed by Cuna et al., the candidate gene approach has been used in most studies on the genetics of NEC (3). Using this approach, a growing number of studies investigated single nucleotide polymorphisms (SNPs) of selected genes based on a-priori hypothesis of relevance to NEC. These studies particularly focused on mediators involved in the regulation of immune/inflammatory responses, such as toll like receptors, interleukins (ILs), tumor necrosis factor, or nuclear factor-kappa beta, and in the control of intestinal microcirculation, such as nitric oxide synthase, or vascular endothelium growth factor (VEGF) (3). However, few of these genetic studies have been replicated in validation cohorts (3). In the present study we aimed to confirm the association with NEC of three candidate SNPs: the *VEGF* C-2578A polymorphism (rs699947) (12, 13), the *IL-18* C-607A polymorphism (rs1946518) (14), and the IL-4 receptor α-chain (*IL-4Rα*) A-1902G polymorphism (rs1801275) (15). We performed our investigation in a cohort of preterm infants from four neonatal intensive care units located in three different European countries (Spain, Italy, and the Netherlands) (16, 17).

MATERIALS AND METHODS

Patients

The present study was performed on DNA samples collected during a previous study on carbamoyl phosphate synthetase SNPs as risk factor for NEC (16). The protocol of the above study was reviewed and approved by the institutional review board (IRB) for each participating center (see names below) and we obtained written informed consent from the parents. The consent also allowed, after anonymization of the DNA samples, the investigation of other SNPs potentially involved in NEC development. The protocol is registered in ClinicalTrials.gov Protocol Registration System (NCT00554866). Additional approval was obtained for the present analysis. All infants with a GA $\leq$30 weeks and birth weight (BW) $\leq$1,500 g born between July 2007 and July 2012 and admitted to the level III neonatal intensive care unit of the Maastricht University Medical Center (the Netherlands, IRB number MEC-07-2-018), Hospital Universitario Materno-Infantil de Canarias (Las Palmas de Gran Canaria, Spain, IRB number CEIC- 276), Carlo Poma Hospital (Mantova, Italy, inclusion until December 2011, IRB number 21366/2007), and Ospedale Maggiore Policlinico (Milan, Italy, inclusion from January 2012, IRB number 220211b) were eligible for participation in the study. As reported in a previous study, buccal cell samples were obtained from 96 healthy term infants (25 in Maastricht, 31 in Las Palmas de Gran Canaria and 40 in Mantova) (18).

Definition of Clinical Characteristics and Outcomes

Definitions were identical to those used in our previous study (16) and are therefore literally reproduced here. Data on clinical characteristics and outcomes were obtained from the medical records. GA was determined by the last menstrual period and early ultrasounds (before 20 weeks of gestation). Small for GA was defined as BW for GA below the sex-specific 10th percentile. Chorioamnionitis was defined as every clinical suspicion of infection of the chorion, amnion, amniotic fluid, placenta, or a combination as judged by the obstetrician. Prolonged rupture of membranes was defined as rupture of membranes >24 h before delivery. Prenatal exposure to a single course of antenatal steroids was defined as two doses of betamethasone administered 24 h apart and exposure to a partial course of antenatal steroids was defined as administration of a single dose of betamethasone <24 h prior to delivery.

NEC was defined as Bell stage II disease or greater. Cases of NEC stage III were also separately analyzed. At the conclusion of the study, all cases of NEC were reviewed in a blinded fashion by a panel of 4 investigators of the study. Cases of spontaneous intestinal perforation (i.e., without pathologic evidence of NEC) were excluded from the NEC group. The control group for NEC was formed by the infants without the condition who survived until 34 weeks postmenstrual age (PMA). In another analysis, we investigated the combined outcome NEC or death before 34 weeks PMA.

Arterial hypotension was defined as the need for volume expansion or inotropic support. A diagnosis of sepsis required signs of generalized infection, a positive blood culture, and antibiotic therapy. Respiratory distress syndrome (RDS) was defined as requirement for oxygen supplementation or respiratory support due to tachypnea, grunting, nasal flaring, retractions, or cyanosis. Bronchopulmonary dysplasia (BPD) was defined as a supplemental oxygen requirement at 36 weeks PMA to maintain oxygen saturation >90% (19). Retinopathy of prematurity (ROP) was defined as stage II or higher. Persistent ductus arteriosus (PDA) was defined as a requirement for indomethacin or ibuprofen and/or surgical ligation. Intraventricular hemorrhage (IVH) was classified by using the 4-level grading system (20). Grade<2 IVHs were not included in the analysis.

Samples and Genotyping

Buccal cell samples for DNA testing were obtained with a sterile OmniSwab (Whatman) and collected in Eppendorf sterile PCR tubes and stored at −80°C until further analysis. The samples obtained in Spain and Italy were transported on

dry ice to Maastricht where all the analyses were performed. DNA was extracted using standard methods and stored at −20°C until genotyping. Discrimination of the *VEGF* rs699947, the *IL-18* rs1946518, and the *IL-4Rα* rs1801275 alleles was performed with TaqMan® SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) ID C_8311602_10 (rs699947), C_2898460_10 (rs1946518), and C_2351160_20 (rs1801275), following the manufacturer's instructions. The TaqMan technique combines DNA amplification and genotype detection in a single assay (21). Probe sequences are depicted in **Supplementary Table 1**. Genotyping assays were performed on an ABI PRISM 7900 Sequence Detection System for allelic discrimination (Applied Biosystems).

Statistical Analysis

Methods for statistical analysis were identical to those used in our previous study (16) and are therefore literally reproduced here. Categorical variables were expressed as counts or percentages and compared using the chi-square test. Continuous variables were expressed as mean (SD) if they followed a normal distribution and compared using unpaired, two-sided *t*-test. If not normally distributed, continuous variables were expressed as median values (interquartile range, IQR; 25th−75th percentile) and compared using the using the Mann–Whitney *U*-test. The Kolmogorov-Smirnov test was used to test for normal distribution of continuous data.

Differences in allelic frequencies and genotype distributions between the investigated populations, as well as Hardy–Weinberg equilibrium (HWE) for genotype distribution were assessed using a chi-square test. The Hardy–Weinberg law states that q2 + 2pq + p2 = 1, where p and q are allele frequencies in a two-allele system. Logistic regression analysis was used to compute the odds ratios (ORs) and their 95% confidence intervals (CI) for NEC and the combined outcome NEC or death before 34 weeks of PMA based on genotype after accounting for the covariates which were significantly different between the groups and are known risk factors for developing NEC. Different genetic models were used to analyze the effect of the risk allele including the general allelic (multiplicative or codominant model), dominant, recessive, and additive models. Assuming a genetic penetrance parameter γ (γ >1), a multiplicative model indicates that the risk of disease is increased γ-fold with each additional copy of the risk allele; an additive model indicates that risk of disease is increased γ-fold for the genotype with one copy of the risk allele and 2 γ-fold for the genotype with two copies of the risk allele; a common recessive model indicates that two copies of the risk allele are required for a γ-fold increase in disease risk, and a common dominant model indicates that either one or two copies of the risk allele are required for a γ-fold increase in disease risk (22). The major allele was considered as a reference and the interactions were tested in the different models by multivariable logistic regression model. All the statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corporation, Armonk, NY, USA) and conducted at the $P < 0.05$ level of significance.

TABLE 1 | Baseline characteristics and neonatal complications in preterm infants with and without NEC.

	NEC-yes (*n* = 26)	*n* data missing	NEC-no (*n* = 332)	*n* data missing	*P*-value
Birth weight (g)	873 (SD 230)	0	1,040 (SD 259)	0	0.002
Gestational age (weeks)	26.8 (SD 1.9)	0	28.1 (SD 1.8)	0	0.000
Male sex	11 (42.3)	0	191 (57.5)	0	0.132
Prenatal steroids	18 (72)	1	278 (87.4)	14	0.097
Preecclampsia	2 (8.0)	1	52 (15.9)	5	0.293
Chorioamnionitis	0 (0.0)	0	35 (10.6)	3	0.080
PROM	6 (23.1)	0	95 (29.1)	5	0.516
Vaginal delivery	17 (65.4)	0	147 (44.5)	2	0.040
Apgar (1 min)	5 [3–8]	1	6 [4–8]	3	0.184
Apgar (5 min)	8 [6–9]	1	8 [7–9]	4	0.161
RDS	22 (84.6)	0	274 (82.8)	1	0.811
Mechanical vent.	23 (88.5)	0	202 (61.8)	5	0.006
BPD	15 (57.7)	0	92 (27.9)	2	0.001
Hypotension	19 (73.1)	0	118 (36.0)	4	0.000
Sepsis	17 (70.8)	2	174 (52.7)	2	0.086
IVH	14 (53.8)	0	93 (28.3)	3	0.006
PVL	3 (11.5)	0	13 (4.0)	4	0.073
PDA	15 (60.0)	1	140 (42.6)	3	0.090
ROP	8 (38.1)	5	51 (16.7)	27	0.014
Mortality	8 (30.8)	0	30 (9.0)	0	0.001
Death before 34 weeks	5 (19.2)	0	25 (7.5)	0	0.038

Results are expressed as mean (SD), median [interquartile range] or absolute numbers of patients (percentage). NEC, necrotizing enterocolitis (≥stage II); PROM, prolonged rupture of membranes; RDS, respiratory distress syndrome; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage (≥grade 2); PVL, periventricular leukomalacia; PDA, patent ductus arteriosus; ROP, retinopathy of prematurity (≥stage II).

RESULTS

Patient Characteristics

A total number of 358 preterm infants (26 NEC cases) were genotyped for the three SNPs, but genotype of *VEGF* C-2578A SNP failed in 9 infants (1 from the NEC group) and genotype of *IL-18* C-607A SNP failed in 1 infant without NEC. Demographic and clinical characteristics of the infants with and without NEC are shown and compared in **Table 1**. Mean GA and mean BW of infants with NEC were significantly lower than in infants without NEC. In addition, infants with NEC showed a higher incidence of vaginal delivery, mechanical ventilation, BPD, hypotension, IVH, ROP, and mortality. We adjusted for GA, BW, mechanical ventilation, hypotension, and death before 34 weeks PMA in the subsequent logistic regression analysis.

The demographic and clinical characteristics of the infants with and without the combined outcome NEC or death before 34 weeks PMA are shown and compared in **Table 2**. Mean GA, mean BW and median Apgar score at 1 and 5 min of infants with NEC or death were significantly lower than in infants without the combined outcome. In addition, infants with the combined outcome NEC or death showed a higher incidence of vaginal delivery, mechanical ventilation, hypotension, IVH, and PDA. We adjusted for GA, BW, Apgar score after at 1 and 5 min,

TABLE 2 | Baseline characteristics and neonatal complications in preterm infants with and without the combined outcome NEC or death before 34 weeks of corrected gestational age.

	NEC/death-yes ($n = 51$)	*n* data missing	NEC/death-no ($n = 307$)	*n* data missing	*P*-value
Birth weight (g)	821 (SD 225)	0	1,063 (SD 250)	0	0.000
Gestational age (weeks)	26.3 (SD 1.9)	0	28.3 (SD 1.6)	0	0.000
Male sex	26 (42.7)	0	176 (57.3)	0	0.397
Prenatal steroids	31 (63.3)	2	259 (88.1)	13	0.060
Preecclampsia	3 (6.0)	1	51 (16.8)	4	0.049
Chorioamnionitis	6 (12.0)	1	29 (9.5)	2	0.584
PROM	15 (30.6)	2	86 (28.3)	3	0.738
Vaginal delivery	32 (62.7)	0	132 (56.7)	2	0.010
Apgar (1 min)	5 [3–6]	1	6 [5-8]	6	0.000
Apgar (5 min)	7 [6–8]	1	8 [7-9]	6	0.000
RDS	43 (84.3)	0	253 (82.7)	1	0.774
Mechanical vent.	48 (94.1)	0	177 (58.6)	5	0.000
BPD	18 (36.0)	1	89 (29.1)	1	0.323
Hypotension	42 (82.4)	0	95 (31.4)	4	0.000
Sepsis	30 (61.2)	2	161 (52.8)	2	0.271
IVH	28 (56.0)	1	79 (25.9)	2	0.000
PVL	4 (8.0)	1	12 (3.9)	3	0.201
PDA	32 (65.3)	2	123 (40.3)	2	0.001
ROP	8 (21.1)	13	51 (17.7)	19	0.615

Results are expressed as mean (SD), median [interquartile range] or absolute numbers of patients (percentage). NEC, necrotizing enterocolitis (≥stage II); PROM, prolonged rupture of membranes; RDS, respiratory distress syndrome; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage (≥grade 2); PVL, periventricular leukomalacia; PDA, patent ductus arteriosus; ROP, retinopathy of prematurity (≥stage II).

mechanical ventilation, hypotension, and PDA in the subsequent logistic regression analysis. We did not observe a significant difference between the NEC and the control group in the rate of exposure to antenatal steroids and therefore we did not control for this exposure.

Analysis of Genotypes

Allele and genotype frequencies of the *VEGF* C-2578A, *IL-18* C-607A, and *IL-4Rα* A-1902G polymorphisms in the total preterm population did not significantly differ from the allele and genotype frequencies observed in the population of 96 healthy term infants (**Table 3**) (18). The distribution of the genotypes of the *VEGF* C-2578A- and the *IL-4Rα* A-1902G polymorphisms in the preterm population and the *IL-4Rα* A-1902G polymorphism in the term population did not fulfill Hardy-Weinberg criteria (**Table 3**). The minor allele frequencies (MAFs) of the studied polymorphisms in the NEC ≥ stage II, and NEC stage III groups were not significantly different from the MAFs observed in the infants without NEC (**Table 4**). The MAF of the *IL-4Rα* A-1902G polymorphism was significantly lower (0.206 vs. 0.331, $P = 0.01$) in the group of infants with the combined outcome NEC or death before 34 weeks PMA than in the infants without the outcome (**Table 4**). The MAFs of the *VEGF* C-2578A, and *IL-18* C-607A SNPS were not significantly different in the group of infants with the combined outcome NEC or death before 34 weeks PMA than in the infants without the outcome (**Table 4**). The distribution of the genotypes of the *VEGF* C-2578A- and the *IL-4Rα* A-1902G polymorphisms in the population of preterm infants without NEC or without the combined outcome NEC/death did not fulfill Hardy-Weinberg criteria (**Table 4**).

We further analyzed the effect of the *VEGF* C-2578A-, *IL-18* C-607A- and *IL-4Rα* A-1902G polymorphism on the occurrence of NEC and NEC or death under different genetic models. Logistic regression analysis could not detect any significant association between the studied polymorphisms and NEC ≥ stage II (**Table 5**), or NEC stage III (**Table 6**) in any of the genetic models. In contrast, the co-dominant, dominant, recessive, and additive model showed a negative significant association of the G-allele of the *IL-4Rα* A-1902G polymorphism with the combined outcome NEC or death before 34 weeks of corrected gestational age (**Table 7**).

DISCUSSION

Given that NEC remains a leading cause of morbidity and mortality, identifying preterm infants at increased risk for developing the condition remains an important but elusive objective. Previous studies suggested an association between the risk of NEC and the SNPs *VEGF* C-2578A, *IL-18* C-607A (14), and *IL-4Rα* A-1902G (15). In the present cohort we could not confirm the association between the *VEGF* C-2578A and *IL-18* C-607A SNPs and the risk of NEC or the combined outcome NEC or death before 34 weeks of corrected gestation. However, the co-dominant, dominant, recessive, and additive models showed

TABLE 3 | Genotype characteristics of the *VEGF* C-2578A, *IL-18* C-607A, and *IL-4 receptor α-chain* A-1902G polymorphism in total preterm study group ($n = 358$) and term control group ($n = 96$).

	Chromosomal location	Allele W/M	Total study group WW/WM/MM alleles (prevalence of mutant allele)	Missing data (*n*)	HWE (*P*-value)	Term control group WW/WM/MM alleles (prevalence of mutant allele)	Missing data (*n*)	HWE in controls (*P*-value)
VEGF C-2578A	6p21.1	C/A	109/155/85 (0.466)	9	0.04*	33/47/16 (0.411)	0	0.92
IL-18 C-607A	11q23.1	C/A	102/177/78 (0.466)	1	0.94	27/48/18 (0.452)	3	0.69
IL-4 R A-1902G	16p12.1	A/G	179/134/45 (0.313)	0	0.01*	56/29/10 (0.258)	1	0.049*

Results are expressed as absolute numbers of patients. Missing data due to technical inability of genotyping sample.
HWE, Hardy–Weinberg Equilibrium; W, wild type allele; M, mutant allele.
*$P < 0.05$.

TABLE 4 | Genotype distribution and minor allele frequency of the *VEGF* C-2578A, *IL-18* C-607A, and *IL-4 R* A-1902G polymorphisms for NEC ≥stage II, NEC stage III and the combined outcome NEC or death before 34 weeks of corrected gestational age.

SNP	Genotype	NEC-no *n* (%)	MAF	NEC (≥stage II)-yes *n* (%)	MAF	NEC (stage III)-yes *n* (%)	MAF	NEC/death-no *n* (%)	MAF	NEC/death-yes *n* (%)	MAF
VEGF C-2578A	CC	101 (31.2)	0.468	8 (32.0)	0.440	2 (20.0)	0.500	95 (31.3)	0.467	14 (31.1)	0.456
	CA	143 (44.1)		12 (48.0)		6 (60.0)		134 (44.1)		21 (46.7)	
	AA	80 (24.7)†		5 (20.0)		2 (20.0)		75 (24.6)†		10 (22.2)	
IL-18 C-607A	CC	96 (29.0)	0.465	6 (23.1)	0.481	5 (45.5)	0.318	85 (27.8)	0.474	17 (33.3)	0.422
	CA	162 (48.9)		15 (57.7)		5 (45.5)		152 (49.7)		25 (49.0)	
	AA	73 (22.1)		5 (19.2)		1 (9.1)		69 (22.5)		9 (17.7)	
IL-4 R A-1902G	AA	165 (49.7)	0.318	14 (53.8)	0.250	7 (63.6)	0.227	147 (47.9)	0.331	32 (62.7)	0.206*
	AG	123 (37.0)		11 (42.3)		3 (27.3)		117 (38.1)		17 (33.3)	
	GG	44 (13.3)††		1 (3.9)		1 (9.1)		43 (14.0)††		2 (4.0)	

SNP, single-nucleotide polymorphism; NEC, necrotizing enterocolitis; MAF, minor allele frequency.
CC denotes homozygosity for the C-encoded VEGF C-2578A and IL-18 C-607A polymorphism variant; AA homozygosity for the A-encoded VEGF C-2578A and IL-18 C-607A polymorphism variant; CA heterozygosity for VEGF C-2578A and IL-18 C-607A polymorphism; AA denotes homozygosity for the A-encoded IL4R A-1902G polymorphism variant; GG homozygosity for the G-encoded IL4R A-1902G polymorphism variant; AG heterozygosity for IL4R A-1902G polymorphism.
$P < 0.05$ vs. NEC/death-no.
†,††$P < 0.05, 0.01$ for deviation of Hardy-Weinberg equilibrium in the disease-free group.

a significant negative association of the G-allele of the *IL-4Rα* A-1902G polymorphism with the combined outcome NEC or death before 34 weeks of corrected gestation.

This study has some limitations that need to be considered. First, we did not investigate the functional consequences of the SNPs. Second, the distribution of the genotypes of the *VEGF* C-2578A- and the *IL-4Rα* A-1902G polymorphisms in the preterm control population and the *IL-4Rα* A-1902G polymorphism in the term population did not fulfill Hardy-Weinberg criteria. Theoretically, disease-free control groups from outbred populations should not deviate from HWE (23). Deviation from HWE may be related to non-random mating, population stratification, selection bias, limited sample size, or genotyping error (23). Of note, deviation from HWE in the control group may increase the chance of detecting a false-positive association, particularly when ORs did not show a strong association (23). This points to the need for caution in interpreting our findings.

VEGF is an angiogenic protein that couples hypoxia sensing to angiogenesis and is necessary for the development and maintenance of capillary networks (9). It is suggested that VEGF-mediated alterations of the intestinal mucosal microvasculature play an important role in NEC pathogenesis (9, 13, 24, 25). In fact, intestinal VEGF protein expression is reduced in human and experimental NEC (9, 26). The *VEGF* gene is highly polymorphic, especially in the promoter, 5′-untranslated and 3′-untranslated regions (27). Some of these SNPs, including the C-2578A, have been related to varying VEGF protein expression and serum VEGFA levels and proposed to be involved in the pathogenesis of various adult diseases (27–30).

As mentioned in the introduction, two previous studies showed that the A allele of the *VEGF* C-2578A SNP increased the risk of NEC in preterm infants (12, 13). Moreover, one of the studies showed that plasma VEGFA levels were significantly lower in carriers of the A allele (13). In contrast, our study could not confirm the association between the *VEGF* C-2578A SNP and NEC. Differences in the inclusion criteria and the ethnic background of the studied populations may account for these different results. The study of Banyasz et al. included infants with all stages of NEC (12), whereas our study and the study of Gao et al. (13) only included infants with confirmed NEC (Bell stage II disease or greater). As highlighted by Cuna et al.,

TABLE 5 | Effects of *VEGF* C-2578A, *IL-18* C-607A, and *IL-4 R* A-1902G polymorphisms on the risk of NEC (≥ stage II) under different genetic models.

SNP	Genotype	Codominant model				Dominant model				Recessive model				Additive model			
		OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
VEGF	CC	1 (Ref.)	–	1 (Ref.)	–	0.96 (0.40–2.30)	0.93	1.08 (0.44–2.67)	0.86	0.76 (0.28–2.10)	0.60	0.88 (0.31–2.49)	0.80	0.90 (0.52–1.57)	0.72	0.99 (0.56–1.76)	0.98
	CA	1.06 (0.42–2.69)	−0.90	1.15 (0.44–3.01)	0.93												
	AA	0.79 (0.25–2.51)	0.69	0.95 (0.29–3.13)	0.86												
IL-18	CC	1 (Ref.)	–	1 (Ref.)	–	1.36 (0.53–3.50)	0.52	1.40 (0.52–3.73)	0.50	0.84 (0.31–2.31)	0.74	0.84 (0.30–2.39)	0.74	1.06 (0.61–1.87)	0.83	1.07 (0.60–1.94)	0.81
	CA	1.48 (0.56–3.95)	0.43	1.52 (0.55–4.23)	0.42												
	AA	1.10 (0.32–3.73)	0.88	1.12 (0.31–3.99)	0.86												
IL-4 R	AA	1 (Ref.)	–	1 (Ref.)	–	0.85 (0.38–1.89)	0.68	0.85 (0.36–1.97)	0.70	0.26 (0.04–1.98)	0.19	0.23 (0.03–1.78)	0.16	0.74 (0.40–1.37)	0.34	0.72 (0.38–1.36)	0.31
	AG	1.05 (0.46–2.40)	0.90	1.09 (0.46–2.61)	0.84												
	GG	0.27 (0.03–2.09)	0.21	0.24 (0.03–1.92)	0.18												

SNP, single-nucleotide polymorphism; NEC, necrotizing enterocolitis (≥stage II); MAF, minor allele frequency; OR, odds ratio; aOR, odds ratio adjusted for birth weight, gestational age, mechanical ventilation, hypotension, and death before 34 weeks.

CC denotes homozygosity for the C-encoded VEGF C-2578A and IL-18 C-607A polymorphism variant; AA homozygosity for the A-encoded VEGF C-2578A and IL-18 C-607A polymorphism variant; CA heterozygosity for VEGF C-2578A and IL-18 C-607A polymorphism; AA denotes homozygosity for the A-encoded IL4R A-1902G polymorphism variant; GG homozygosity for the G-encoded IL4R A-1902G polymorphism variant; AG heterozygosity for IL4R A-1902G polymorphism.

TABLE 6 | Effects of *VEGF* C-2578A, *IL-18* C-607A, and *IL-4 R* A-1902G polymorphisms on the risk of NEC stage III under different genetic models.

SNP	Genotype	Codominant model				Dominant model				Recessive model				Additive model			
		OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
VEGF	CC	1 (Ref.)	–	1 (Ref.)	–	1.81 (0.38–8.68)	0.46	2.76 (0.51–14.9)	0.24	0.76 (0.16–3.67)	0.74	1.20 (0.21–6.74)	0.84	1.13 (0.48–2.62)	0.79	1.56 (0.60–4.08)	0.36
	CA	2.12 (0.42–10.7)	0.36	2.93 (0.51–16.9)	0.23												
	AA	1.26 (0.17–9.16)	0.82	2.38 (0.28–20.2)	0.43												
IL-18	CC	1 (Ref.)	–	1 (Ref.)	–	0.49 (0.15–1.64)	0.25	0.44 (0.12–1.69)	0.23	0.35 (0.05–2.81)	0.33	0.26 (0.03–2.43)	0.24	0.54 (0.22–1.34)	0.18	0.48 (0.18–1.28)	0.14
	CA	0.59 (0.17–2.10)	0.42	0.57 (0.14–2.31)	0.43												
	AA	0.26 (0.03–2.30)	0.23	0.19 (0.02–1.99)	0.34												
IL-4 R	AA	1 (Ref.)	–	1 (Ref.)	–	0.57 (0.16–1.97)	0.37	0.72 (0.18–2.82)	0.64	0.66 (0.08–5.24)	0.69	0.65 (0.07–5.91)	0.70	0.67 (0.26–1.73)	0.41	0.77 (0.29–2.08)	0.61
	AG	0.58 (0.15–2.27)	0.43	0.77 (0.17–3.50)	0.74												
	GG	0.54 (0.06–4.47)	0.56	0.60 (0.06–5.69)	0.65												

SNP, single-nucleotide polymorphism; NEC, necrotizing enterocolitis (stage III); MAF, minor allele frequency; OR, odds ratio; aOR, odds ratio adjusted for weight, gestational age, mechanical ventilation, hypotension, and death before 34 weeks.

CC denotes homozygosity for the C-encoded VEGF C-2578A and IL-18 C-607A polymorphism variant; AA homozygosity for the A-encoded VEGF C-2578A and IL-18 C-607A polymorphism variant; CA heterozygosity for VEGF C-2578A and IL-18 C-607A polymorphism; AA denotes homozygosity for the A-encoded IL4R A-1902G polymorphism variant; GG homozygosity for the G-encoded IL4R A-1902G polymorphism variant; AG heterozygosity for IL4R A-1902G polymorphism.

TABLE 7 | Effects of *VEGF* C-2578A, *IL-18* C-607A and *IL-4 R* A-1902G polymorphisms on the risk of the combined outcome NEC or death before 34 weeks of corrected gestational age under different genetic models.

SNP	Genotype	Codominant model			Dominant model			Recessive model			Additive model		
		OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
VEGF	CC	1 (Ref.)	–	1 (Ref.)	–	1.01 (0.51–1.98)	0.99	1.34 (0.58–3.09)	0.50	0.87 (0.41–1.85)	0.72	1.24 (0.51–3.02)	0.63	0.96 (0.63–1.46)	0.85	1.21 (0.72–2.02)	0.48
	CA	1.06 (0.52–2.20)	0.87	1.29 (0.52–3.17)	0.59												
	AA	0.91 (0.38–2.15)	0.82	1.44 (0.51–4.11)	0.49												
IL-18	CC	1 (Ref.)	–	1 (Ref.)	–	0.77 (0.41–1.45)	0.42	0.73 (0.34–1.56)	0.41	0.74 (0.34–1.59)	0.43	0.66 (0.26–1.65)	0.37	0.81 (0.53–1.24)	0.33	0.77 (0.46–1.27)	0.30
	CA	0.82 (0.42–1.61)	0.57	0.80 (0.36–1.80)	0.59												
	AA	0.65 (0.27–1.55)	0.34	0.58 (0.21–1.63)	0.30												
IL-4 R	AA	1 (Ref.)	–	1 (Ref.)	–	0.55 (0.30–1.00)	0.05	0.44 (0.21–0.92)	0.03*	0.25 (0.06–1.07)	0.06	0.15 (0.03–0.74)	0.02*	0.56 (0.34–0.91)	0.02*	0.46 (0.26–0.80)	0.01*
	AG	0.68 (0.35–1.26)	0.21	0.61 (0.28–1.32)	0.21												
	GG	0.21 (0.05–0.93)	0.04*	0.13 (0.03–0.63)	0.01*												

SNP, single-nucleotide polymorphism; NEC, necrotizing enterocolitis (≥stage II); MAF, minor allele frequency; OR, odds ratio; aOR, odds ratio adjusted for birth weight, gestational age, Apgar score at 1 min, Apgar score at 5 min, mechanical ventilation, hypotension and PDA.

CC denotes homozygosity for the C-encoded VEGF C-2578A and IL-18 C-607A polymorphism variant; AA homozygosity for the A-encoded VEGF C-2578A and IL-18 C-607A polymorphism variant; CA heterozygosity for VEGF C-2578A and IL-18 C-607A polymorphism; AA denotes homozygosity for the A-encoded IL4R A-1902G polymorphism variant; GG homozygosity for the G-encoded IL4R A-1902G polymorphism variant; AG heterozygosity for IL4R A-1902G polymorphism.

*$P < 0.05$.

a precise phenotypic definition of NEC is essential in the design of genetic studies and only confirmed cases of NEC in preterm infants should be included. The study of Gao et al. (13) involved a Chinese Han population and a simple association may not translate to a population of different ethnic backgrounds (31). An important point of consideration is that the Chinese population has a lower prevalence of the A allele of the *VEGF* C-2578A SNP (32–34) than the Caucasian population (28).

Excessive inflammation is a hallmark in the pathogenesis of NEC and, therefore, many investigators have examined the potential association between several SNPs of pro-and anti-inflammatory cytokine genes and NEC (3, 10). Altered IL-18 levels are present in patients with inflammatory bowel disease (35) and IL-18 deficient mice are showed decreased intestinal damage following experimental NEC (36). Alterations of IL-18 production have been associated with the C-607A SNP of the *IL-18* gene (37). Heninger et al. showed in a case-control studies including 136 preterm newborns (46 NEC, 90 control) that the frequency of the AA genotype of the C-607A SNP was higher in infants with stage III NEC compared to those with stages I–II and those without NEC (14). We could not replicate their results in our cohort.

IL-4 is another cytokine with powerful anti-inflammatory actions. Th1-cell proliferation is inhibited by IL-4 and IL-4 opposes the effects of pro-inflammatory cytokines on macrophages. Isolated lamina propria mononuclear cells from inflamed intestine expressed IL-4 mRNA and secreted this cytokine in lower amounts than control cells (38, 39). The *IL-4Rα* A-1902G polymorphism has been shown to affect receptor signaling and the anti-inflammatory effect of IL-4 is more pronounced in peripheral blood mononuclear cells from adults carrying the G-variant of the SNP (40). Trezl et al. showed that carriers of the mutant allele of the *IL-4Rα* A-1902G polymorphism had a lower risk of all stages of NEC (15). In contrast, our study could not demonstrate an association between the *IL-4Rα* A-1902G SNP and the risk of NEC (≥ Bell stage II). Nevertheless, we found a significant negative association of the G-allele of the *IL-4Rα* A-1902G polymorphism with the combined outcome NEC or death before 34 weeks of corrected gestation in all genetic models. We analyzed this combined outcome because epidemiological studies indicate that the time to onset of NEC follows a bimodal distribution with a first peak around at 1 week of age and a second peak around at the end of the third week of life (41, 42). Therefore, early death is a competing outcome for NEC.

As reported by Treszl et al. (15, 38), the *IL-4Rα* G1902 variant does not influence IL-4 levels, but its presence is associated with enhanced transduction of IL-4 signals. This enhanced IL-4 transduction is known to shift the development of lymphocytes to a more pronounced Th2 proliferation. They speculate that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC (15, 38). Accordingly, our data suggest that the minor G variant of the IL-4RαA-1902G SNP might protect against the combined outcome NEC/early death. However, there is still a need for further studies to determine the mechanisms of the protective role of the *IL-4Rα* G1902 variant in NEC.

CONCLUSION

This is one of the largest cohort studies investigating the association of SNPs involved in the homeostasis of intestinal microcirculation and inflammatory response with NEC. We observed that the *IL-4Rα* A-1902G genotype was associated with the risk of developing the combined outcome of NEC or death before 34 weeks PMA. Nevertheless, NEC is a condition with a complex and multifactorial pathogenesis and, therefore, an isolated genetic derangement may not be sufficient to account for the entire complexity of the disease. Many genetic variations associated to prematurity, intestinal function, microcirculatory regulation, inflammatory signaling, or immune defense could protect or increase the susceptibility of preterm infants to NEC (3, 43). The future challenge resides in identifying the haplotypes that confer increased risk for NEC and in understanding their interaction with environmental risk factors. In addition, further investigation is required to determine functional consequences of each SNP.

DATA AVAILABILITY STATEMENT

This article contains previously unpublished data. Data are available upon request to the authors.

ETHICS STATEMENT

The present study was performed on DNA samples collected during a previous study on carbamoyl phosphate synthetase SNPs as risk factor for NEC. The protocol of the above study was reviewed and approved by the Research Ethics Committees of the participating centers and we obtained written informed consent from the parents. The protocol is registered in ClinicalTrials.gov Protocol Registration System (NCT00554866). Additional approval was obtained for the present analysis.

AUTHOR CONTRIBUTIONS

RM participated in the design of the study, collection of data, analysis and interpretation of data, and drafted the first version of the manuscript. MH, GG-L, GC, and FM collected data, participated in the design of the study and the interpretation of the data, and helped to draft the manuscript. EV conceived the study, participated in the design of the study, collection of data, analysis and interpretation of data, and drafted the final version of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00045/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Identification of Two Novel Mutations in *COG5* Causing Congenital Disorder of Glycosylation

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Objective: This study reports a Chinese patient with a Congenital Disorder of Glycosylation (CDG) caused by compound-heterozygous mutations in the Conserved Oligomeric Golgi 5 (*COG5*) gene and thereby offers concrete evidence for early diagnosis.

Methods: The clinical manifestations, the results of laboratory examinations and genetic analysis of a 4-year-old Chinese girl with CDG are reported. We also reviewed previous CDG cases that involved *COG5* mutations by comparing the phenotypes and genotypes in different cases.

Results: The patient was admitted to our hospital due to ataxia and psychomotor delay. The major clinical manifestations were postural instability, difficulty in walking, psychomotor delay, hypohidrosis, hyperkeratosis of the skin, and ulnar deviation of the right-hand fingers. Biochemical analyses revealed coagulation defect and liver lesions. Vision tests showed choroidopathy and macular hypoplasia. Whole-exome sequencing identified the hitherto unreported compound-heterozygous *COG5* mutations, c.1290C > A (p.Y430X) and c.2077A > C (p.T693P). Mutation p.Y430X is nonsense, leading to a truncated protein. Mutation p.T693P is located at a highly conserved region, and thus the polar-to-non-polar substitution presumably affects the structure and function of COG5. According to the Human Genome Mutation Database Professional, there have been totally 13 CDG cases caused by 13 *COG5* mutations. They are mainly characterized by psychomotor delay, hypotonia, ataxia, microcephaly, and hearing and visual abnormalities.

Conclusion: The clinical manifestations of the patient are mild but consistent with the clinical characteristics of the published COG5-CDG cases. The results of this study extend the spectrum of clinical and genetic findings in COG5-CDG.

Keywords: *COG5* gene, congenital disorder of glycosylation, psychomotor delay, ataxia, visual abnormalities, genetic sequencing

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BACKGROUND

Congenital Disorders of Glycosylation (CDGs) represent a group of metabolic disorders resulting from defects in the glycosylation of proteins. Most of CDGs are autosomal recessive, but autosomal dominant and X-linked ones have also been reported. The classification of CDGs depend on the defective enzyme and its function. More than 130 types of CDGs characterized to date involve

enzymes participating in various steps along glycosylation pathways (Chang et al., 2018). Conserved Oligomeric Golgi 5 (COG5) is a subunit of the COG complex which functions in trafficking and glycosylation as well as the maintenance of structure of Golgi apparatus (Blackburn et al., 2019). The deficiency of COG5 leads to COG5-CDG, which is a rare type and usually caused by mutations in the *COG5* gene. Its clinical manifestations may include neurological problems among other abnormalities and the pathogenesis is unclear. Only 7 of 13 cases have provided clinical information in detail to date. Here, we report a 4-year-old Chinese patient carrying two novel compound-heterozygous mutations in *COG5*, whereby we expand the mutation database.

CASE PRESENTATION

The patient was admitted to the department of preventive health care in the Children's Hospital of Capital Institute of Pediatrics due to ataxia and psychomotor delay. She was the first child of her parents, and her mother had no pregnancy before. The birth occurred 38 weeks post-gestation. The height and weight of the patient at birth were 49 cm and 3000 g, respectively. She presented with neonatal jaundice but it completely subsided 35 days after birth. In addition, the patient had recurrent upper respiratory infections approximately five times a year.

Upon admission, her height, weight and BMI were 104.7 cm (35th percentile), 18.1 kg (76th percentile), and 17.42 kg/m^2 (91st percentile), respectively. Her head circumference was 48 cm. She had convergent strabismus and could not respond to any communication attempt. Hypohidrosis, hyperkeratosis of the skin at the dorsa of the hands and ulnar deviation of the right-hand ring finger and litter finger (**Figure 1A**) were identified, but no hypotonia was observed in the examination. We could not perform an ataxia text due to the psychomotor delay of the patient.

Biochemical examinations showed the elevated ALT and AST levels (ALT 48.3 U/L and AST 55.3 U/L, respectively; reference values [r.v.] are 0–40.0 U/L), but liver ultrasound revealed nothing abnormal. The activated partial thromboplastin time (APTT) was 40 s ([r.v.] 24–37 s). The lymphocyte count and immunoglobin level of the patient were normal. No abnormalities were found in brain MRI (**Figure 1B**).

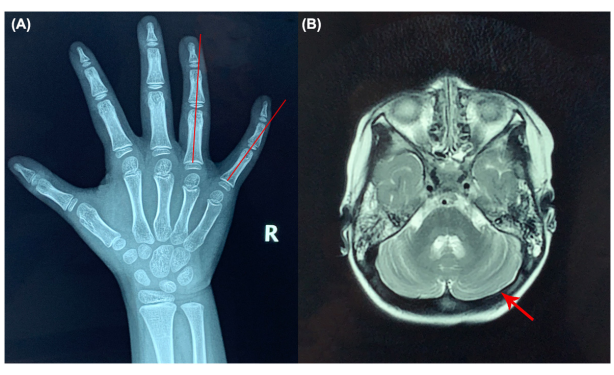


FIGURE 1 | X-ray of the right hand and brain MRI of the patient. **(A)** Ulnar deviation of the right-hand ring finger and little finger (red lines). **(B)** Brain MRI results (red arrow shows the cerebella).

The Gesell Development Scale (Conoley and Impera, 1995) results revealed that the developmental quotient of the patient was 43 and her intelligence age was 23.4 months. Her Clancy Autism Behavior Scale (CABS) (Zachary Warren and Howell Dohrmann, 2013) and Infants-Junior High School Students' Social Life Abilities Scale (S-M scale) (Zhang et al., 1995) were 9 and 10, respectively.

METHODS

To confirm the diagnosis, genetic sequencing was performed with 5-ml peripheral blood samples from the patient and her parents. The samples were collected in our hospital and then sent to Running Gene Inc. (Beijing, China) for sequencing. DNA samples were extracted and purified by DNA Isolation Kit (AU1802, Bioteke) and Agencourt AMPure XP kit (Beckman Coulter, Inc., United States), respectively. Hybridization was performed using the IDTxGen Exome Research Panel v1.0 (Integrated DNA Technologies, Inc., United States). Targeted DNA samples were sequenced by the Illumina Novaseq 6000 system (Illumina, United States), and quality control was applied to the raw data (stored in FASTQ format) by Illumina Sequence Control Software (SCS). High-quality data were aligned to the human reference genome sequence hg19[1] using BWA tool[2]. Consensus single nucleotide variants (SNV) and insertion/deletions (INDEL) were filtered via GATK[3]. All SNV and INDEL information was annotated by the software ANNOVAR[4]. The pathogenicity of candidate mutations was analyzed based on the American College Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015). Sanger sequencing was used to validate the family segregation of mutations.

The expression level of COG5 proteins has been demonstrated by western blot. Leukocytes of the patient and control were extracted from their peripheral blood samples by human peripheral blood leukocyte separation kit (P8670, Solarbio, China). Extracted cells were mixed with RIPA buffer and broken by ultrasonication. After centrifugation, the concentrations of supernatants were measured by BCA protein assay kit (CW0014S, Cowin Bio, China). Proteins were separated by SDS-PAGE and immunoblotted. Signals were detected by ECL kit (P0018FM, Beyotime, China), and the quantification was performed using ImageJ2 (Rueden et al., 2017). Anti-COG5 (ab229830), anti-GAPDH (ab8245), goat anti-rabbit IgG H&L (HRP) (ab205718), and goat anti-mouse IgG H&L (HRP) (ab205719) were purchased from Abcam, United Kingdom. All antibodies were diluted as 1:1000.

[1]http://genome.ucsc.edu/
[2]http://bio-bwa.sourceforge.net/
[3]https://software.broadinstitute.org/gatk/
[4]http://annovar.openbioinformatics.org/en/latest/

RESULTS

Laboratory Examinations

An extension of APTT revealed defective coagulation. Lymphocyte count and quantitation of immunoglobin level indicated a normal immune system. Acoustic impedance tests showed normal results. Multispectral retinal image analysis of binoculus revealed abnormal fluorescence on the retina and mass shadows in the macular region of the choroid (**Figure 2A**). Optical Coherence Tomography (OCT) detected no fovea centralis in the center of the macula lutea of the retina (**Figure 2B**). Pattern visual evoked potential (PVEP) test revealed that the latencies of P100 waves at 1-degree checks were roughly normal but the P100 amplitudes were moderately reduced in both eyes; the latencies at 0.25-degree checks were significantly longer, and the amplitudes were severely reduced in both eyes, suggesting choroiditis in the retina and hypoplasia in the macula lutea (**Figure 2C**).

Genetic Sequencing

We identified two novel compound-heterozygous mutations in the *COG5* gene (NM_006348.3). Sanger sequencing of the DNA samples from the parents of the proband validated this result (**Figures 3A–D**). She inherited the nonsense mutation, c.1290C > A (p.Y430X), from her father and the missense mutation, c.2077A > C (p.T693P), from her mother. According to the Human Genome Mutation Database Professional version (HGMD Pro, 20190711 updated) (Richards et al., 2015), neither of these mutations has been reported before.

Western Blotting

The expression levels of COG5 proteins and GAPDH were shown by western blotting (**Figure 3F**). The protein levels were quantified and normalized (**Figure 3G**). It illustrated that the expression level of COG5 proteins in the patient was lower compared with a healthy control. Meanwhile, a band with smaller size (around 45 kDa) showed a stronger signal in the patient's sample but control.

DISCUSSION

Congenital Disorders of Glycosylation are disorders in the glycosylation of proteins, classified as CDG I and II. CDG I encompasses defects in the biosynthesis of lipid oligosaccharide chains or in the transformation of sugar chains to protein precursors, whereas CDG II involves defects in subsequent trimming of the oligosaccharide chains and glycosylation of terminal sugars (Wu et al., 2004; Kim et al., 2017). COG5-CDG is a subtype of CDG II, affecting modification pathways of N-linked oligosaccharide (Sparks and Krasnewich, 2017). The COG complex, as a membrane protein, plays an essential role in the maintenance of the Golgi structure, glycosylation of proteins and retrograde transport of vesicles in the Golgi apparatus (Zeevaert et al., 2008; Jaeken, 2011; Blackburn et al., 2019). The COG complex is composed of 8 subunits, including lobe A (from subunit COG1 to COG4) and lobe B (from subunit COG5 to COG8) (Rymen et al., 2012). Lobe A and B bind each other via interactions between subunits COG1 and COG8. In humans, mutations in gene *COG1*, *COG4*, *COG5*, *COG6*, *COG7*, and *COG8* have been reported to be associated with CDG (Wu et al., 2004; Foulquier et al., 2006; Foulquier et al., 2007; Reynders et al., 2009; Rymen et al., 2015; Kim et al., 2017).

In this study, the patient carried two novel mutations in *COG5*, c.1290C > A (p.Y430X) and c.2077A > C (p.T693P). The nonsense mutation p.Y430X presumably leads to the loss-of-function of gene *COG5* (PVS1) and is extremely infrequent in GnomAD, ExAC and 1000 Genomes (PM2) (Genomes Project et al., 2015; Lek et al., 2016; Karczewski et al., 2019). Several mutations downstream of p.Y430X have previously been reported as disease-causing, including p.V594F, p.I640T, p.P775L, and p.E840X. Since p.Y430X is a nonsense mutation, a truncated COG5 protein that lacks residues V594, I640, P775, and E840, forms and likely causes the disease. Therefore, p.Y430X is classified as "likely pathogenic," according to the ACMG guidelines. Moreover, the missense p.T693P is absent from 1k Genome database and extremely infrequent in ExAC and GnomAD databases (PM2). Additionally, it was located in *trans* with p.Y430X in the patient (PM3). Thus, p.T693P is considered as a variant of uncertain significance (VUS). However, this missense mutation was in a highly conserved region (**Figure 3E**), indicating the importance of the mutated residue. Whereas threonine is an uncharged polar amino acid, but Proline carries a charged residue. Thus, the alteration of the residue characteristics might impair the structure and functions of the final product. We considered both p.Y430X and p.T693P are disease-causing mutations.

As shown in **Figures 3F,G**, the expression levels of COG5 proteins were declined in the patient, who carried two mutations. Based on the location of the nonsense mutation (p.Y430X) and the size of COG5 (93kDa) and the small band (45kDa), we considered it as the aberrant COG5 protein. Our reasonable speculation is supported by previous research which also identified a small band around 40kDa in the patient with p.M403IfsX3 (Kim et al., 2017). The amino acid sequence of the smaller protein could be determined to find out whether or not it is the truncated COG5 in the further research. Although we cannot prove it is the aberrant COG5 for now, the results from western blot indeed indicate the deficiency of normal COG5 in our patient.

According to the HGMD pro, there have been totally 13 CDG cases caused by 13 COG5 mutations. Only 8 COG5-CDG cases carrying 10 COG5 mutations have been reported in detail to date, including the one characterized in this study (**Table 1**) (Paesold-Burda et al., 2009; Fung et al., 2012; Rymen et al., 2012). The patients in all the cases had intellectual disability (ID) and developmental delay, and some patients suffered from unstable gait and delayed speech functions, similar to the manifestations observed in this study. Most of the patients had hypotonia (7/8). Four of patients also had abnormal MRI results, including myelination delay, cerebellar atrophy and reduced white matter. However, these symptoms are absent in the case reported here, and the brain MRI showed a generally normal result. These

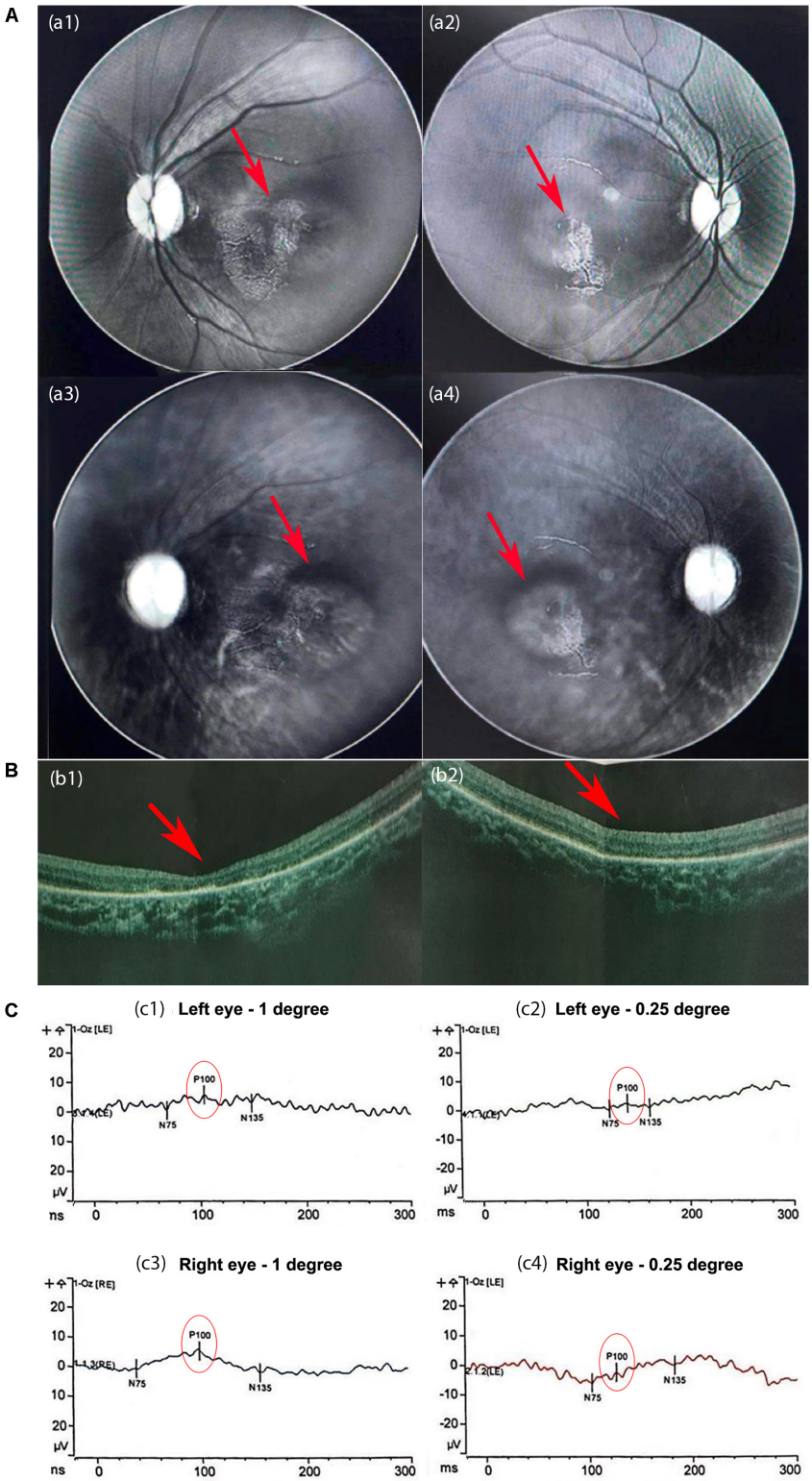


FIGURE 2 | The results of the eye examination. **(A)** Multispectral retinal image analysis. Abnormal fluorescence on the left **(a1)** and right **(a2)** retinas. Mass shadows in the macular region of the choroid in the left **(a3)** and right **(a4)** eyes. **(B)** Optical coherence tomography (OCT) images. The fovea centralis was absent from the center of the macula lutea of the left **(b1)** and right **(b2)** retinas (red arrows). The retinal pigment layers were intact. **(C)** The results of the pattern visual evoked potential (PVEP). At 1-degree checks, the latencies of P100 were normal but the amplitudes of P100 were moderately reduced in the left eye **(c1)** and slightly in the right eye **(c3)**. **(c2**,**c4)** At 0.25-degree checks, the latencies of P100 were significantly longer, and the amplitudes were severely reduced in both eyes.

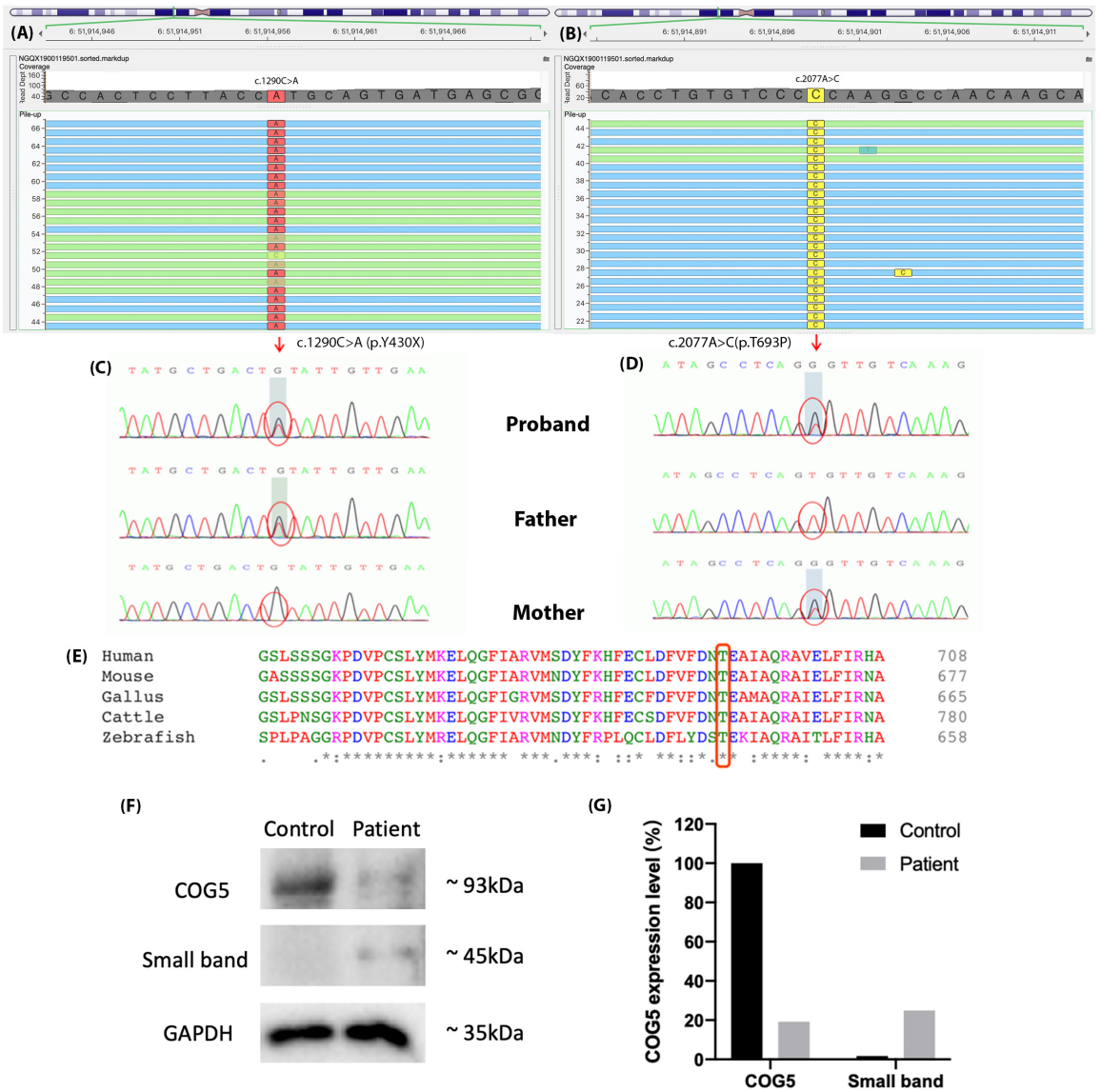


FIGURE 3 | The results of genetic sequencing. Two mutations were identified in *COG5*, **(A)** c.1290C > A (p.Y430X) and **(B)** c.2077A > C (p.T693P). **(C**,**D)** The father of the proband carried mutation c.1290C > A (p.Y430X), and her mother carried c.2077A > C (p.T693P). The compound-heterozygous mutations in the proband were inherited from her parents. **(E)** Thr693 is highly conserved across species and located in a highly conserved region. **(F)** Expression levels of COG5 (93 kDa), small band (around 45 kDa) and GAPDH (Reference, 35 kDa) were shown by western blotting. **(G)** Quantification of the protein levels. The expression level of COG5 proteins was lower in the patient compared with control. A unknown small band appeared in the patient's sample but control.

observations are consistent with the statement that hypotonia might be correlated with brain lesion (Paesold-Burda et al., 2009; Fung et al., 2012; Rymen et al., 2012).

Other clinical manifestations are microcephaly (6/8), visual abnormalities (strabismus, cortical blindness, etc.) (5/8), neurogenic bladder (4/8), ataxia (3/8), liver lesion (3/8), and facial dysmorphism (2/8). Microcephaly was absent in this case. Since hearing and visual abnormalities, such as sensorineural deafness, strabismus and cortical blindness have been reported in COG5-CDG patients before (Rymen et al., 2012), we assessed the hearing and visual capabilities of the patient. Convergent strabismus, choroidopathy, and macular hypoplasia were diagnosed. Since there have hitherto been 5/8 patients diagnosed with visual abnormalities, we herein recommended a regular ophthalmic examination upon admission of COG5-CDG patients and follow-ups every year. The examination is widely used in other CDG patients. The major ophthalmological manifestations of PMM2-CDG (phosphomannomutase2-CDG, the commonest type) are also strabismus, nystagmus, pigmentary retinopathy, reduced visual acuity, and myopia (Altassan et al., 2019). All these symptoms may share a similar unknown pathogenic mechanism with COG5-CDG. Although there is no

TABLE 1 | Genetic and clinical features of COG5-CDG patients whose data are available.

Case	Ref	M/F	Age of onset	Race	Mutation	Allele frequency in GnomAD	ID/ development delay 8/8	Hypotonia 7/8	Brain MRI 4/8	Microcephaly 6/8	Vision abnormalities 5/8	Neurogenic bladder 4/8	Ataxia 3/8	Liver lesion 3/8	Facial dysmorphism 2/8	Others
1	Present case	F	4 years	Chinese	c.2077A > C, p.T693P; c.1290C > A, p.Y430X	0.000004; 0.000012	+/+	−	−	−	Choroidopathy; hypoplasia of macula; convergent strabismus	−	+	Increased ALT and AST levels	−	Hypohidrosis, hyperkeratosis; deviation of finger; coagulation defect
2	Paesold-Burda et al. (2009)	F	8 years	Iraqi	c.1669-15A > G	0	+/+	+	Diffuse atrophy of cerebellum and brain stem	−	Ocular motor apraxia	−	+	−	−	−
3	Fung et al. (2012) and Rymen et al. (2012) P2	F	1 month	Chinese	c.556_560 delAGTAAinsCT; c.1919T > C, p.I640T; c.95T > G, p.M32R	0; 0.00156037; 0.0000323206	+/+	+	Delayed myelination	+	−	−	−	Liver cirrhosis; mild hepato-splenomegaly	−	Mild thrombocytopenia; persistent mild hyperlactacidemia; portal hypertension; fixed flexion contractures of all fingers
4	Rymen et al. (2012) P1.1	F	1 year	Moroccan	c.2518G > T, p.E840X	0	+/+	+	Global decrease of white matter; enlarged lateral ventricles	+	−	−	−	−	Posteriorly rotated, low set ears, a prominent nose and low hair line	−
5	Rymen et al. (2012) P1.2	F	N/A	Moroccan	c.2518G > T, p.E840X	0	+/+	+	N/A	+	−	+	+	−	−	Slight dysmorphism; autistic behavior
6	Rymen et al. (2012) P1.3	F	8 months	Moroccan	NA	−	+/+	+	−	+	Strabismus	+	−	−	−	Autistic behavior
7	Rymen et al. (2012) P3	M	3 months	Italian	c.189delG, p.C64Vfs*6; c.2338_2340 dupATT, p.I780dup	0.0000322872; 0	+/+	+	Severe supra- and subtentorial brain atrophy	+	Strabismus; cortical blindness	+	N/A	hepatomegaly	−	Sensorineural deafness; recurrent urinary tract infection; spastic quadriplegia; scoliosis
8	Rymen et al. (2012) P4	M	At birth	Belgian	c.1780G > T, p.V594F	0	+/+	+	−	+	Cortical blindness	+	N/A	−	Low set, posteriorly rotated ears, a prominent nose with a broad root and retrognathia	Sensorineural deafness; hypohidrosis, epilepsy; micropenis with cryptorchidism; campodactyly and clinodactyly; flexion contractures of knees and elbows

NA = not available. All mutations are stated as in NM_006348.

targeted therapy for these visual abnormalities, eyeglasses or eye surgery can be applied to correct visual acuity.

Moreover, ALT and AST levels were elevated in the patient presented here, suggesting a metabolic disorder in the liver. The liver is the major location of glycosylation, and the majority of glycosylated serum proteins are generated by it. The dysfunction of glycosylation can impair the structure and functions of the liver, elevating the mRNA and protein levels of ALT and AST and subsequently exacerbating the liver lesion (Marques-da-Silva et al., 2017). Thus, the impaired liver function is commonly observed in CDG patients. Although there is no radical cure, associated complications, such as ascites, liver failure, bleeding tendency and hepatopulmonary syndrome can be treated. Therefore, assessment of liver function and imaging of the abdomen are suggested to be regularly performed for prevention and early diagnosis.

In addition, glycosylation plays critical roles in the maturation of immune cells and the recognition between antibodies and antigens, modulating innate and adaptive immunities (Rudd et al., 2001). Recurrent infections have been found in COG6-CDG patients with simultaneous T-/B-cell and neutrophil dysfunctions (Huybrechts et al., 2012). Recurrent infections were noted in the present case (upper respiratory tract) and case 7 (urinary tract) as well. However, the immune system of the patient reported here had no defect. Furthermore, no immune deficiency has been reported in any COG5-CDG patients.

The patient described in this study is the only COG5-CDG patient reported to have a coagulation defect. Previously, coagulation disturbance has been presented in MPI-CDG, ALG12-CDG, ALG2-CDG, ALG1-CDG, and B4GALT1-CDG, and it has been one of the most commonly reported clinical symptoms in CDG patients. Both bleeding and thrombosis risks are high in CDG patients due to the altered levels of serum coagulation factors, such as factors IX and XI, Protein C, Protein S, and antithrombin III. However, the internal mechanism is still elusive, and there is no targeted treatment. Thus, it is strongly recommended that the coagulation statues of CDG patients are regularly monitored (Krasnewich et al., 2007).

In our study, the child had mild symptoms, mild ID and ataxia without hypotonia, microcephaly and facial dysmorphism. The clinical characteristics among COG5-CDG patients revealed a massive difference from mild ID and normal facies to severe ID, hypotonia, microcephaly, facial dysmorphism and multiple organ involvement. The declined protein levels of COG5 and COG7 has been identified via western blot in COG5-CDG patients with different clinical features, and the patients with severe symptoms had clinical manifestations overlapped with COG7-CDG patients (Rymen et al., 2012). Since protein COG5 and COG7 are responsible for the formation of the stable complex lobe B, the interactions of protein levels might affect the severity of patients' phenotypes.

Currently, there is no specific therapy for COG5-CDG patients. However, following symptomatic treatments can be performed: surgical treatment of patients who have finger contracture affecting finger function, gastrostomy or nasal feeding of children who suffer from feeding difficulties, and bladder ostomy on patients who have neurological bladder. No death has been reported in any of the cases we reviewed. In 4/8 cases, the intelligence level gradually improved, and the myelination delay was improved in the present case (Jaeken, 2011; Fung et al., 2012; Rymen et al., 2012). The follow-up visit of the patient reported here revealed that her intelligence development progressed after a 2-month intervention.

To date, 8 CDG cases with detailed clinical data have been reported to be associated with *COG5* mutations. Here, we diagnosed a 4-year-old Chinese girl as COG5-CDG based on clinical manifestations and whole-exome sequencing. The clinical manifestations of this patient were mild ID, ataxia, convergent strabismus, choroidopathy, macular hypoplasia, coagulation defect, and elevated AST and ALT levels. All these manifestations were mild but consistent with the clinical characteristics of described COG5-CDG cases. Genetic sequencing can offer concrete evidence for early assessment, diagnosis and treatment for CDG patients with similar symptoms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Children's Hospital of Capital Institute of Pediatrics. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XW and LW designed the study and cared the patient. XW and LH wrote the manuscript. XW, LH, X-ML, and C-HJ collected and analyzed the clinical data and published cases. LH contributed to the experiment. XW, LH, and LW reviewed the manuscript. LW, X-YW, and J-HW supervised this study. All authors read and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any other commercial or financial relationships that could be construed as a potential conflict of interest.

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Genotype and Phenotype Analysis of Chinese Children With Tuberous Sclerosis Complex: A Pediatric Cohort Study

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Tuberous sclerosis complex (TSC) is a genetic condition characterized by the occurrence of hamartomatous wounds stemming from the dysfunction of the mammalian target of rapamycin (mTOR) pathway. We investigated the clinical phenotypes and genetic variants in 243 unrelated probands and their families in China. Exome sequencing, targeted sequencing or multiplex ligation-dependent probe amplification (MLPA) was performed in 174 children with TSC, among whom 31 (17.82%) patients/families were identified as having pathogenic or likely pathogenic variants in the *TSC1* gene, 120 (68.97%) as having pathogenic or likely pathogenic variants in the *TSC2* gene and 23 (13.21%) as having no pathogenic or likely pathogenic variants identified (NMI). In the 31 patients with pathogenic or likely pathogenic *TSC1* variants, 10 novel variants were detected among 26 different variants. In all 120 patients with *TSC2* variants, 39 novel variants were found among a total of 107 different variants. We compared the phenotypes of the individuals with *TSC1* pathogenic variants, *TSC2* pathogenic variants and NMI. Patients with *TSC2* variants were first diagnosed at a younger age ($p = 0.003$) and had more retinal hamartomas ($p = 0.003$) and facial angiofibromas ($p = 0.027$) (age $\geq$ 3 years) than individuals with *TSC1* variants. Compared with individuals with *TSC1/TSC2* pathogenic variants, NMI individuals had fewer cortical tubers ($p = 0.003$). Compared with individuals with *TSC1* pathogenic variants, NMI patients had more retinal hamartomas ($p = 0.035$), and compared with individuals with *TSC2* pathogenic variants, they had less epilepsy ($p = 0.003$) and fewer subependymal nodules (SENs) ($p = 0.004$).

Keywords: tuberous sclerosis complex, genotype, phenotype, children, Chinese

Abbreviations: MLPA, multiplex ligation-dependent probe amplification; mTOR, mammalian target of rapamycin; NMI, no variants identified; SDs, standard deviations; SENs, subependymal nodules; TAND, tuberous sclerosis-associated neuropsychiatric disorders; TSC, tuberous sclerosis complex; WES, whole-exome sequencing.

INTRODUCTION

As an autosomal dominant disease, tuberous sclerosis complex (TSC, OMIM #191100, #613254) is characterized by a wide range of disease severities involving hamartomatous tumors in multiple organs. The incidence of TSC is 1 in 6,000–10,000 live births, and it affects nearly 2 million individuals worldwide (Curatolo et al., 2008; Peron and Northrup, 2018). TSC is the result of mutations in either the *TSC1* (OMIM #605284) or *TSC2* (OMIM #191092) gene, triggering the hyperactivation of the mechanistic target of rapamycin (mTOR) signaling pathway, and subsequent cell proliferation deregulation (Cheadle et al., 2000; Crino et al., 2006; Henske et al., 2016; Franz and Krueger, 2018).

In this study, we combined the latest TSC gene testing with the clinical data of patients to evaluate the phenotype-genotype correlation.

MATERIALS AND METHODS

Patients

A retrospective chart review was carried out at the Children's Hospital of Fudan University. The review included children (age < 16 years) with TSC seen between August 2013 and July 2018. In total, 243 unrelated probands met the clinical diagnostic criteria for TSC (Northrup and Krueger, 2013). Exome sequencing or targeted sequencing was performed in 174 probands and their families. Our research was carried out in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Fudan University Children's Hospital. Signed informed consent was provided by the patients' parents.

Clinical Data

We studied the clinical information obtained from the medical records and phone calls with the families, including information on each patient's birth history, family history, age at seizure inception, seizure forms, treatments, neurological development, central nervous system manifestations, renal disease, cutaneous manifestations, and tuberous sclerosis-associated neuropsychiatric disorders (TAND).

Genetic Analysis

Extraction of genomic DNA from whole blood of patients and their patients was performed using the Agilent (Santa Clara, CA, United States) SureSelectXT Human All Exon 50 Mb kit according to the manufacturer's instructions. *TSC1* and *TSC2* variants were detected in individuals by clinical exome sequencing (emphasis on 2742 genes) or whole-exome sequencing (WES). Our databank and public databases (the exome aggregation consortium, the 1000 Genome Project, and dbSNP137 reported in the UCSC Genome Browser) were utilized for variant screening. Candidate variants were verified using Sanger sequencing. Detection of significant deletions/repeats in TSC1 and TSC2 was performed using multiplexed-dependent probe amplification (MLPA) (MRC-Holland, Probemix *TSC2* P046, Probemix *TSC1* P124) following the manufacturer's instructions. The variants were considered to be pathogenic on the basis of the following (Richards et al., 2015; Yang et al., 2018): (1) this variant would likely explain the indication for TSC and may be responsible for the clinical manifestation; (2) there is a null variant (non-sense, frameshift, canonical ± 1 or 2 splice sites, initiation codon, single or multiexon deletion) in the *TSC1/TSC2* gene causing a loss of function (LOF); or the same amino acid change as a previously established pathogenic variant regardless of nucleotide change is a known mechanism of TSC; and (3) the mutation is inherited from the affected parents or is *de novo* (both maternity and paternity confirmed) in the proband with a negative TSC family history.

Statistical Analysis

Continuous variables are expressed as medians and ranges or as the means and standard deviations (SDs), while categorical data are presented as percentages and numbers where appropriate. TSC individuals were classified into the following three groups according to the results of the molecular analyses: variants in *TSC1*, variants in *TSC2*, and NMI. Then, we compared the clinical characteristics among the three groups using the Kruskal-Wallis tests for continuous variables and Fisher's exact tests for categorical data. P values < 0.05 were considered statistically significant. Analyses were performed using Stata 15.1[1].

RESULTS

Cohort Characteristics

We identified and enrolled 174 unrelated patients with a definite clinical diagnosis of TSC. There were 88 (50.57%) females and 86 (49.43%) males. The median age at first presentation was 24.35 months (interquartile range: 9.56–68.83 months), and the median duration of follow-up was 28.81 months (interquartile range: 14.09–51.35 months). Thirty-one patients (17 males and 14 females; median age at first presentation: 34.79 months) had variants in the *TSC1* gene; 120 (58 males and 62 females; mean age at first presentation: 21.70 months) had variants in *TSC2* (*TSC2: TSC1* = 7.06:1); and 23 (11 males and 12 females; mean age at first presentation: 19.15 months) had NMI.

Clinical Outcomes

Phenotype data regarding the major and minor diagnostic criteria for TSC and neurobehavioral features were collected from 174 affected individuals who met the criteria for a definite diagnosis of TSC. There are myriad neurologic sequelae of TSC, including neurodevelopmental disorders, epilepsy, and intellectual impairment, as well as many structural lesions. The most common manifestation leading to a diagnosis of TSC was the presence of epilepsy (140/174, 80.46%). During the follow-up period, a total of 148/174 (85.06%) patients suffered from epilepsy (*TSC1*:*TSC2*: NMI = 24:109:15), with 65.54% of those patients manifesting epilepsy within the first year of life. The seizure manifestations were variable, and 27.70% of patients

[1]www.stata.com

presented with more than one seizure type. In our cohort, 44.59% (66/148) of the patients had infantile spasms, 52.03% (77/148) had focal seizures, and 33.11% (49/148) had another type of seizure. On average, patients were treated with ≥2 AEDs (median: 2; range: 0–7).

Compared with the general population, patients with TSC are at higher risk of developing neuropsychiatric disorders (de Vries et al., 2018). According to DSM-5, we defined all patients with IQ < 70 as intellectual disability (ID) patients, which accounted for 65.52% (114/174) of the population, including those with mild ID (IQ 51–70) and moderate ID (IQ 36–50), who accounted for 50% (87/174), and those with severe ID (IQ 20–35) and profound ID (IQ < 20), who accounted for 15.52% (27/174). In 97.70% (170/174) of the patients in this cohort, brain imaging abnormalities were found. Cortical tubers were found in up to 93.68% (163/174) of the patients and were located throughout all regions of the cortex and superficial white matter, especially the frontal lobe. As a major clinical feature of TSC, subependymal nodules (SENs) were found in up to 91.95% (160/174) of the patients, especially in the ependymal lining of the lateral ventricles. SEGA was diagnosed in 9/174 (5.17%) of the patients.

Retinal astrocytic hamartomas were detected in 38.89% (35/90) of the patients and were nearly universally benign.

In our cohort, 43.68% (76/174), 95.40% (166/174), 32.76% (57/174) and 3.05% (5/164) of TSC patients had facial angiofibromas, hypomelanotic macules, shagreen patches, and periungual fibroma, respectively. The presence of multiple facial angiofibromas in adolescence is characteristic of TSC.

Moreover, we found 61 patients with cardiac rhabdomyoma (35.06%) and 9 patients with liver hamartoma (5.17%).

Genetic Analysis

In our study, 133 pathogenic or likely pathogenic variants were identified. Of these, 36.84% (49/133) were novel. Among all of the variants, most *TSC1* variants were truncating mutations, including non-sense mutations (38.71%) and frame shifts (29.03%), and most of the variants were located in crucial regions (N-terminal protein interaction domain and tuberin interaction coiled-coil domain). Most *TSC2* variants were missense mutations (24.17%), non-sense mutations (25.00%) and frame shifts (22.50%), and most of the variants were located in crucial regions (N-terminal hamartin interaction GTPase activation protein domain) (**Table 1**).

A total of 31 patients (17.82%) had pathogenic variants or likely pathogenic variants of *TSC1*, 120 (68.97%) had pathogenic variants or likely pathogenic variants of *TSC2*, and 23 (13.21%) had NMI. In the *TSC1* group, we found 10 (39.29%) novel variants and 16 (60.71%) known variants. In our cohort, exon 15 of the *TSC1* gene had a relatively high probability of mutation (15/31, 48.39%). In the *TSC2* group, 39 novel pathogenic or likely pathogenic variants (36.45%) and 68 known variants (63.55%) were found. Among the 42 exons in the *TSC2* gene, exon 34 (13/120, 10.83%) and exon 41 (17/120, 14.17%) of the *TSC2* gene had relatively high mutational probabilities (**Supplementary Table 1**).

TABLE 1 | Description of the types of mutations detected (*n* = 151).

	TSC1		TSC2	
Mutation type	**n**	**%**	**n**	**%**
Missense	5	16.13	29	24.17
Non-sense	12	38.71	30	25.00
Splicing	2	6.45	17	14.17
Frame shift	9	29.03	27	22.50
Small deletion	1	3.23	8	6.67
Large deletion	2	6.45	7	5.83
Deletion-insertion	0	0	2	1.66
Total	31	100.00	120	100.00

TSC1, patients with TSC1 mutation; TSC2, patients with TSC2 mutation.

Genotype-Phenotype Correlations in Patients With TSC

The main features of the three groups (*TSC1, TSC2,* and NMI) were compared in 174 patients who underwent complete genetic testing (point mutations and deletions/repetitions), as shown in **Table 2**. Compared with individuals with *TSC1* pathogenic variants, individuals with *TSC2* pathogenic variants were first diagnosed at a younger age ($p = 0.003$) and had more retinal hamartomas ($p = 0.003$) and facial angiofibromas ($p = 0.027$) (age ≥ 3 years). Compared with individuals with *TSC1/TSC2* pathogenic or likely pathogenic variants, NMI individuals had fewer cortical tubers ($p = 0.003$). Compared with individuals with *TSC1* pathogenic of likely pathogenic variants, NMI individuals had more retinal hamartomas ($p = 0.035$), and compared with individuals with *TSC2* pathogenic of likely pathogenic variants, NMI individuals were less likely to have epilepsy ($p = 0.003$) and had fewer SENs ($p = 0.004$).

After comparing multiple clinical phenotypes in individuals with different *TSC1/TSC2* mutation types, we found no significant differences in CNS, cutaneous manifestations, cardiac rhabdomyoma, renal system disorders, or TAND (**Table 3**).

DISCUSSION

Tuberous sclerosis is a hereditary multisystem disease characterized by hamartomas in multiple organs, including the skin, brain, liver, kidneys, heart and eyes. After the discovery of the involvement of the *TSC1* and *TSC2* genes in TSC in 1997 and 1993, respectively, the genetic basis of the disease was studied to better understand its clinical manifestations. Unfortunately, despite the continuous development of new molecular detection techniques, understanding the complex clinical manifestations of TSC that predict the observed individual clinical phenotypes is still challenging. TSC1 and TSC2 encode hamartin and tuberin, respectively, which form heterodimers that act synergistically to regulate cell growth and proliferation (Plank et al., 1998; van Slegtenhorst et al., 1998; Han and Sahin, 2011). *TSC1* and *TSC2* variants are thought to result in a loss of function, leading to multiple organ hamartias and hamartomas (Au et al., 2004). In our cohort, all patients fulfilled the current diagnostic criteria for

TABLE 2 | Comparison of clinical manifestations among *TSC1*, *TSC2*, and NMI patients.

Phenotype	*TSC1*	*TSC2*	NMI	*p* value		
				TSC1 vs. *TSC2*	*TSC1* vs. NMI	*TSC2* vs. NMI
N	31	120	23			
Demographic characteristic						
Male: female	17:14	58:62	11:12			
Family history of TSC	5/31	19/120	1/23	1.000	0.224	0.312
Central nervous system						
Epilepsy	24/31	109/120	15/23	0.059	0.369	0.003*
Age at seizure onset, months (median ± SD)	39.00 ± 45.12	14.10 ± 29.06	25.79 ± 35.37	0.003*	0.308	0.308
Seizure onset before age 1 year	9/24	81/109	8/15	0.001*	0.508	0.124
Spasm	10/24	51/109	5/15	0.821	0.496	0.173
SENs	27/31	112/120	21/23	0.304	0.204	0.004*
Cortical tubers	30/31	111/9	22/23	1.000	0.003*	0.013*
SEGA	0/31	7/120	2/23	0.347	0.186	0.645
Retinal hamartomas	1/15	28/59	6/14	0.003*	0.035*	1.000
Cutaneous manifestation						
Hypomelanotic macules	28/31	117/120	21/23	0.101	1.000	0.183
Facial angiofibromas (≥3 years)	9/27	56/94	7/17	0.027*	0.749	0.189
Shagreen patch	12/31	41/117	4/23	0.833	0.133	0.142
Periungual fibroma	0/30	5/111	0/23	0.580	Null	0.590
Cardiac rhabdomyoma	12/30	40/112	4/23	0.675	0.141	0.240
Renal system						
Multiple renal cyst	0/31	10/120	1/22	0.216	0.426	1.000
Renal angiomyolipoma	2/29	31/112	5/23	0.025*	0.219	0.796
TAND						
Intellectual disability	19/31	81/120	14/23	0.529	1.000	0.631
Autism	3/15	31/126	2/18	0.390	0.650	0.140

*SENs, subependymal nodules; SEGA, subependymal giant cell astrocytoma; autism, autism spectrum disorder; *p < 0.05; Wilcoxon rank-sum tests for continuous variables, Fisher's exact tests for categorical data.*

TSC. In total, 42.86% (6/14) of the *TSC1* variants were located in crucial regions (N-terminal protein interaction domain and tuberin interaction coiled-coil domain), and 51.35% (19/37) of the *TSC2* variants were located in crucial regions (N-terminal hamartin interaction GTPase activation protein domain). We compared the correlation between the clinical features obtained in our cohort with the different mutation types found in previous studies (Dabora et al., 2001; Sancak et al., 2005; Au et al., 2007; Yang et al., 2017).

As shown in **Table 1**, patients with *TSC2* variants were identified at a younger age ($p = 0.003$) and showed more retinal hamartomas and facial angiofibromas compared with patients with *TSC1* variants (age ≥ 3 years) ($p = 0.027$). NMI patients had fewer cortical tubers ($p = 0.003$) compared to patients with *TSC1*/*TSC2* variants. Compared with patients with *TSC1* variants, NMI patients exhibited more retinal hamartomas ($p = 0.035$), were less likely to have epilepsy ($p = 0.003$), and had fewer cortical tubers ($p = 0.013$) and SENs ($p = 0.004$) than patients with TSC2 variants.

As shown in **Table 4**, the frequency of ID (65.52% vs. 46.47∼82.41%), seizures (85.06% vs. 74.77∼96.58%) and SENs (91.95% vs. 83.39∼91.71%) in our cohort was consistent with that of previous studies. Compared with the cohorts in previous reports, our cohort may have more cortical tubers (93.68% vs. 83.97∼89.74%), retinal hamartomas (39.77% vs. 23.96∼37.68%) and hypomelanotic macules (95.40% vs. 89.10∼94.87%). Compared with reports from other countries, our TSC patients were more similar to those in the reports of Yang et al. Compared with patients in reports from other countries, our patients were less likely to develop SEGA (5.17∼9.40% vs. 11.06∼30.00%), facial angiofibromas (26.50∼43.68% vs. 60.06∼84.54%), shagreen patches (19.66∼33.33% vs. 38.94∼53.57%), multiple renal cysts (6.32∼14.53% vs. 24.79∼42.03%), and renal angiomyolipomas (23.01∼28.21% vs. 41.89∼54.11%), possibly due to the younger age of our cohort.

This study has some limitations. Exome sequencing, targeted sequencing or MLPA was performed in 174 children. However, we have not performed further deep-depth sequencing of the 23 clinically confirmed NMI cases, nor have we sequenced tissue samples (such as brain tissue, etc.). The cases of somatic mosaicism are not yet clear. Assuming a somatic mosaicism detection rate of 5% (Kozlowski et al., 2007) and given that no mutation was identified in *TSC1* or *TSC2* by the sequence analyses in 13.21% of individuals with TSC, we conclude that fewer than 1% of persons with TSC have somatic mosaicism for a *TSC1* or *TSC2* mutation. Improving sequencing depth and collecting more tissue samples will lead to better somatic mosaicism detection.

TABLE 3 | Clinical manifestations of different mutation types in TSC1 and TSC2 genes.

Phenotype	*TSC1*								*TSC2*							
	Mis	**Non**	**Sp**	**Fs**	**Sd**	**Ld**	**Di**	***p***	**Mis**	**Non**	**Sp**	**Fs**	**Sd**	**Ld**	**Di**	***p***
N	5	12	9	2	2	1	0		29	30	17	27	8	7	2	
Central nervous system																
Epilepsy	3 (60%)	8 (67%)	8 (89%)	2 (100%)	2 (100%)	1 (100%)	0 (0%)	0.720	26 (90%)	26 (87%)	16 (94%)	26 (96%)	8 (100%)	6 (86%)	1 (50%)	0.350
Spasm	0 (0%)	5 (63%)	2 (25%)	1 (50%)	1 (50%)	1 (100%)	0 (0%)	0.310	10 (38%)	14 (54%)	7 (44%)	12 (46%)	5 (63%)	3 (50%)	0 (0%)	0.850
SENs	4 (80%)	11 (92%)	8 (89%)	2 (100%)	1 (50%)	1 (100%)	0 (0%)	0.590	27 (93%)	29 (97%)	15 (88%)	26 (96%)	7 (88%)	6 (86%)	2 (100%)	0.590
Cortical tubers	4 (80%)	12 (100%)	9 (100%)	2 (100%)	2 (100%)	1 (100%)	0 (0%)	0.320	26 (90%)	30 (100%)	16 (94%)	24 (89%)	7 (88%)	6 (86%)	2 (100%)	0.360
SEGA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	Null	3 (10%)	3 (10%)	0 (0%)	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0.370
Retinal hamartomas	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1.000	7 (54%)	5 (31%)	4 (44%)	7 (54%)	3 (60%)	2 (67%)	0 (0%)	0.740
Cutaneous manifestation																
Hypomelanotic macules	4 (80%)	11 (92%)	8 (89%)	2 (100%)	2 (100%)	1 (100%)	0 (0%)	0.870	28 (97%)	30 (100%)	17 (100%)	25 (93%)	8 (100%)	7 (100%)	2 (100%)	0.560
Facial angiofibromas	3 (60%)	5 (42%)	1 (11%)	1 (50%)	0 (0%)	0 (0%)	0 (0%)	0.310	10 (34%)	19 (63%)	10 (59%)	11 (41%)	3 (38%)	4 (57%)	2 (100%)	0.180
Shagreen patch	2 (40%)	4 (33%)	4 (44%)	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0.510	9 (31%)	16 (55%)	6 (38%)	8 (31%)	1 (13%)	1 (14%)	0 (0%)	0.170
Periungual fibroma	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	Null	2 (7%)	2 (7%)	1 (6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.810
Cardiac rhabdomyoma	1 (20%)	4 (36%)	1 (11%)	2 (100%)	1 (50%)	0 (0%)	0 (0%)	0.190	10 (37%)	11 (41%)	8 (47%)	11 (41%)	3 (38%)	1 (17%)	0 (0%)	0.880
Renal system																
Multiple renal cyst	0 (0%)	0 (0%)	1 (11%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.620	4 (15%)	4 (15%)	2 (12%)	2 (7%)	2 (33%)	3 (50%)	0 (0%)	0.200
Renal angiomyolipoma	0 (0%)	1 (9%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	0 (0%)	0.330	6 (23%)	13 (48%)	3 (18%)	7 (26%)	1 (14%)	1 (17%)	0 (0%)	0.280
TAND																
Intellectual disability	2 (40%)	7 (58%)	8 (89%)	0 (0%)	1 (50%)	1 (100%)	0 (0%)	0.120	18 (62%)	19 (63%)	10 (59%)	22 (81%)	6 (75%)	5 (71%)	1 (50%)	0.600
Autism	0 (0%)	2 (40%)	1 (25%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.820	13 (52%)	5 (21%)	3 (23%)	7 (35%)	1 (20%)	3 (43%)	0 (0%)	0.290

SENs, subependymal nodules; SEGA, subependymal giant cell astrocytoma; autism, autism spectrum disorder; Mis, Missense; Non, Non-sense; Sp, splicing; Fs, frame shift; Sd, small deletion; Ld, large deletion; Di, deletion-insertion. Fisher's exact tests for categorical data.

TABLE 4 | Frequencies of major clinical characteristics in TSC patients in previous and current studies.

	TSC1: TSC2: NMI	ID	Epilepsy	SENs	Cortical tubers	SEGA	Retinal hamartomas	Hypomelanotic macules	Facial angiofibromas	Shagreen patch	Cardiac rhabdomyoma	Multiple renal cyst	Renal angiomyolipoma
Dabora et al., 2001	28:158:38	65.81 (102/155)	90.58 (202/223)	91.71 (177/193)	88.50 (100/113)	11.06 (23/208)	23.96 (46/192)	92.24 (202/219)	69.20 (155/224)	46.95 (100/213)	51.28 (100/195)	42.03 (87/207)	54.11 (112/207)
Sancak et al., 2005	53:182:27	82.41 (89/108)	90.91 (110/121)	90.32 (84/93)	88.89 (56/63)	30.00 (9/30)	37.68 (26/69)	92.16 (94/102)	84.54 (82/97)	53.57 (30/56)	39.66 (23/58)	32.84 (22/67)	41.89 (31/74)
Au et al., 2007	61:182:74	46.47 (112/241)	74.77 (243/325)	83.39 (231/277)	83.97 (220/262)	18.00 (45/250)	30.62 (64/209)	89.10 (286/321)	60.06 (191/318)	38.94 (118/303)	46.15 (114/247)	24.79 (60/242)	46.58 (109/234)
Yang et al., 2017	16:101	71.79 (84/117)	96.58 (113/117)	84.62 (99/117)	89.74 (105/117)	9.40 (11/117)	–	94.87 (111/117)	26.50 (31/117)	19.66 (23/117)	35.04 (41/117)	14.53 (17/117)	28.21 (33/117)
Present study	24:109:15	65.52 (114/174)	85.06 (148/174)	91.95 (160/174)	93.68 (163/174)	5.17 (9/174)	39.77 (35/88)	95.40 (166/174)	43.68 (76/174)	33.33 (57/171)	36.97 (61/165)	6.32 (11/174)	23.17 (38/164)

ID, intellectual disability; TSC1, patients with TSC1 mutation; TSC2, patients with TSC2 mutation; SENs, subependymal nodules; SEGA, subependymal giant cell astrocytoma.

As reported by Yang et al. (2017) despite these general associations, there is significant clinical variability among TSC patients, even within the same family. In our clinical experience, severe and mild cases can occur in patients with different genotypes; therefore, it is challenging to predict the clinical phenotype of TSC patients based on their genotype. Therefore, we recommend that clinicians use caution when counseling patients. For patients who receive a genetic diagnosis early in life, their genotypes should never be used to predict clinical outcomes unless there is an accurate genotype-phenotype correlation.

CONCLUSION

TSC1 or TSC2 variants are key causes of TSC. The overall mutation rate of the TSC patients in our cohort was approximately 86–87%. TSC exhibits variability in clinical findings, with TSC2 variants producing a more severe phenotype than that observed in patients with TSC1 variants or NMI. Compared with TSC1 patients and NMI patients, those with TSC2 variants have a greater chance of developing renal abnormalities, intracranial lesions, and skin diseases, and their age at the onset of epilepsy is younger.

DATA AVAILABILITY STATEMENT

The genotyping data generated for this study can be found in the LOVD database (https://databases.lovd.nl/shared/variants?search_owned_by_=%3D%22Yifeng%20Ding%22), with associated accession numbers ranging from #0000642979 to #0000643105, #0000642877, #0000642883, #0000642972, #0000642973, #0000642974, and #0000642976. The whole exome sequencing data cannot be made publicly available because it is not allowed according to the national requirements about human genetic resource of research: The Regulations of the People's Republic of China on the Management of Human Genetic Resources (http://www.moj.gov.cn/government_public/content/2019-06/10/593_236557.html). Requests to access the datasets should be directed to corresponding author.

ETHICS STATEMENT

Our research was carried out in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Fudan University Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YD and YW designed the research. YD, JW, SZ, YZ, LZ, and LY performed the collection and analysis of data. YW wrote the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00204/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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A Sphingosine-1-Phosphate Lyase Mutation Associated With Congenital Nephrotic Syndrome and Multiple Endocrinopathy

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Background: Loss of function mutations in *SGPL1* are associated with Sphingosine-1-phosphate lyase insufficiency syndrome, comprising steroid resistant nephrotic syndrome, and primary adrenal insufficiency (PAI) in the majority of cases. *SGPL1* encodes sphingosine-1-phosphate lyase (SGPL1) which is a major modulator of sphingolipid signaling.

Case Presentation: A Pakistani male infant presented at 5 months of age with failure to thrive, nephrotic syndrome, primary adrenal insufficiency, hypothyroidism, and hypogonadism. Other systemic manifestations included persistent lymphopenia, ichthyosis, and motor developmental delay. Aged 9 months, he progressed rapidly into end stage oligo-anuric renal failure and subsequently died. Sanger sequencing of the entire coding region of *SGPL1* revealed the novel association of a rare homozygous mutation (chr10:72619152, c.511A>G, p.N171D; MAF−1.701e-05) with the condition. Protein expression of the p.N171D mutant was markedly reduced compared to *SGPL1* wild type when overexpressed in an *SGPL1* knockout cell line, and associated with a severe clinical phenotype.

Conclusions: The case further highlights the emerging phenotype of patients with loss-of-function *SGPL1* mutations. Whilst nephrotic syndrome is a recognized feature of other disorders of sphingolipid metabolism, sphingosine-1-phosphate lyase insufficiency syndrome is unique amongst the sphingolipidoses in presenting with multiple endocrinopathies. Given the multi-systemic and progressive nature of this form of PAI/ nephrotic syndrome, a genetic diagnosis is crucial for optimal management and appropriate screening for comorbidities in these patients.

Keywords: *SGPL1*, sphingosine-1-phosphate lyase, congenital nephrotic syndrome, primary adrenal insufficiency, multiple endocrinopathy

INTRODUCTION

Sphingosine-1-phosphate lyase insufficiency syndrome (SPLIS) is a recently described condition, which in the main, incorporates steroid resistant nephrotic syndrome, and primary adrenal insufficiency (PAI). The disease is associated with loss of function mutations in *SGPL1*, encoding sphingosine-1-phosphate lyase that irreversibly binds sphingosine 1-phosphate (S1P) and commits it to the final degradative step in sphingolipid metabolism (1–3). Phenotypic expression of the condition is variable, from severe multi-systemic forms, associated with fetal hydrops and early mortality, to isolated single organ disease (1, 2, 4–9).

Nephropathy is associated with other genetic disorders of sphingolipid metabolism, such as Fabry and Gaucher disease, and sphingolipid accumulation is reported in glomerular disease of non-genetic origin (10). Endocrinopathy, including PAI, on the other hand is not commonly reported in the sphingolipidoses (11).

We report the novel association of a rare variant in *SGPL1* in a patient presenting with a severe combination of primary adrenal insufficiency and infantile onset nephrotic syndrome. This case further highlights the emerging multi-systemic phenotype in this rare disorder of sphingolipid metabolism, including its unique association with wider endocrinopathy.

CASE PRESENTATION

A Pakistani male infant with 46XY karyotype, born to consanguineous parents, presented at 5 months of age with failure to thrive, metabolic acidosis and nephrotic syndrome, unmasked by a transient diarrhoeal illness. Postnatal growth failure was evident at presentation with a length of 58.5 cm (−3.05 SDS) and weight of 5.25 kg (−3.16 SDS) and a history of normal birthweight of 3.3 kg at 36 + 6 weeks gestation (+0.69 SDS). He was noted to have palmar hyperpigmentation, ichthyosis, under-virilized male genitalia and generalized developmental delay. Laboratory investigations revealed initially normal renal function, hypoalbuminaemia, mild lymphopenia, and nephrotic range albuminuria. There was an initial high anion gap metabolic acidosis which became normal anion gap acidosis following intravenous rehydration. Transient glycosuria and hypokalaemia prompted the consideration of proximal renal tubular acidosis, however these values normalized with no specific treatment other than rehydration and oral bicarbonate medication (**Table 1**). Ultrasound scanning demonstrated bilateral large, echogenic kidneys and calcification in both adrenals. The patient was treated with captopril, 1-alfacalcidol, sodium feredetate, ranitidine, bicarbonate 8.4% solution and energy-dense oral and nasogastric feeding. Oedema was relatively mild and managed with *as required* diuretic medication; 20% Human albumin infusions were not required.

The clinical phenotype and laboratory findings prompted early endocrine review and the additional diagnosis of PAI was made (low serum cortisol 61 nmol/L, markedly elevated ACTH 999 ng/L and low normal aldosterone 190 pmol/L). A wider endocrine phenotype included primary hypogonadism with microphallus and bilateral cryptorchidism (with raised basal gonadotrophins FSH 71 IU/L, LH 27 IU/L) and primary hypothyroidism (elevated TSH 25 mU/L and low fT4 10.7 pmol/L). Endocrine treatments included hydrocortisone, fludrocortisone and L-thyroxine replacement. Multiple hospital admissions ensued for nutritional and therapeutic interventions.

TABLE 1 | Results of laboratory investigations at initial (5 months) and last presentation (9 months).

Test (reference range)	Acute presentation (district hospital)	At presentation (Tertiary hospital)	Last acute presentation (9 months old)
UREA (2.5–6.0 mmol/L)	1.4	1	22.6
CREATININE (14–34 umol/L)	30	21	323
CYSTATIN C (0.7–1.5 mg/L)	N/A	1.52	5.26
SODIUM (133–146 mmol/L)	137	139	138
POTASSIUM (3.5–5.0 mmol/L)	3.3-4.5	4.5	6.2
BICARBONATE (19–28 mmol/L)	13	16	33
CHLORIDE (95–108 mmol/L)	109	113	87
eGFR BY CREATININE (ml/min/1.73m^2)	N/A	107	8
eGFR BY CYSTATIN-C	N/A	48	15
CALCIUM (mmol/L)	2.31	2.11	1.51
PHOSPHATE (1.2–2.2 mmol/L)	1.97	1.79	2.74
PTH (1.6–6.9 pmol/L)	60	93.1	195.4
ALP (77–540 u/L)	524	505	198
ALBUMIN (28–40 g/L)	17	17	22
URINE PCR (0–30 mg/mmol)	>2,000	2,189	1,975
URINE ACR (0–3.0 mg/mmol)	N/A	1978.5	n/a
HEMOGLOBIN (100–130 g/L)	90	84	83
LYMPHOCYTES (3.3–11.5 × 10^9/L)	0.9–1.6	1.52	0.58
PLATELETS (150–560 × 10^9/L)	391	457	415
CHOLESTEROL (1.2–4.7 mmol/L)	N/A	4.8	7.6
ACTH (0–46 ng/L)*	999		
CORTISOL (133–537 nmol/l)*	61		
LH (1.7–8.6 IU/L), FSH (1.5–12.4 IU/L.)*	71, 27		
TSH (0.2–5 mu/L)*	27		
Free T4 (9–24 pmol/l)*	10.5		

**Results prior to starting endocrine replacement therapy.*

Renal function continued to deteriorate and the eGFR reduced from 107 mls/min/1.73m^2 at 5 months to 31 mls/min/1.73m^2 by 8 months (Chronic Kidney Disease (CKD) stage IIIb). Aged 9 months, the patient presented with acute oligo-anuric renal failure and pulmonary oedema following a brief diarrhoeal illness. Renal replacement therapy was declined by the family given the burden of morbidity to date and the patient subsequently died.

Initial genetic screening did not identify mutations within the standard SRNS genetics panel *(Bristol SRNS exome screen),* noting only a likely non-pathogenic, heterozygous missense mutation in COL4A4 (NM_000092.4); Intron 41, c.3974-4dupT which was not consistent with phenotype. The unusual combination of nephrotic syndrome and primary adrenal insufficiency prompted consideration of the recently published multi-systemic syndrome of SGPL1 deficiency as a causative disease mechanism (1, 2).

MATERIALS AND METHODS

Sanger Sequencing

SGPL1 exons of interest (coding region) were amplified by PCR using specific primers as previously described (2).

Sequence Interpretation and *in silico* Analysis of Variants

Using an autosomal recessive disease model, variants were assigned causality if they segregated within the proband family and were deemed pathogenic based on several *in silico* prediction software tools (SIFT; PolyPhen-2; Human Splicing Finder, version 3.0). Minor Allele Frequency was determined from the ExAC and Genome Aggregation Database (gnomAD) browsers.

Protein Structure Modeling

An online tool, G23D (http://www.sheba-cancer.org.il/G23D) was used to map the genomic coordinates of pathogenic variant and predict functional alterations to mutant protein structure.

In vitro Splicing Assay

An *in vitro* splicing assay was designed using the commercially obtained Exontrap cloning vector pET01 (MoBiTec GmbH, Göttingen, Germany) containing an intronic sequence interrupted by a multiple cloning site. DNA fragments of interest (insert length of 1,129 bp; exon 7 + 500 bp intronic sequence at both 5′ and 3′ ends) were amplified using a standard PCR protocol and specifically designed primers containing a restriction enzyme target site for XbaI. PCR products were sequenced, column purified using the QIAquick® PCR Purification Kit, and cloned into the Exontrap cloning vector pET01 (MoBiTec GmbH). The cloned sequences were verified by Sanger sequencing to ensure the fragment was in the correct orientation using pET01 specific primers. Wild-type (pET01-WT) and pET01 mutant plasmids were transfected into HEK293T cells using Lipofectamine 2000® reagent (Thermo Fisher Scientific). Total RNA, obtained from cells 24 h after transfection, was subjected to RT-PCR to generate cDNA with primer GATCCACGATGC (MoBiTec GmbH) and amplified with primers within the 5′ and 3′ exons in the pET01 vector. Amplification products were assessed on a 2% agarose gel.

Site Directed Mutagenesis

Site directed mutagenesis of *SGPL1* Human Tagged ORF Clone (Origene) and oligonucleotide primer design was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) and web based QuikChange program in keeping with the manufacturer's guidelines.

Western Blotting

CRISPR/ Cas9 engineered *SGPL1* knockout H295R cells were seeded into 6 well plates and transfected with wild type *SGPL1* and p.N171D mutant constructs using Lipofectamine 2000® reagent (Thermo Fisher Scientific). After 48 h whole cell lysates were prepared by addition of RIPA buffer (Sigma Aldrich) supplemented with protease and phosphatase inhibitor tablets (Roche). Protein concentrations were quantified using a Bradford protein assay (Bio-Rad). Lysates were denatured by addition of Laemmli sample buffer 2X (Sigma Aldrich) and boiled for 5 min at 98°C. 15–20 μg of protein were loaded into wells of a 4–20% SDS-PAGE gel (Novex) prior to electrophoretic separation using MOPS buffer. Protein transfer to PVDF membrane was achieved by electro-blotting at 15V for 45 min. The membrane was blocked with 5% fat free milk in TBS/0.1% Tween-20 and left to gently agitate for 1 h. Primary antibody (Human SGPL1 Antibody; AF5535, R&D Systems) was added at a concentration of 1:500 and β-actin (1:10,000) used as a housekeeping control. Primary antibody incubation was left overnight at 4°C with gentle agitation. The membrane was then washed for 5 min (X3) with Tris Buffered saline-Tween20 (TBST). Secondary anti-goat and anti-mouse antibodies were added at a concentration of 1:5,000 to blocking buffer and the membrane incubated at 37°C for 60–90 min. The membrane was subsequently washed three times (5 min each) with TBST and visualized with the LI-COR Image Studio software for immune-fluorescent detection.

Study Approval

This study was approved by the Outer North East London Research Ethics Committee, reference number 09/H0701/12.

Consent

Written informed consent was obtained from the parents or guardians of the participant for participation in the study and the publication of this case report.

RESULTS

Sanger sequencing of the entire coding region of *SGPL1* revealed a very rare homozygous missense mutation in *SGPL1* (chr10:72619152, c.511A>G; p.N171D) (gnoMAD MAF−1.701e-05; no homozygotes described) which was heterozygous in the unaffected consanguineous parents and siblings (**Figures 1A,B**). Partial alignment of SGPL1 protein sequences showed conservation of asparagine (N) at position 171 across several species (**Figure 1C**). Single nucleotide change c.511A>G (p.N171D), whilst denoted as deleterious across several platforms (SIFT, PolyPhen), showed minor conformational change on protein modeling that was likely to lead to protein instability but with a relatively low reliability score (**Figure 2A**). *In silico* prediction tools (Mutation Taster and Human Splicing Finder 3.0) suggested creation of an exonic splicing silencer site and aberrant splicing as the underlying pathogenic mechanism. An *in vitro* splicing assay revealed the mutant p.N171D heterologous mini-gene construct demonstrated unaltered splicing with normal incorporation of exon 7 between the constitutional exons of the exon trap vector with removal of the intervening sequences (**Figure 2B**). The p.N171D mutant plasmid generated by mutagenesis of an *SGPL1* Human ORF clone was expressed in a eukaryotic CRISPR/Cas9 engineered *SGPL1* KO H295R adrenocortical cell line and protein expression quantified by western blotting after 48 h. When compared to wild type, p.N171D was markedly reduced suggesting reduced stability and increased susceptibility to degradation in the mutant protein (**Figure 2C**).

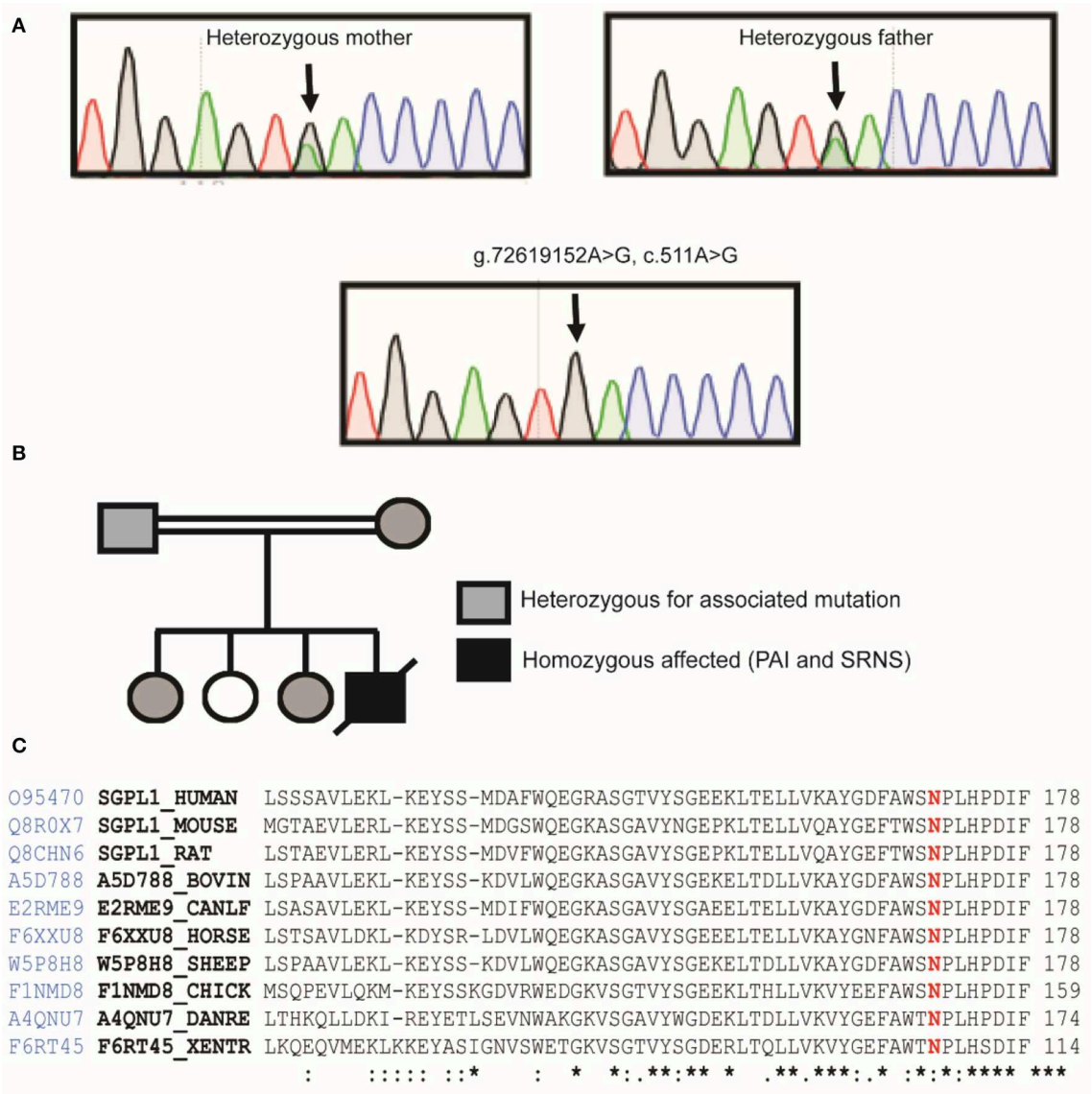


FIGURE 1 | Our patient harbors a homozygous mutation in *SGPL1*, c.511A>G, p.N171D. **(A)** Partial sequence chromatograms of genomic DNA from the parents who are heterozygote carriers and the homozygote patient, showing the base change from A to G in exon 7. **(B)** Pedigree of affected patient. Black-filled symbols indicate individual homozygous, half-filled indicate individuals heterozygous for the mutation. White-filled symbols indicate wild-type individuals. **(C)** Partial alignment of SGPL1 protein sequences, showing conservation of asparagine (N) at position 171 across several species, highlighted in red, numbering relative to human sequence. Sequence conservation is beneath the alignment, *total conservation, : partial conservation.

DISCUSSION

SGPL1 encodes sphingosine-1-phosphate lyase (SGPL1), which provides the single exit point for the sphingolipid metabolic pathway, irreversibly converting S1P to hexadecanal and phosphoethanolamine. Sphingosine-1-phosphate lyase insufficiency syndrome (SPLIS) describes the recently reported multi-systemic disease associated SGPL1 deficiency (clinical features summarized in **Table 2**) (1–9). Other mutations in the sphingolipid pathway are related to the sphingolipidoses, which include Gaucher, Fabry, and Niemann-Pick disease amongst others. Sphingolipids play an integral role as structural components of cell membranes, and sphingolipid intermediates such as S1P, sphingosine, and ceramide are signaling molecules involved in cell migration, differentiation, and cell survival.

SGPL1 deficiency leads to an accumulation of upstream bioactive sphingolipids, often in a tissue-specific manner (1, 2, 9). SGPL1 is found in the endoplasmic reticulum of renal podocytes, mesangial, and endothelial cells and immunofluorescence studies show co-localization in the renal proximal tubules, which may explain the initial biochemical picture in our patient consistent with proximal renal tubular dysfunction (1). Furthermore,

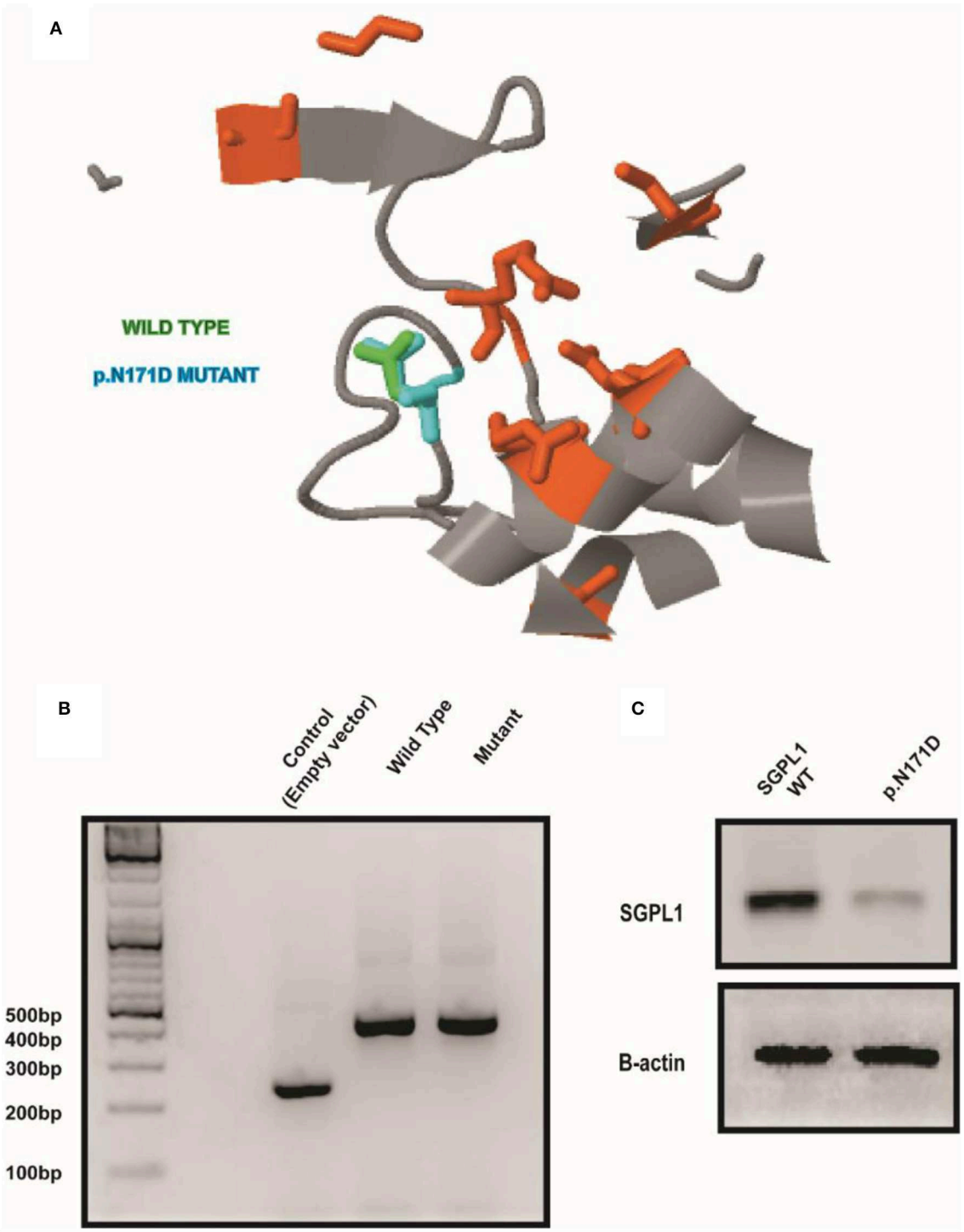


FIGURE 2 | A conformational change in SGPL1 occurs with the p.N171D mutation, resulting in reduced protein expression. **(A)** Protein modeling showed a minor conformational change in SGPL1 due to amino acid substitution at position 171, predicted to result in reduced protein stability. **(B)** An *in vitro* splicing assay showed effective splicing of both wild type and mutant *SGPL1* exon 7 constructs providing no evidence of aberrant splicing. **(C)** Expression of p.N171D mutant in an *SGPL1* KO cell line is reduced when compared to wild type suggesting increased susceptibility to degradation.

it has been hypothesized that S1P accumulation leads to a signaling defect of angiogenic factors, resulting in additional changes within the renal parenchyma (6). The enzyme is moderately expressed in the adrenal cortex, testes and thyroid gland (2); the endocrine organs affected in our patient. The sphingolipid intermediates have been implicated in the

TABLE 2 | Summary of main clinical features of patients described with genetically confirmed sphingosine-1-phosphate lyase insufficiency syndrome (SPLIS) to date (1, 2, 4–9), including our own patient.

	Patient numbers	Patient details
Total patients	35 (+ 4 fetal demise)	Initial presentation at ages varying postnatally to 19 years
Sex (M:F)	21:14	
Deaths	15	Age at demise varying postnatally, with fetal hydrops, to 9 years; several precipitated by septic episodes
Nephrotic syndrome	29	Congenital to oldest presentation at 19 years with SRNS, with 19 patients presenting in infancy, six patients were reported to have renal transplantation (two of these required a second transplant).
Adrenal insufficiency	23* (five additional cases with adrenal calcification on imaging)	Glucocorticoid deficiency in all cases ± mineralocorticoid deficiency. Age at presentation varying from early postnatal to oldest at 11 years, 11 patients presenting in infancy. 11 patients noted to have adrenal calcifications on imaging.
Hypothyroidism	12	Primary hypothyroidism with mildly raised TSH levels in all cases where biochemistry was reported
Gonadal dysfunction	7	Male patients presenting postnatally with cryptorchidism ± microphallus, all with raised gonadotrophins indicating primary gonadal failure.
Ichthyosis	12	
Neurological/ Developmental delay	18	Phenotypically heterogenous, varying from microcephaly, seizures, sensorineural deafness to later presentation with abnormal gait, peripheral neuropathy, progressive neurological deficit. Central and peripheral nervous system pathology variably reported. Neuroimaging findings varied from corpus callosum atrophy, cortical atrophy, cerebellar hypoplasia to basal ganglia involvement.
Immunodeficiency	13	Most consistently this is an absolute lymphopenia, some patients additionally with hypogammaglobulinemia and neutropenia.
Other features	<5 patients	Dysmorphic features, skeletal abnormalities, liver dysfunction are reported.

**Indicates biochemically proven.*

regulation of acute steroidogenesis and expression of steroid responsive transcriptional elements while SGPL1 deficient mice have abnormal adrenal and testicular morphology (2, 12, 13), in keeping with the phenotype seen in the human disease.

We present a novel association of a rare variant within *SGPL1* associated with a severe phenotype of SGPL1 deficiency, encompassing infantile-onset nephrotic syndrome with a wider endocrine phenotype including PAI, primary hypothyroidism and primary hypogonadism. In addition, our patient also demonstrated persistent mild lymphopenia, ichthyosis, and neurodevelopmental delay, features similar to previously described cases.

To date, there have been 35 published genetically confirmed cases involving 20 kindreds and 20 different mutations in *SGPL1* (including this patient, not including those with fetal demise) (1, 2, 4–9) (see **Tables 2**, **3**). Patients present with congenital or steroid resistant nephrotic syndrome (see **Tables 2**, **3**) and progress to end-stage renal disease with renal biopsy histological findings of focal segmental glomerulosclerosis and diffuse mesangial sclerosis. Similarly, the majority of affected individuals have adrenal disease (see **Tables 2**, **3**). SGPL1 has a potential role to play in other endocrine tissues with emerging reports of wider endocrinopathy (thyroid and testicular), as seen in our patient (1, 2, 5–9). This appears to be unique to SPLIS in comparison to other sphingolipidoses. In common with several disorders of sphingolipid metabolism, ichthyosis is reported (1, 2, 9). Neurological dysfunction can affect both the central and peripheral nervous system and is described with progressive morbidity. Interestingly, mutations inherited in compound heterozygosity have been associated with isolated peripheral neuropathy akin to Charcot-Marie Tooth; the adult siblings described do not have additional disease (4). Lymphopenia is seen in some patients and is associated variably with an increased frequency of infections (1, 2, 5, 9). SPLIS therefore encompasses a clinically heterogenous phenotype, with the most severe cases presenting with fetal hydrops, whilst others are affected with single organ disease alone.

Despite *in silico* predictions, the p.N171D *SGPL1* mutant showed unaltered splicing in comparison to wild-type, however protein expression was markedly reduced as was anticipated by the instability predicted by protein modeling. Whilst all functional characterizations of *SGPL1* variants, described in this condition so far, demonstrate markedly reduced protein expression, clinical severity has not always correlated (1, 2). It has been proposed that individuals with most severe phenotypes harbor mutations within the active pyridoxal-dependent decarboxylase domain (5), which is home to the variant in our presented case (**Table 3**, **Figure 3**). However, milder phenotypes characterized solely by axonal polyneuropathy (Charcot Marie Tooth) due to mutations p.S361* and p.I184T also occur within this region. As yet, there is no evident genotype- phenotype correlation in SPLIS, with equally severe clinical phenotypes seen in mutations outside the active region and in both frameshift and missense mutations (**Table 3**, **Figure 3**). Phenotypic heterogeneity is also evident in individuals with the same *SGPL1* variants, even within kindreds. Further investigation of the genotype together with detailed clinical phenotyping (at presentation and longitudinally) is required, as more individuals are diagnosed. Establishing a genotype/ phenotype correlation is further complicated by the consanguinity of the affected kindreds and the likely presence of other genetic modifiers of disease.

The recent description of SPLIS finally provides a diagnostic link for patients presenting with congenital NS and PAI. In view of the reports of isolated adrenal or renal disease at presentation it would be pertinent for *SGPL1* to be considered in diagnostic genetic panels for PAI/SRNS and certainly if seen in combination. Given the phenotypic variability of this disease, the genetic diagnosis may be crucial for confirming the clinical suspicion and facilitating initiation of appropriate screening and treatment for patients. Importantly, early consideration of this disease will hasten identification of associated co-morbidities. This could, for

TABLE 3 | Phenotypic characterization of mutations in *SGPL1*.

Mutation	Deaths	SRNS	PAI	Hypothyroidism	Gonadal dysfunction	Ichthyosis	Neurological features	Immunodeficiency
p.S3Kfs*11	1/4	+	+	+	−	+	±	±
c.261+1G>A	−	+	+	+	+	+	+	+
p.E132G[¥]	−	+	+	−	−	+	−	−
p.N171D	1/1	+	+	+	+	+	+	+
p.I184T[†]	−	−	−	−	−	−	+	−
p.S202L[Ψ]	−	+	−	+	−	−	+	+
p.R222W	2/2	+	+	−	−	−	±	±
p.R222Q	1/9	+	+	−	−	−	+	±
p.R278Gfs*17[¥]	−	+	+	−	−	+	−	−
p.F290L[δ]	1/1	+	+	+	+	+	+	+
p.L312Ffs*30	−	+	+	−	+	−	−	−
p.A316T[Ψ]	−	+	−	+	−	−	+	+
p.Y331*[δ]	1/1	+	+	+	+	+	+	+
p.R340W	2/2	+	+	+	−	−	−	−
p.S346I	3/3	+	+	+	−	+	+	+
p.S361*[†]	−	−	−	−	−	−	+	−
p.F411Lfs*56	1/1	+	+	+	+	−	+	+
p.Y416C	−	+	+	+	−	−	+	+
p.R505*	2/2	+	+	−	+	−	−	−
p.F545del	1/1	+	+	+	−	+	+	+

[¥†Ψδ]*denotes compound heterozygous mutations.*

Sources of published mutations: Lovric et al. (1), Prasad et al. (2); Atkinson et al. (4); Bamborschke et al. (5); Janecke et al. (6); Linhares et al. (7); Settas et al. (8); Taylor et al. (9). Our patient's mutation is denoted in red. + denotes presence in all; ± denotes presence in some but not all patients; – denotes absence of clinical feature. Deaths reported all occurred at <10 years of age.

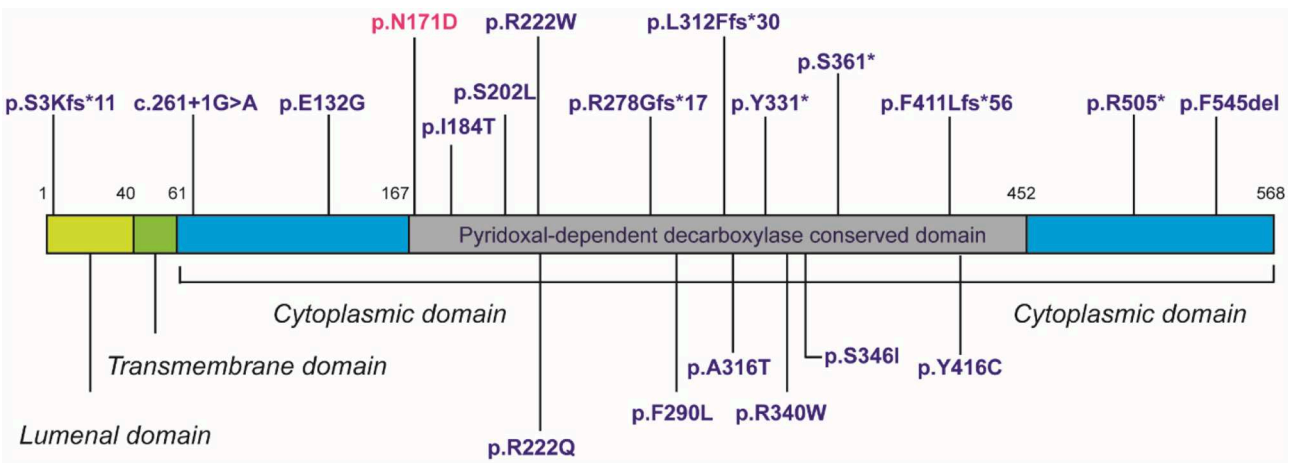


FIGURE 3 | Domain topology showing distribution of 20 published mutations.

instance, reduce the rate of CKD progression and minimize the long-term complications of the associated endocrinopathy. The published literature suggests that the prognosis for these patients is poor with just under 50% mortality (15/35 reported cases, with 4 additional cases of fetal demise), the majority of those deaths in the first year of life. Future research will potentially lead to targeted genetic therapies but for now, a heightened awareness of the syndrome will allow earlier recognition, reducing the time to diagnosis and appropriate intervention and inform genetic counseling for affected families.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Outer North East London Research Ethics Committee, reference number 09/H0701/12. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

DW, DT, and IB recruited and clinically characterized patient. AM, RP, and LM conducted Sanger sequencing and analysis of data. AM conducted protein modeling and functional analysis of the *SGPL1* mutant described. DW, AM, and RP prepared the draft manuscript. All authors contributed to the discussion of results, edited, and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Novel Compound Heterozygous Variants of *ETHE1* Causing Ethylmalonic Encephalopathy in a Chinese Patient: A Case Report

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Ethylmalonic encephalopathy (EE) is a very rare autosomal recessive metabolic disorder that primarily affects children. Less than one hundred EE patients have been diagnosed worldwide. The clinical manifestations include chronic diarrhea, petechiae, orthostatic acrocyanosis, psychomotor delay and regression, seizures, and hypotonia. The *ETHE1* gene has been shown to be associated with EE, and genetic sequencing provides concrete evidence for diagnosis. To date, only 37 variants of *ETHE1* have been reported as disease-causing in EE patients. We identified two novel *ETHE1* variants, i.e., c.595+1G>T at the canonical splice site and the missense variant c.586G>C (p. D196H), in a 3-year-old Chinese boy with EE. The patient had mild symptoms with only chronic diarrhea. The typical symptoms, including spontaneous petechiae, acrocyanosis, and hypotonia, were all absent. Herein, we report on the clinical, biochemical, and genetic findings of our patient and review the phenotypes and genotypes of all patients with EE caused by *ETHE1* variants with available information. This study supports the early assessment and diagnosis of EE.

Keywords: ethylmalonic encephalopathy, *ETHE1*, elevated ethylmalonic acid, chronic diarrhea, genetic sequencing

INTRODUCTION

Ethylmalonic encephalopathy (EE, OMIM#602473) is a rare, early-onset metabolic disorder of infancy that affects the development of the brain, gastrointestinal tract, and peripheral blood vessels. EE was first identified in 3 unrelated Italian children as a branched-chain acyl-CoA oxidation defect in 1991 (Burlina et al., 1991), but most patients diagnosed with EE were of Mediterranean and Arabic descent (Tiranti et al., 2004). To date, only two EE patients have been reported in China (Li et al., 2014; Zhang et al., 2018). However, EE always leads to death within the first decade of life in the most of children (Walsh et al., 2010).

In 2004, for the first time, recessive variants of *ETHE1* (ethylmalonic encephalopathy protein 1) were identified as the causes of EE (Tiranti et al., 2004). *ETHE1* (chromosome 19q13.32) encodes a mitochondrial sulfur dioxygenase involved in sulfide detoxification. The impairment of sulfur dioxygenase leads to the accumulation of hydrogen sulfide (H_2S) and its derivatives, and the major clinical manifestations of EE are mediated by hydrogen sulfide accumulation, which damages mucosal cells in the large intestines and vascular endothelial cells and is associated with chronic diarrhea. Hydrogen sulfide is also vasoactive and vasotoxic, which could explain the symptoms of petechiae and orthostatic acrocyanosis in EE (Tiranti et al., 2009). Vasculopathy is also associated with multiple necrotic brain lesions, resulting in hypotonia, psychomotor delay and regression, predominant pyramidal signs, seizures, and eventually global neurological failure (Walsh et al., 2010; Dionisi-Vici et al., 2016). Regarding biochemical examinations, the disorder is characterized by elevated urinary ethylmalonic acid (EMA), methylsuccinic acid (MSA), lactic acid, C4-/C5-acylcarnitine esters and plasma thiosulfate. The toxic levels of hydrogen sulfide are responsible for these biochemical alterations because they inhibit the activities of cytochrome *c* oxidase and short-chain acyl-CoA dehydrogenase, especially in skeletal muscle (Tiranti et al., 2009; Di Meo et al., 2011).

Generally, next-generation sequencing is a good approach used to support the clinical diagnosis of EE. According to the Human Gene Mutation Database professional version (HGMD pro) (Stenson et al., 2003), only 37 variants of *ETHE1* have been identified as disease-causing. Expanding the variant spectrum of *ETHE1* is necessary for the diagnosis of and further research investigating EE. Herein, we report the clinical, biochemical and genetic findings of a 3.5-year-old Chinese boy with EE who carries two novel *ETHE1* variants.

METHODS

This research was approved by the Medical Ethics Committee of Wuhan Children's Hospital. The patient's parents were informed and provided written consent for the study and the publication of this report.

Case Presentation

The patient, a 2-year-old Chinese boy referred to our hospital in 2018, was born to non-consanguineous parents. When the child was born, he presented with chronic diarrhea (3–6 times per day) which lasted for two years. Four months before the patient was admitted to our department, he was diagnosed with superficial gastritis and colitis at the Gastroenterology Department. Oral probiotic supplements were given to the patient, but he failed to respond to the treatment. Chronic diarrhea remained, but no developmental delay was observed. A urine analysis showed obviously elevated EMA [71.84, 61.89, and 28.96 μmol/L; reference value (r.v.) 0.00–6.20], slightly increased lactic acid (9.05 μmol/L, normal, normal; r.v. 0.00–4.70) and increased isobutyryl glycine (1.85 μmol/L, r.v. 0.00–0.40). After admission to our department, tandem mass spectrometry revealed increased C4-acylcarnitine (0.91 Um, r.v. 0.06–0.50) in the blood. The assessment based on Gesell Developmental Schedules[1] showed that the developmental age was approximately 18 months and indicated overall developmental delay, especially in adaptive skills, fine motor skills, and language (**Figure 1a**). Brain MRI (**Figure 1b**) showed abnormal signal shadows in the temporal horn of the left lateral ventricle, revealing demyelination in the brain. Petechiae on the skin caused by falling appeared on the patient (**Figure 1c**). However, spontaneous petechiae, acrocyanosis, and hypotonia were absent in the patient. Regarding treatment, L-carnitine, vitamin B1 and vitamin B2 were given, but this treatment was ceased due to due aggravation of diarrhea. *Clostridium butyricum* tablets were given continuously but chronic diarrhea remained. Except for a language delay, the patient did not exhibit any other evident abnormalities during a telephone follow-up. In early 2020, a combination of metronidazole and N-acetylcysteine was given based on previous studies (Viscomi et al., 2010). The patient will be continuously followed up, and liver transplantation will be considered if the disease cannot be controlled.

Whole-Exome Sequencing

Whole-exome sequencing (WES) was applied to support the diagnosis. Peripheral blood samples were collected from the patient and his parents and sent to Running Gene Inc. (Beijing, China) for WES. DNA samples were extracted and qualified using a DNA Isolation Kit (AU1802, Bioteke) and Qubit dsDNA HS Assay Kit (Q32851, Invitrogen, United States). The qualified DNA samples were fragmented into 200–300 bp fragments and then prepared with a KAPA Library Preparation Kit (KR0453, Kapa Biosystems) according to the manufacturer's protocol. Hybridization of the prepared libraries to the capture probes was carried out according to the instructions for the IDT and xGen Lockdown® Probes (Integrated DNA Technologies Inc., United States). The captured DNA samples were sequenced on a HiSeq X10 (Illumina, United States). High-quality paired-end reads were aligned to the human reference genome sequence hg19 from the UCSC genome browser (Kent et al., 2002) with the BWA tool (Li and Durbin, 2009). Single-nucleotide variants (SNVs) and insertions and deletions (INDELs) were filtered using GATK (Van der Auwera et al., 2013). All called variants were annotated based on public databases, including the 1000 Genomes Project (Genomes Project et al., 2015), ExAC (Lek et al., 2016), and gnomAD (Karczewski et al., 2019). China National GeneBank (CNGB)[2] was also used to validate the novelty of the variants in the Chinese population. We analyzed the pathogenicity of the variants mainly based on criteria following the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015). Sanger sequencing was applied to validate the familial segregation of the variants.

[1] https://gesellinstitute.org/

[2] https://db.cngb.org/

(a) Gesell Developmental Schedules					
Strands	Adaptive skills	Large motor skills	Fine motor skills	Language strand	Social behavior
Developmental Age (month)	**18**	**21**	**18**	**18**	**21**
Developmental Quotient	**62**	**72**	**62**	**62**	**72**

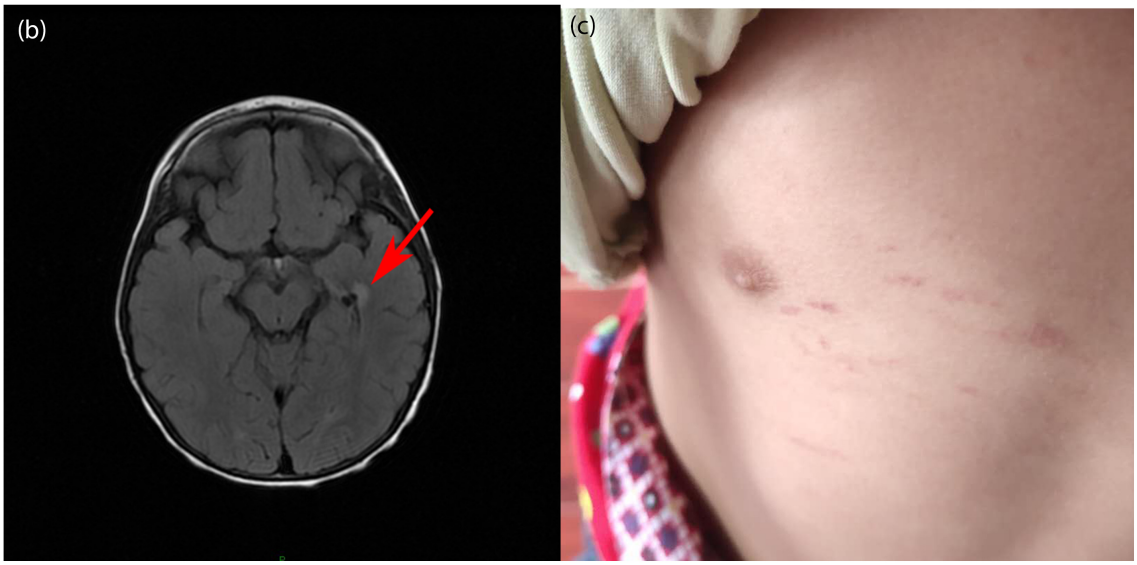


FIGURE 1 | (a) Results based on the Gesell Developmental Schedules. Chronological age is 29 months. Developmental quotient<75 indicates developmental delay. **(b)** Brain MRI. The red arrow indicates shadows in the temporal horn of the left lateral ventricle. **(c)** Petechiae on the skin caused by falling appeared on the patient.

RESULTS

The patient carried two novel compound heterozygous variants of *ETHE1* (NG_008141.1, NM_014297.5, NP_055112.2) (**Figures 2a,b**). The variant with a paternal origin, i.e., c.595+1G>T, is a null variant located at the canonical +1 splice site of exon 5 (PVS1) and likely affects splicing, resulting in the loss of protein function. The variant is absent from controls (1000 Genomes, ExAC, gnomAD, and CNGB) (PM2). Multiple computational software programs predicted that the variant probably has deleterious effects on splicing (PP3). Human Splicing Finder v3.1 predicted that the variant may alter the WT donor site, most likely affecting splicing (Desmet et al., 2009). Similarly, the NetGene2 Server[3] suggested the disappearance of a donor splice site after the alteration. Thus, c.595+1G>T is considered a pathogenic variant. In addition, the maternal missense variant c.586G>C in exon 5 leads to an amino acid change (p.D196H). This recessive variant is absent from controls (PM2) and is in *trans* with the pathogenic variant c.595+1G>T (PM3). This variant is also predicted to be a deleterious variant by several computational software programs (PP3). Mutation Taster, SIFT, Provean and Polyphen-2 predicted it as disease-causing (probability 1.000), damaging (score 0.001<0.05), deleterious (score −6.93<−2.5) and probably damaging (score 1.000), respectively (Choi, 2012; Choi et al., 2012; Adzhubei et al., 2013; Schwarz et al., 2014). Although c.586G>C is a novel variant, c.586G>A (p. D196N) has been reported in the HDMG pro database as a disease-causing variant at the exact same site (PM5) (Mineri et al., 2008), indicating the pathogenicity of the novel variant of *ETHE1*. In addition, the site of this residue is highly conserved in the *ETHE1* sequence across species (**Figure 2c**). Therefore, c.586G>C is classified as a likely pathogenic variant.

[3]http://www.cbs.dtu.dk/services/NetGene2/

DISCUSSION

EE is a devastating recessive mitochondrial disorder with only approximately 100 patients diagnosed worldwide. There is no specific treatment for EE patients, and research concerning disease is quite rare. Currently, developing a targeted therapy for EE is not an economical idea, but it is necessary to improve the processes used for the diagnosis of EE. Compared with physical and biochemical examinations, the sequencing of the responsible gene *ETHE1* is an essential and direct approach used to diagnose patients with EE, although the results still need support from conventional examinations. Genetic sequencing also helps in differential diagnosis between EE and multiple acyl-CoA dehydrogenase deficiency (MADD), which is caused by *SCAD* variants (Zafeiriou et al., 2007).

Only 37 disease-causing variants of *ETHE1* are listed in HGMD pro, including 19 missense variants, 8 insertion and deletion variants, 4 exon deletion variants and 5 splicing variants, including 3 at canonical splicing sites (**Figure 2d**). Since 9 variants are located in the 4th exon and the 4th exon is involved in all exon deletions, we consider the 4th exon a hot region for disease-causing variants, highlighting the importance of its

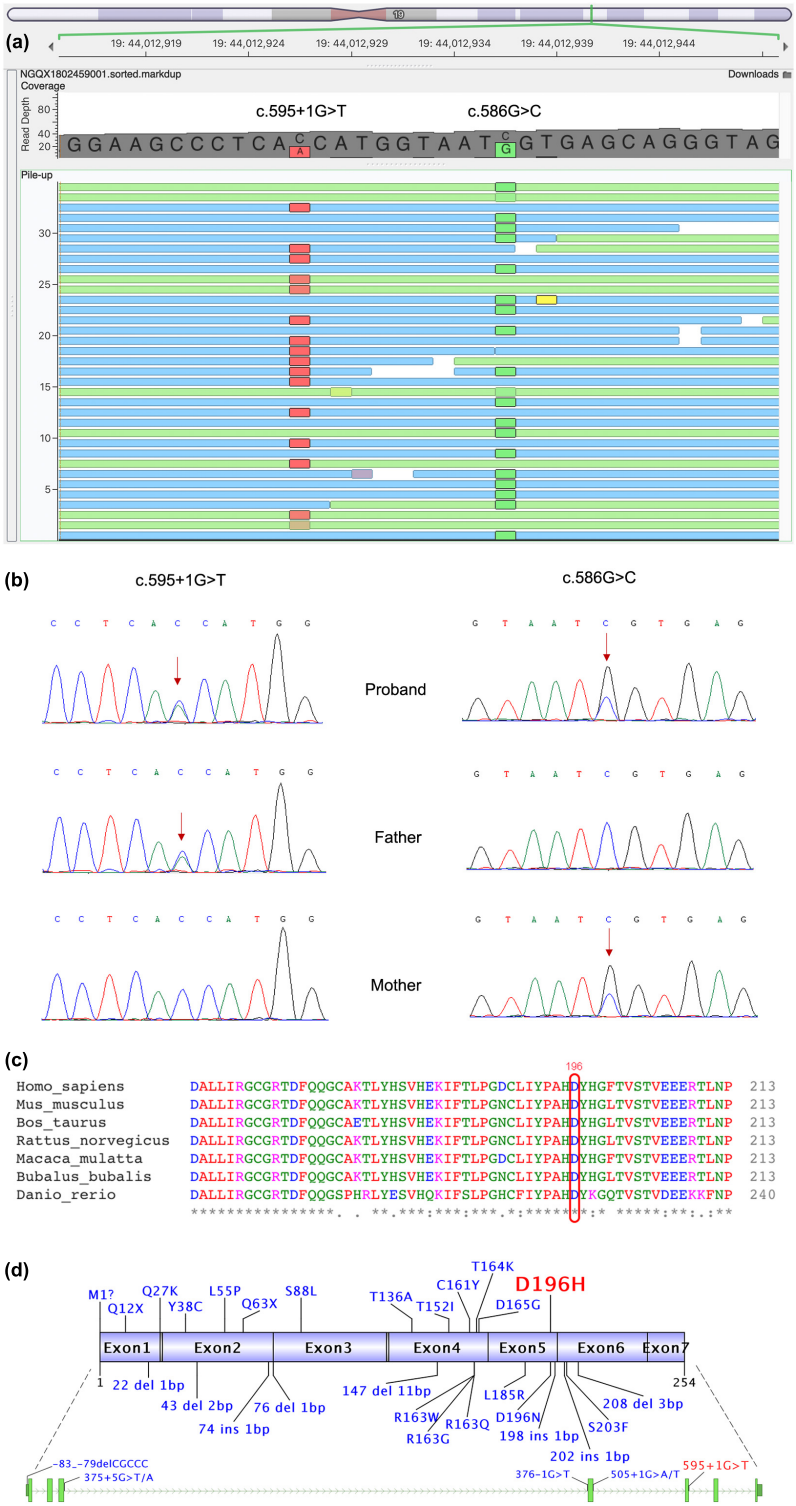


FIGURE 2 | Whole-exome sequencing and Sanger sequencing results. **(a)** Two variants of *ETHE1*, c.586G>C and c.595+1G>T, were identified. **(b)** Sanger sequencing of *ETHE1* performed to verify the variants in the proband. Sanger sequencing confirmed that the c.595+1G>T variant was inherited from the father and that c.586G>C (p. D196H) was of maternal origin. **(c)** Multiple alignment of the ETHE1 protein sequence across several species. Variant site Asp196 is labeled by a red circle. * in the last row indicates "highly conserved." **(d)** Nineteen missense variants, 8 insertions/deletions, 5 splicing variants and 1 variant in the noncoding region are labeled in blue (two variants leading to p. M1?). Our case shows c.586G>C (p. D196H) in exon 5 and c.595+1G>T at the canonical splicing site (red). The upper panel is the ETHE1 protein sequence (exon n indicates the exon n-encoding region). The bottom panel shows the *ETHE1* gene sequence, including the exons, introns, and noncoding regions.

structural and functional integrity. In addition, in our case, we identified two novel variants, including one missense variant (c.586G>C; p. D196H) and one splicing variant (c.595+1G>T) to enrich the database of *ETHE1* variants.

In the active form of ETHE1, a mitochondrial sulfur dioxygenase is a dimer of two identical monomers, chain A and chain B (**Figure 3a**) (Pettinati et al., 2015). ETHE1 contains an αββα metallo-β-lactamase fold, which supports metal binding by catalytic histidine clusters and aspartate (Papetti et al., 2015). The 4th exon encodes a β-sheet as a part of the active site, which explains the significance of its integrity. Asp196 is located near the catalytic histidine cluster (His195) and interacts with several residues (Cys34, Tyr197, His198, Gly199, and Phe200), which might contribute to the maintenance of the monomeric or dimeric structure (**Figure 3b**). The alteration from Asp196 to His196 generates a powerful repulsion between His196 and Phe200, probably reducing the stability of the EHTE1 protein structure (**Figures 3c,d**). Unfortunately, this speculation cannot be verified since it is not possible to retrieve biological material from the patient. However, it has been reported that the missense variant c.586G>A (p. D196N) does not influence the expression level of ETHE1 protein; thus, this variant likely affects the catalytic activity of EHTE1 (Mineri et al., 2008). Therefore, it is probable that the novel variant c.586G>C (p. D196H) in our case alters the conformation of the catalytic histidine cluster or the structure of the dimer, thus indirectly interfering with substrate recognition and catalysis.

EE is characterized by chronic diarrhea, petechiae, orthostatic acrocyanosis, hypotonia, pyramidal signs, psychomotor delay and seizures. Biochemically, elevated urinary EMA, MSA, lactic acid and C4-/C5-acylcarnitine ester are signs of EE. However, the clinical manifestations of patients vary depending on the individuals and the variants they carry. The **Table 1** reviews the published variants and their corresponding clinical phenotypes and includes the current case. Elevated EMA and C4-acylcarnitine ester are present in all cases (15/15) and are necessary conditions for an EE diagnosis. Psychomotor delay (14/15), chronic diarrhea (10/15), hypotonia (11/15) and elevated serum lactic acid (12/15) are typical features. Half of all patients also experience symptoms, including petechiae (8/15), acrocyanosis (6/15), pyramidal signs (6/15), and seizures (6/15). The clinical synopsis of EE in OMIM[4] recorded that retinal lesions with tortuous vessels is among the manifestations, but only the present case was associated with abnormal visual evoked potentials (1/15). Moreover, allelic heterogeneity is likely present in our case and case 2. These two patients shared the same locus but different variants and showed relatively mild symptoms compared to that of the other patients, supporting the opinion that the variant on Asp196 is associated with a benign course of EE. Since orthostatic acrocyanosis and the pyramidal signs in case 2 were identified at the age of 5, follow-ups of our patient will be necessary to determine the presence of allelic heterogeneity. Since our patient has already shown petechiae caused by falling at 3 years of age, it is likely that spontaneous petechiae or acrocyanosis will emerge in the near future.

[4]https://www.omim.org/entry/602473

In addition, the patients in the present case, case 14 and case 15 were of Chinese origin. Although the patient in case 14 died at the age of 4.25 years, no symptoms other than chronic diarrhea, motor delay, language delay, and regression appeared. All patients of Chinese origin exhibited chronic diarrhea and developmental delay, but orthostatic acrocyanosis, hypotonia, and pyramidal signs were absent. It seems that the phenotypes in the patients of Chinese descent exhibited fewer and milder symptoms associated with vasculopathy. Since the onset-age of some patients was only a week old, we considered that different intestinal bacterium from breast milk might influence hydrogen sulfide production, and that intestinal sources of sulfur are unlikely the major factor. However, as children grown up, the exacerbation of hydrogen sulfide production and accumulation is likely associated with the increased uptake of food sources of sulfur, such as dried fruits, breads, brassica vegetables and nuts (Carbonero et al., 2012). To validate these hypotheses, more studies are required to determine the correlations between EE and dietary lifestyles, different gut bacteria or sulfur sources.

Although the pathogenesis of EE is clear, there is no well-established standard therapy for EE patients. Palliative treatments are commonly given based on clinical symptoms as follows: maintenance of hydration or caloric intake for chronic diarrhea, muscle relaxants for dystonia, antiepileptic medications for seizures, physical therapy for contractures, and L-carnitine or coenzyme Q_{10} supplements and vitamin therapies for poor energy metabolism (Gorman et al., 2016). Moreover, the combined therapy of oral metronidazole and N-acetylcysteine has been found to be an effective in EE treatment by reducing the high levels of hydrogen sulfide (Viscomi et al., 2010). Dual treatment caused marked improvement in 5 EE patients, almost without side effects as follows: body weight increased; diarrhea occurred less frequently; petechiae, acrocyanosis and seizures decreased or disappeared; hypotonia showed marked improvement; and plasma EMA significantly decreased in all 5 affected children. In addition, liver transplantation is considered as a viable therapeutic option for EE (Dionisi-Vici et al., 2016; Tam et al., 2019). Although only 3 EE patients achieved psychomotor developmental improvement and marked reversion of biochemical abnormalities after liver transplantation, their outcomes support the promising approach as a standard treatment for EE. In the present case, liver transplantation will be considered if the symptoms worsen in the future follow-ups.

In conclusion, this study described a Chinese EE patient whose disease was confirmed by WES. Two novel variants, c.586G>C (p. D196H) and c.595+1G>T, were identified and classified as "pathogenic" and "likely pathogenic." The clinical manifestations and genetic information of all EE patients with available data were reviewed. We found that elevated EMA and C4-acylcarnitine ester are necessary conditions for an EE diagnosis and that Chinese patients have comparatively mild symptoms. Thus, for future patients with psychomotor delay, chronic diarrhea, petechiae, orthostatic acrocyanosis or hypotonia, especially those with elevated EMA and C4-acylcarnitine ester on biochemical examination, EE should be the first consideration. Using Sanger

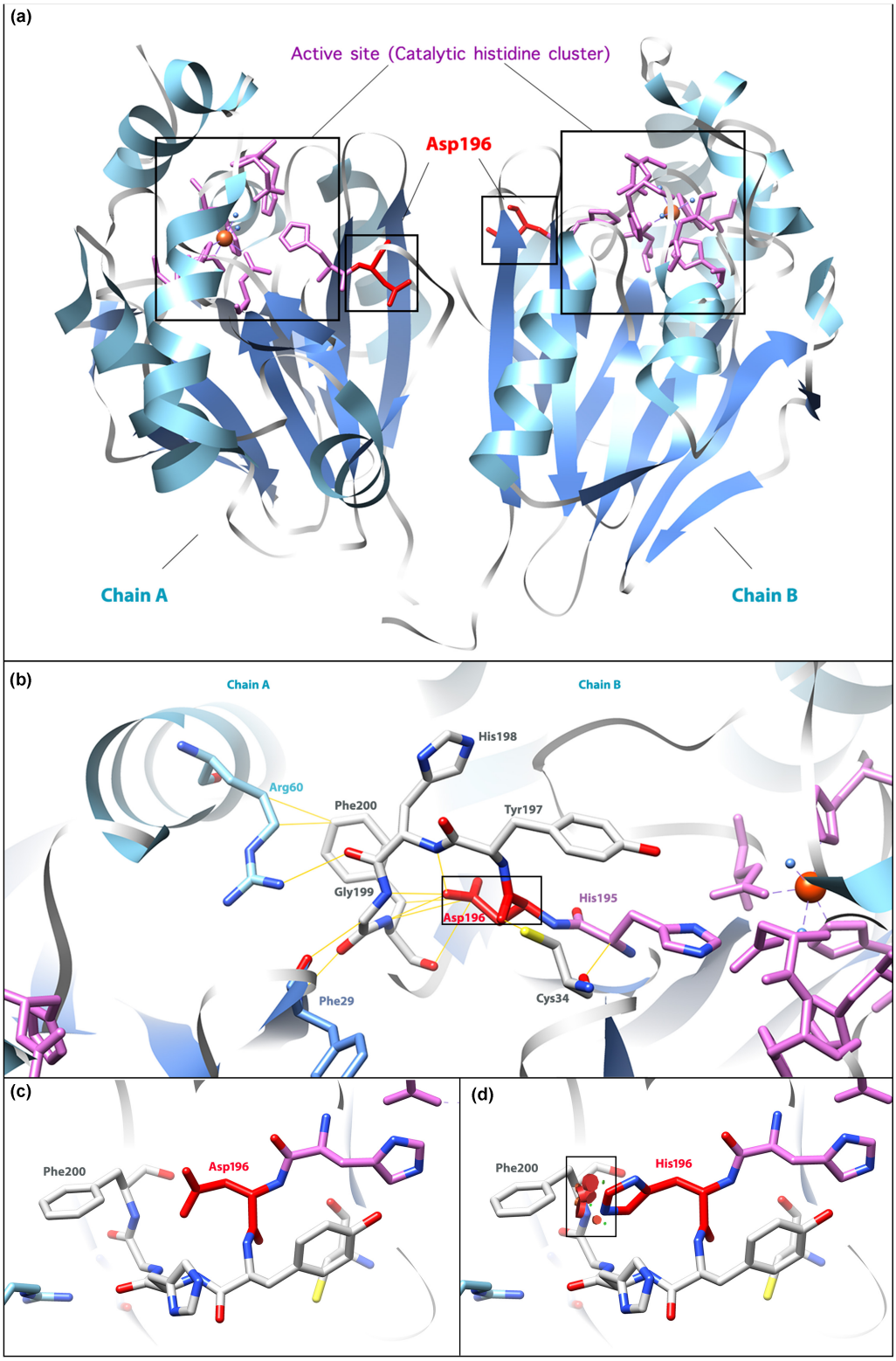


FIGURE 3 | Molecular model of the human ETHE1 protein. **(a)** The active form of the ETHE1 protein is a dimer, including chains A and B. α-helices and β-sheets are labeled in light blue and cornflower blue, respectively. The active site (catalytic histidine cluster) is the iron-binding region. The residue affected in the missense variant, Asp196, is shown in red. **(b)** Interactions between Asp196 and surrounding residues. Yellow lines show interactions between labeled residues in ETHE1. **(c)** Wild-type Asp196 and Phe200. **(d)** Mutant His196 and Phe200. Powerful repulsions between two residues are labeled in the black square. Three-dimensional structures were generated based on the data from Pettinati et al. (2015) by the UCSF Chimera (Pettersen et al., 2004).

TABLE 1 | Variants and clinical manifestations of EE patients.

Case	Variant	Age at onset	Development	Chronic diarrhea	Petechiae	Acrocyanosis	Hypotonia	Pyramidal sign	Seizures	Cytochrome c oxidase deficiency	EMA↑	MSA↑	Lactic acid↑	C4 – carnitine↑	C5 – carnitine↑	Eyes	Brain MRI	Other clinical findings	Exitus	References
1	c.586G>C p.D196H and c.595+1G>T splice in ETHE1	2 years 5 m	Global developmental delay	+	+ (only after falling, 3 years)	–	–	–	–	N/A	+	+	+	+	–	Abnormal visual evoked potentials	Abnormal shadow next to the left lateral ventricle	Abnormal auditory evoked potentials in the brainstem	–	Present case
2	Homozygous c.586G>A p.D196N in ETHE1	6 m	Psychomotor delay and regression	+	–	+ (5 years)	+	+ (5 years)	–	58%	+	–	+	+	N/A	–	Bilateral asymmetrical high T2-weighted signal intensity in the globus pallidus, capsula extrema, and amygdala	Vomiting	–	Mineri et al., 2008
3	c.221–222insA p.Y74X and c.491C>A p.T164K in ETHE1	1 week	Psychomotor delay and regression and poor growth	+	+	+	+	+	+	28%	+	+	+	+	+	–	Symmetrical bilateral lesions in the striatum and globus pallidus	–	17 m	Mineri et al., 2008
4	Homologous c.164T>C p.L55P in ETHE1	6 m	Psychomotor delay	–	+	+	+	–	+	30%	+	–	+	+	+	–	Abnormal signal in the white matter with Leigh-like lesions	–	–	Mineri et al., 2008
5	c.487C>T p.R163W and c.455C>T p.T152I in ETHE1	6 m	Psychomotor delay and neurological regression	–	–	–	+	–	+	N/A	+	–	N/A	+	+	–	N/A	–	–	Mineri et al., 2008
6	Homologous del ex.4–7 in EHTE1	1 week	Psychomotor delay and regression	+	+	+	+	–	–	N/A	+	+	+	N/A	N/A	–	Brain atrophy	Upper respiratory infection	–	Mineri et al., 2008
7	c.66delC stop codon in ETHE1 and c.625G>A in SCAD	5 m	Failure to thrive	+	+	–	+	–	–	+	+	+	+	+	+	–	Cortical atrophy (EEG)	Enlarged kidneys and diffuse mesangial sclerosis	–	Merinero et al., 2006
8	c.488G>A p.R163Q and c.375+5G>T splice, in ETHE1	2 years	Developmental regression	+	+	+	+ (Peripheral hypertonia at 4 years)	–	–	N/A	+	+	+	+	+	–	Abnormal hyperintense signals in T2W/FLAIR in bilateral putamen and caudate nuclei	Abnormal acyl-carnitine profile and metabolites on urinary analysis; easy bruising	–	Bijarnia-Mahay et al., 2016

(Continued)

TABLE 1 | Continued

Case	Variant	Age at onset	Development	Chronic diarrhea	Petechiae	Acrocyanosis	Hypotonia	Pyramidal sign	Seizures	Cytochrome c oxidase deficiency	EMA↑	MSA↑	Lactic acid↑	C4 – carnitine↑	C5 – carnitine↑	Eyes	Brain MRI	Other clinical findings	Exitus	References
9	c.622_624 delGAG p.E208del (paternal), c.340A>T p.I114F and c.488G>A p.R163Q (maternal) in ETHE1	3 m	Psychomotor delay	+	+	+	+	+	–	10%	+	–	+	+	+	–	Abnormal signal intensity involving the lenticular and caudate nuclei bilaterally, the brainstem and cerebellar dentate nuclei	Bilateral and symmetrical spasms of the neck, trunk and extremities with West syndrome	9 m	Papetti et al., 2015
10	c.79C>A p.Q27K and c. 554T>G p.L185R in ETHE1	15 m	Psychomotor delay	–	–	–	+	+	+	N/A	+	+	+	+	+	–	Abnormalities in the white matter, corpus callosum, and basal ganglia	Mild gastroenteritis; status epilepticus; decreased level of consciousness	–	Pigeon et al., 2009
11	c.79C>A p.Q27K and c. 554T>G p.L185R in ETHE1	6 week	Psychomotor delay	–	–	–	+	+	–	N/A	+	+	+	+	+	–	Abnormalities in the white matter, corpus callosum, and basal ganglia	Intercurrent viral illness; gastroenteritis	–	Pigeon et al., 2009
12	Homologous c.494A>G p.D165G in ETHE1	6 m	Global development delay	N/A	N/A	N/A	N/A	N/A	N/A	+	+	+	+	+	–	–	Nonspecific abnormalities	Spastic diplegia; deficiency of mitochondrial complex IV	–	Walsh et al., 2010
13	Homologous c.487C>G p.R163G in ETHE1 and c.625G>A in SCAD	2 m	Psychomotor delay	+	+	–	+	+	+	+	+	–	N/A	+	–	–	Symmetrical hyperdense lesions in T2-weighted images in the basal ganglia, the periventricular white matter, the corpus callosum and the cerebellum	Head lag at the age of 5 months; convergent strabism; no auditory interest	3 years	Zafeiriou et al., 2007
14	c.2T>A p.M1? and c.488G>A p.R163Q in ETHE1	2 years	Motor delay and language delay and regression	+	–	–	–	–	–	N/A	+	–	–	+	–	–	Lesions in the basal ganglia, bilateral lateral ventricles and corpus callosum	Hypertonia	4.25 years	Zhang et al., 2018
15	c.203T>C p.L68P and c.488G>A p.R163Q in ETHE1	7 m	Psychomotor delay and regression	+	–	–	–	–	+	N/A	+	+	+	+	+	–	Bilateral basal ganglia lesion	Hypertonia	–	Li et al., 2014

sequencing for *ETHE1* only or next-generation sequencing could provide supportive evidence to confirming the diagnosis. Our study and review are also helpful for the early and rapid diagnosis and treatment of EE patients in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was approved by the Medical Ethics Committee of Wuhan Children's Hospital. An informed consent form was signed by the patient's parents for the study and the publication of this case report.

AUTHOR CONTRIBUTIONS

XC cared for the patient and collected the clinical data of the patient. LH interpreted the results of the genetic sequencing, reviewed the published cases, and wrote the manuscript. HY supervised the study. All authors read and approved the submitted version.

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Conflict of Interest: LH was employed by Running Gene Inc., Beijing, China.

The remaining authors declare that the research was conducted in the absence of any other commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pediatrics

CASE REPORT
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Congenital Myasthenic Syndrome Caused by a Novel Hemizygous *CHAT* Mutation

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Congenital myasthenic syndrome (CMS) is a neuromuscular transmission disorder caused by mutations in genes encoding neuromuscular junction proteins. CMS due to choline acetyltransferase (*CHAT*) gene mutation is characterized by episodic apnoea. To date, 52 cases of CMS caused by *CHAT* gene mutations have been reported. Here, we report a neonate with the third hemizygous mutation [a 4.9 Mb deletion [10q11.22–10q11.23 (chr10: 46123781–51028772)] containing the whole *CHAT* gene and c.1976A>T (p.Gln659Leu in the *CHAT* gene)]. The c.1976A>T (p.Gln659Leu) variant had not been reported in the ExAC or gnomAD databases and was predicted to be pathogenic. The alignment of amino acid sequences revealed that glutamine at codon 659 is highly conserved in different species and causes structural changes in the substrate-binding site. Our female patient with neonate-onset CMS presented with apnoea, dyspnoea, feeding difficulties, weak crying, and seizure-like episodes, and her respiration was ventilator dependent. The prostigmine test was positive. This case may help to further elucidate clinical features and treatment methods in neonate-onset CMS caused by *CHAT* gene mutations.

Keywords: congenital myasthenic syndrome, CHAT mutation, hemizygous, apnoea, genetic diagnosis

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INTRODUCTION

Congenital myasthenic syndromes (CMSs) are rare genetically heterogeneous diseases caused by abnormal signal transduction due to mutations in genes coding for proteins expressed at the neuromuscular junction (NMJ). According to the site of mutation, CMS can be divided into presynaptic, synaptic or postsynaptic (1). Individuals with presynaptic types commonly present with apnoea and can also present with dyspnoea, ventilator dependence, ptosis, bulbar paralysis, and fatigue. The above symptoms may be triggered by fever, cold, stress, infection, excitement, and excessive exercise and may lead to apnoea crisis. Arthrogryposis can also be seen in presynaptic types of CMS. Individuals with synaptic types can present with delayed pupillary light reflex; renal and ocular malformations; Pierson syndrome; and severe ocular, respiratory, and proximal limb muscle weakness. Patients typically present with apnoea and generalized weakness in the neonatal period or infancy that persists throughout life. Individuals with postsynaptic types can present with ptosis and ophthalmoparesis; micrognathia; lesser face symptoms; and weakness of the masticatory, limb girdle and neck muscles in the first year of life, and arthrogryposis at birth occurs in close to one-third of patients. Patients presenting later in childhood often have a milder clinical course. A useful clinical clue is selectively severe involvement of cervical, wrist, and extensor muscles (1–3).

CMSs, with the exception of slow channel syndrome, SNAP25-CMS and synaptotagmin 2–CMS, are due to autosomal recessive hereditary mutations (4–6). Several novel CMS mutations have been discovered since 2011 due to the development of whole exome sequencing. Protein glycosylation with enzymatic defects, that is, posttranslational addition of glucose or mannose residues that occurs in the Golgi apparatus and endoplasmic reticulum, has been identified as a new subtype of CMS (7). To date, more than 30 CMS disease genes have been identified (8, 9).

CHAT gene mutations are presynaptic types. They account for approximately 5% of CMS probands (1). The *CHAT* gene is located on the long arm of human chromosome 10 (10q11.23) and encodes the biosynthetic enzyme of the neurotransmitter acetylcholine, namely, choline acetyltransferase. *CHAT* gene mutations can result in deficient resynthesis of acetylcholine following its reuptake at the nerve terminal (10). The choline acetyltransferase protein belongs to the family of eukaryotic acetyltransferases (11). To date, 48 *CHAT* gene mutations have been identified as related to CMS (http://www.hgmd.cf.ac.uk/ac/dipx.php). Mutations located near the active-site tunnel, impairing substrate binding, result in more severe phenotypic effects in patients with *CHAT*-related CMS (12–14). Fifty-two cases of CMS caused by *CHAT* gene mutations have been reported, and only two of them have been identified as hemizygous mutations (12, 13, 15–29). In 2011, Shen et al. revealed the first hemizygous *CHAT* mutation in a patient with CMS by quantitative RT-PCR (12). Schwartz et al. reported the second hemizygous *CHAT* mutation in a patient with CMS in 2018 (16).

Here, we report a third case of hemizygous mutation. A neonate with CMS carried a 4.9 Mb deletion and a c.1976A>T (p.Gln659Leu) mutation in the *CHAT* gene. The deleted region, q11.22–q11.23 (chr10: 46123781-51028772) × 1, included 91 genes. Only five were associated with abnormal phenotypes in the Online Mendelian Inheritance in Man (OMIM®) database: *CHAT* (choline acetyltransferase), *ERCC6* (chromatin remodeling factor), *SLC18A3* (solute carrier family 18, member 3), *GDF2* (growth differentiation factor 2), and *RBP3* (retinol binding protein 3). Whole exome sequencing showed a hemizygous mutation in *CHAT* (c.1976A>T, p.Gln659Leu), which likely accounts for the patient's phenotype. The clinical features and treatment of this neonate were discussed below.

CASE REPORT

Medical history

The proband was a 13-day-old female, the second child of healthy non-consanguineous parents. She was born as the first of fraternal twins at 37 weeks via cesarean section due to reduced heart rate of the twins. Her birth weight was 2.4 kg (<10th p). Her older brother and the other neonate twin were both normal, and there was no relevant medical family history. After birth, she required a laryngeal mask to pressurize the oxygen. The treatment relieved her cyanosis. She was then immediately transferred to the local neonatal intensive care unit where she was intubated and ventilated. She was also treated with anti-infectious and excitatory respiration center drugs (nalmefene hydrochloride injection). However, the effect was poor. She failed to extubate after multiple attempts due to carbon dioxide retention. The patient was hospitalized in our neonatal intensive care unit for dyspnoea for 13 days with failure to extubate for 1 day.

Physical Examination

The patient had a full-term appearance, a weight of 2.35 kg, poor response to physical examinations, weak crying, labored breathing, shortness of breath, and a positive three-concave sign. Both lungs were clear on auditory percussion. Neither obvious wet nor dry rattling was heard. The heart rate was strong, and no obvious murmurs were heard in the precordial region. There was no abdominal guarding upon palpation, and abdominal rumbling was weak. There was reduced movement of the limbs, decreased muscle tone and absent primitive reflexes.

Main Laboratory Examinations

The results of routine blood examination were as follows: white blood cell count 27.90×10^9/L, erythrocyte count 3.61×10^{12}/L, hemoglobin 113.0 g/L, platelet count 390×10^9/L, percentage of neutrophils 55.8%, and percentage of lymphocytes 32.8%. The levels of inflammatory markers were as follows: procalcitonin 0.390 ng/mL and C-reactive protein 7.60 mg/L. The N-terminal pro b-type natriuretic peptide level was 1,334.00 pg/mL. The prostigmine test was positive. The fungal glucan level was 405.05 pg/mL. The myocardial enzyme levels were as follows: creatine kinase 70.00 U/L and creatine kinase isoenzyme 20.10 U/L. The chest plain film indicated a double-lung texture growing thick and heavy, and CT images showed an increasing density of patchy shadows with blurry margins, which was consistent with the manifestations of pneumonia (**Figures 1A,B**). No abnormalities were found on cranial MRI (**Figure 1C**). In regard to brain function, the upper and lower peaks were generally normal, but the differences in the sleep-wake cycle were not obvious. The acetylcholine receptor antibody level was 0.045 mmol/L (within the normal range).

Therapeutic Process

Upon transfer to our neonatal intensive care unit, the patient's oxygen saturation decreased frequently and at times was accompanied by convulsions. Subsequently, the patient was intubated for mechanical ventilation, was fed milk through a nasogastric tube and later showed difficulty in weaning from the ventilator. We administered a neostigmine (0.05 mg/kg) intramuscular injection, which relieved the dyspnoea and improved her feeding. We then orally administered pyridostigmine (5 mg/kg) to replace the intramuscular injection, which controlled her dyspnoea. We did not observe any side effects from these drugs. Later, she was weaned successfully from mechanical ventilation and could gradually breathe independent of oxygen therapy. However, episodes of dyspnoea accompanied by cyanosis and decreased oxygen saturation occurred upon reducing the dosage of pyridostigmine. We failed to make a definitive diagnosis at the initial stage of treatment due to rejection of genetic testing by the family members. She was transferred to a hospital with better equipment and treatment capacity, according to the parent's wishes, where she died.

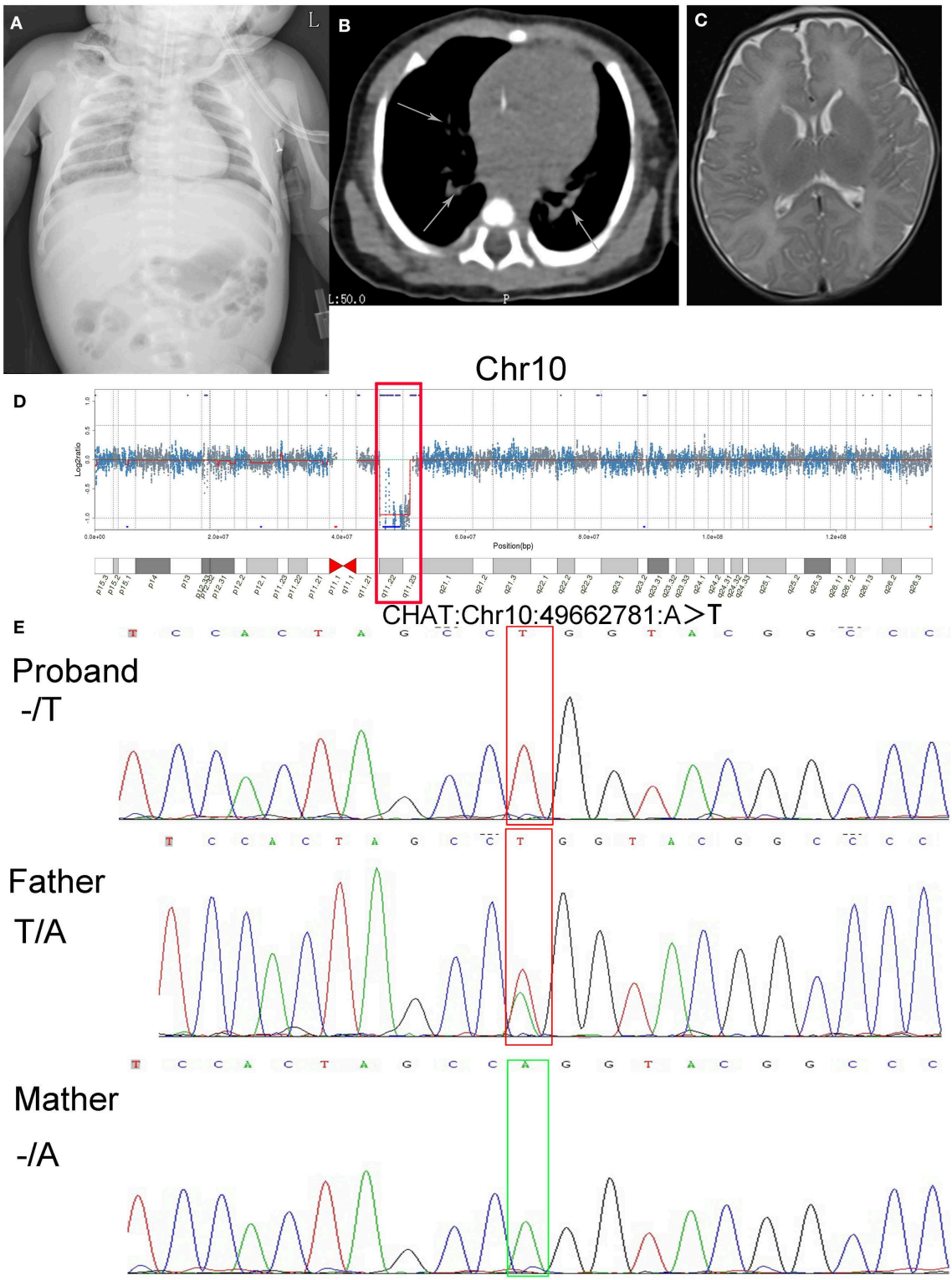


FIGURE 1 | Results of imaging tests and genetic tests, alignment of amino acid sequences, and a schematic of gene structure and protein structure. **(A)** Chest plain film indicated double-lung texture growing thick and heavy. **(B)** CT images showed an increasing density of patchy shadows with blurry margins (arrows). **(C)** The brain MRI showed no abnormalities. **(D)** The results of CNV-Seq from the patient's genomic DNA sample (q11.22–q11.23 deletion on chromosome 10 is shown in the box). **(E)** Sanger sequencing of the CHAT gene showed the hemizygous mutation c.1976A>T (p.Gln659Leu) in the proband, absence of the mutation in the mother and heterozygous mutation in the father.

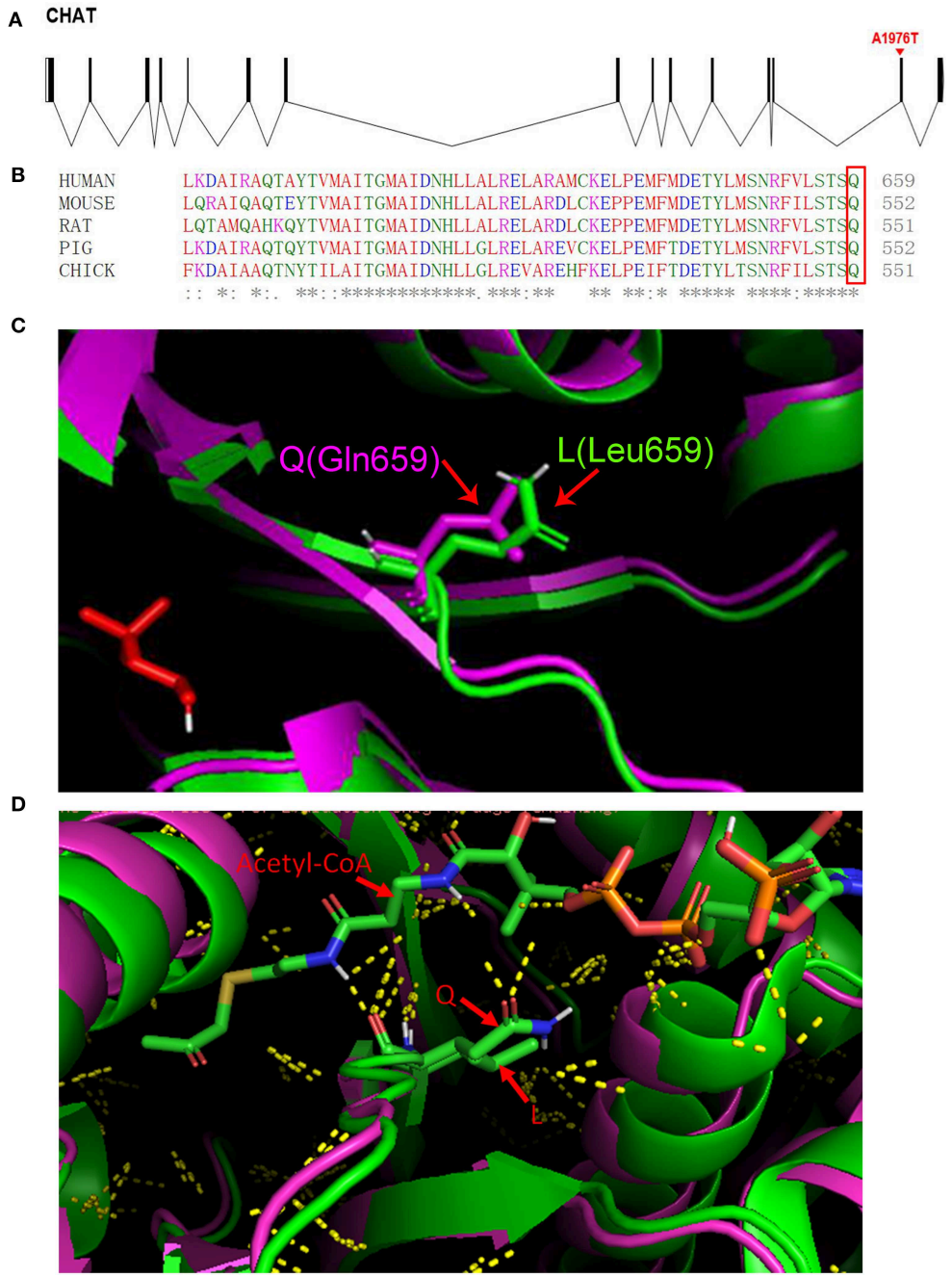


FIGURE 2 | (A) The c.1976A>T (p.Gln659Leu) mutation is shown in a schematic of the gene structure. **(B)** (Q) Glutamine at position 659 is highly conserved in CHAT in various species (the box marks the amino acid of interest). **(C)** Homology models for *CHAT* protein generated using the crystal structures of *CHAT* protein (PDB accession code: 2FY4) as the template. **(D)** Cartoon structure representation of the subtle changes (Gln-to-Leu) at position 659. The green cartoon represents the wild-type protein, and the purple cartoon represents the mutant protein. Yellow dots represent polar contacts.

Genetic Tests

In consideration of the clinical course of the neonate, we suspected that she had CMS. To explore the potential genetic etiology, we communicated with the patient's parents repeatedly and ultimately obtained informed consent for genetic tests 15 days after admission. The genetic testing was performed when she was 28 days old (2018-05-14). Two milliliters of peripheral blood was extracted from the proband and her parents. The blood was sent to Cipher Gene LLC, and the following tests were conducted. Whole exome sequencing was performed on the proband and her parents using the Illumina NovaSeq 6000 platform. Agilent SSELXT Human All Exon V6 was used to

capture and construct the library, and a paired-end sequencing strategy was used. CNV-Seq was performed on the proband using the Illumina NovaSeq 6000. A TruSeq Library Construction Kit was used to capture and construct the library, and a paired-end sequencing strategy was used. CNV-Seq indicated a 4.9 Mb deletion [10q11.22-10q11.23 (chr10: 46123781-51028772)], which contained the whole *CHAT* gene (**Figure 1D**). The whole-exome sequencing validated by Sanger sequencing indicated that the patient carries a hemizygous mutation in exon14 of *CHAT* (c.1976A>T, p.Gln659Leu), which is inherited from her father (**Figure 1E**).

DISCUSSION

Our patient presented with dyspnoea, ventilator dependence, and apnoea. The prostigmine test was positive. Pyridostigmine relieved dyspnoea in our patient, and she weaned successfully from mechanical ventilation and could gradually breathe independent of oxygen therapy. The clinical course of the neonate and whole exome sequencing indicated *CHAT*-related CMS.

fifty-two cases of *CHAT*-related CMS have been reported to date (**Table S1**), and our literature search revealed a total of 48 genetic variations that have been identified to date. Our patient carried a 4.9 Mb deletion [10q11.22-10q11.23 (chr10: 46123781-51028772)] and a hemizygous mutation in *CHAT* (p.Gln659Leu). The copy number variation of the 4.9 Mb deletion in this study and its highly overlapping deletion fragment could not be found in the Genetic Variation Database. Highly overlapping pathogenic or suspected pathogenic CNVs were found in the Decipher patient database (https://decipher.sanger.ac.uk/syndromes#syndromes/overview (**Table S2**). The mutation of p.Gln659Leu was located on the 14 exon of *CHAT* (**Figure 2A**). The variant had not been reported in the ExAC (http://exac.broadinstitute.org/) or gnomAD databases (http://gnomad.broadinstitute.org/about) and was predicted to be pathogenic by multiple prediction software programs (SIFT, Polyphen2, LRT, Mutation Taster, FATHMM, etc.). The alignment of amino acid sequences revealed that glutamine at codon 659 is highly conserved in different species (https://www.ebi.ac.uk/Tools/msa/), which suggests p.Gln659Leu is likely pathogenic (**Figure 2B**). Three-dimensional structures of the wild-type and mutant *CHAT* protein of human were constructed by the homologous modeling database SWISS-MODEL (http://swissmodel.expasy.org/). Residual changes between the wild-type and mutant *CHAT* proteins were mapped to the model by PyMol v1.3 software. This mutation causes a structural change in the substrate-binding site (**Figures 2C,D**). Mutations located near the active-site tunnel impair substrate binding and result in more severe phenotypic effects in patients with *CHAT*-related CMS (12–14). Glutamine possesses features of polarity compared with non-polar leucine due to the amide group in the residue side chain. As shown in **Figure 2D**, higher numbers of polar contacts between Q (amide group) and acetyl coenzyme A (amino group in the alanine residue and hydroxyl group in diphosphate) might indicate stronger substrate binding in the wild type. However, the hydrophobic alkyl group in the leucine residue side chain might impair substrate binding. Therefore, we speculate the mutation reduces catalytic activity of the enzyme resulting in the defect of neuromuscular transmission.

The current treatment for CMS is dependent on the CMS gene involved. The treatments of *CHAT*-associated CMS are mainly cholinesterase inhibitors and respiratory support, which can help improve clinical symptoms and prognosis. The most commonly used cholinesterase inhibitor is pyridostigmine, which can be used for the treatment of *CHAT*-related CMS pediatric patients. Apnoeic episodes can be relieved with oral or parenteral neostigmine administration (30). In our patient, apnoea and dyspnoea were significantly improved with intramuscular injection of neostigmine, and she was weaned from the ventilator successfully after pyridostigmine (4 mg/kg/day) administration. After 14 days of treatment, we reduced the dosage of pyridostigmine (2 mg/kg/day); however, the oxygen saturation was reduced. We considered that the crisis may have been caused by uncontrolled infection, insufficient drug administration or rapid drug reduction. Patients with *CHAT*-related CMS may have symptoms accompanied by convulsions, which may be easily mistaken for epileptic seizures (27). Our patient's symptoms were initially accompanied by convulsions. Sedative drugs with bolus injection had poor anticonvulsive effects in our case, a finding that warrants attention. It is important to carefully select drug therapy because the same drug can be effective, invalid, or even harmful for different CMSs. For instance, the use of cholinesterase inhibitor in CMS caused by collagen-like tail subunit of acetylcholinesterase mutation is invalid and worsens the condition by enhancing the effect of cholinesterase (30). Patients diagnosed with CMS are rare, and clinical trials to determine optimal pharmacotherapeutic combinations for patients remain difficult to perform. Therefore, a standard treatment for patients with *CHAT* mutations has not been established.

We suggest that all patients suspected of having CMS receive precise genetic diagnosis as soon as possible. We report here a case of CMS caused by a novel hemizygous mutation of the *CHAT* gene and hope this report helps improve physicians' knowledge of *CHAT*-related CMS.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://gnomad.broadinstitute.org/about.

ETHICS STATEMENT

We obtained written informed consent to publish this case. This study was approved by the First Affiliated Hospital of Zhengzhou University ethics committee (ID No: 2019-LW-010).

AUTHOR CONTRIBUTIONS

QZ conceived and designed the work. YZ drafted the manuscript and collected the data. XC contributed to the

conception of the work and played an important role in interpreting the clinical data. CL contributed to the manuscript revision. ML contributed to figure preparation. FM contributed to the adjustment of the structure of the manuscript. ZS contributed to figure preparation. WC contributed to table preparation. JZ contributed to data collection.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00185/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Chromosome 15 Imprinting Disorders: Genetic Laboratory Methodology and Approaches

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Chromosome 15 imprinting disorders include Prader-Willi (PWS) and Angelman (AS) syndromes, which are caused by absent expression from the paternal and maternal alleles in the chromosome 15q11. 2–q13 region, respectively. In addition, chromosome 15q duplication caused by the presence of at least one additional maternally derived copy of the 15q11.2–q13 region can lead to seizures, cognitive and behavioral problems. We focus on PWS and AS in the report, and expand the discussion of clinical care and description with genetic testing to include high-resolution studies to more specifically characterize the molecular mechanisms of disease. The importance of early diagnosis with the necessity for accurate molecular characterization through a step-wise algorithm is emphasized in an era of targeted therapeutic interventions. We present a flowchart to aid in ordering specialized genetic testing as several methods are available for patients presenting with features of PWS and/or AS.

Keywords: Prader-Willi syndrome, Angelman syndrome, imprinting disorders, genetic testing flowchart, targeted genetic treatment approaches, duplication 15q, chromosome 15 disorders

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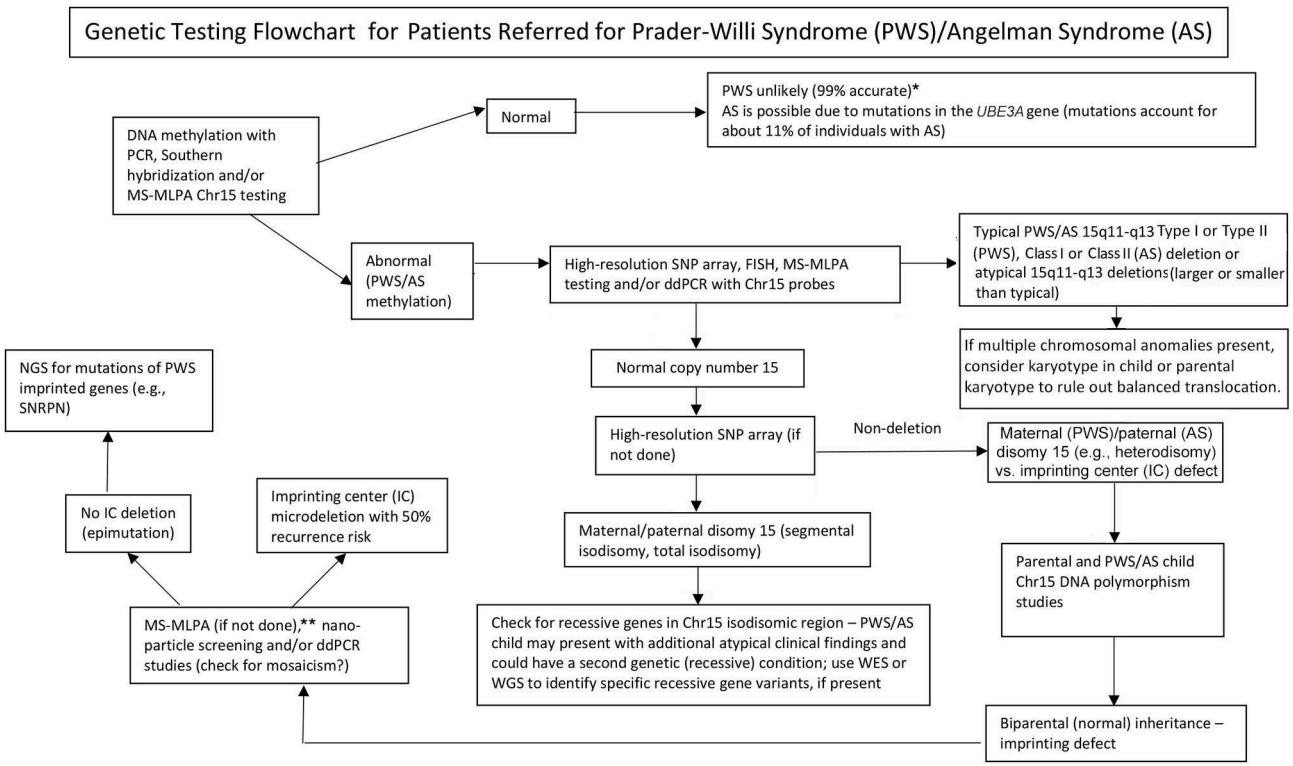
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INTRODUCTION

The chromosome 15 imprinting disorders include Prader-Willi (PWS) and Angelman (AS) syndromes (1–6) and chromosome 15q duplications. Diagnosis of PWS or AS depends on the parent of origin and whether expression is aberrantly limited to the maternal or the paternal imprinted genes. Duplication 15q is caused by an additional copy of the maternally-derived 15q11.2–q13 region which can lead to seizures, cognitive and behavioral problems including autism spectrum disorder (ASD), but not a PWS or AS phenotype. PWS arises from loss of maternally imprinted and paternally expressed genes from the chromosome 15q11–q13 region, while AS is caused by loss of imprinted and maternally expressed genes in this region, specifically impacting the *UBE3A* gene. Due to the imprinted nature of the responsible genes, both genetic and epigenetic errors can be causative.

In 1989, those with both PWS and non-deletion status were found to have maternal disomy 15 or both 15s from the mother when using polymorphic DNA markers from the proximal 15q11–q13 region (7). Later in the mid-1990s, the development of fluorescent *in situ* hybridization (FISH) DNA probes were used to identify deletions of the 15q11–q13 region in both PWS and AS. Methylation DNA testing was developed during this time period and an abnormal methylation pattern was seen in PWS and AS. Methylation DNA testing is ∼99% accurate in identifying the diagnosis of PWS, but will not identify the individual PWS molecular class (2). For AS, DNA methylation testing identifies ∼80% of individuals, but again does not distinguish between the molecular classes or detect a mutation in the *UBE3A* gene causing AS.



GRAPHICAL ABSTRACT | Genetic testing flowchart for patients referred for Prader-Willi syndrome (PWS)/Angelman syndrome (AS). *Rule out Chr15 translocations or inversions by routine chromosome studies; consider other obesity-related genetic disorders; may require fragile X syndrome DNA screening for FMR1 gene repeat expansion or advanced genetic testing with next-generation sequencing (NGS) for FMR1 or other candidate gene variants using whole-exome sequencing (WES) or whole-genome sequencing (WGS; e.g., monogenic causes of obesity). **Can be used to check on methylation status of other Chr15 imprinted genes; ddPCR, droplet digital PCR can be used for mosaicism screening.

Microarray technology was developed in the early to mid-2000s and advanced the diagnostic yield. Now the new SNP microarrays include over two million DNA probes and are useful in detecting deletion subtypes and UPD15 subclasses. Other technology such as droplet digital PCR (ddPCR) quantitates copy number using chromosome 15 DNA probes and can diagnose genetic defects in PWS or AS (8). Furthermore, SNP microarrays can identify LOHs defined as >8 Mb in size and when present on chromosome 15 supports the diagnosis of maternal disomy 15 or paternal disomy 15 in the presence of an abnormal DNA methylation pattern for PWS or AS, respectively. Imprinting defect confirmation may require not only SNP microarrays to identify small microdeletions but also parental DNA samples with genotyping to identify the presence of normal (biparental) inheritance of chromosome 15s supporting the presence of an epimutation imprinting defect in PWS or AS thereby impacting recurrence risks. Differentiation of an IC microdeletion from a non-deletion epimutation status is clinically important for families as a 50% recurrence risk is present for additional children if an IC microdeletion is found in the parent (9).

There are over one dozen genes and transcripts in the 15q11–q13 region which appear to play a role in the causation of PWS and/or AS. Genes and transcripts included in the area from the proximal 15q11.2 breakpoints BP1 and the distal 15q13 breakpoint BP3 are *TUBGCP5*, *CYFIP1*, *NIPA1*, *NIPA2*, *MRKN3*, *MAGEL2*, *NDN*, *NIPAP1*, *SNURF-SNRPN*, non-coding RNAs (SNORDs), *UBE3A*, *ATP10A*, *GABRB3*, *GABRA5*, *GABRG3*, *OCA2*, and *HERC2*. Imprinted *MRKN3*, *MAGEL2*, *NDN*, *NIPAP1*, and *SNURF-SNRPN* genes are paternally expressed and when disturbed may cause features of PWS [e.g., (10)]. For example, *MAGEL2* gene mutations can cause neonatal hypotonia, developmental delay, arthrogryposis, autistic features, a poor suck, and obesity [Schaaf-Yang syndrome; (11)]. Patients have also been reported with features of PWS as a result of small deletions of the non-coding *SNORD116* transcript (12) and other similar deletions in the region (10, 13).

We focused on AS and PWS in this report as both syndromes are detected via DNA methylation testing, which allows for determination of the active parental gene allele and definitive diagnosis in individuals with PWS and in most individuals with AS (2). However, DNA methylation testing will not identify the molecular class in either syndrome. High-resolution chromosome analysis was developed and used in the early 1980s and became a standard laboratory genetic-based test to evaluate for the chromosome 15q11–q13 deletion identified in the majority of patients with PWS at that time (14) and later for AS. The paternal origin of the 15q11–q13 deletion was reported in 1983 (15) and found to be *de novo* but the size of 15q11–q13

deletion or type (typical vs. atypical) could not be determined. Accurate and early diagnosis with identification of the molecular class is essential not only to confirm the clinical diagnosis but also for genetic counseling, to inform care and treatment and to guide expectations. With the intention of ongoing clinical trials, a better understanding of molecular etiology may impact opportunities for patient participation. Furthermore, imminent trials include antisense oligonucleotides to reactivate the silenced paternal copy of the chromosome 15 in individuals with AS.

PWS and AS are complex rare neurodevelopmental disorders due to errors in genomic imprinting. PWS is recognized as the most common genetic cause of life-threatening obesity, if left uncontrolled (2, 4, 6). There are three recognized PWS molecular classes including a paternal 15q11–q13 deletion about 5–6 Mb in size (60% of cases) and maternal disomy 15 (UPD15) in which both chromosome 15s are inherited from the mother (36%) originating from trisomy 15 with loss of the paternal chromosome 15 in early pregnancy leading to two chromosome 15s from the mother (16). The third class is an imprinting center defect. If a microdeletion or epimutation of the imprinting center (IC), which controls the expression status of selected imprinted genes on chromosome 15, is present on the paternal allele then PWS occurs. This imprinting defect is seen in 4% of individuals with PWS (8, 16). Most cases of PWS are sporadic with an approximate equity among ethnic groups and sex. The estimated prevalence of PWS is one in 10,000 to one in 30,000 (2). The number of individuals worldwide with PWS is thought to be ∼400,000 with about 20,000 individuals living in the USA (2, 17).

PWS is characterized by infantile hypotonia, a poor suck reflex with feeding difficulties, short stature with small hands and feet, hypogonadism secondary to hormone deficiencies, mild intellectual disability, behavior problems, and hyperphagia often with onset between 6 and 8 years of age that persists into adulthood and results in obesity if environmental controls are not in place. During infancy, characteristic craniofacial features are seen including a narrow bifrontal diameter, strabismus, small upturned nose with a thin upper lip, and down-turned corners of mouth, sticky saliva, and enamel hypoplasia (2, 4, 6, 18). Cognition is generally reduced based on the family background and behavior problems beginning in childhood include self-injury (skin picking), outbursts, stubbornness, and temper tantrums with psychiatric problems occurring during this time or later in adolescence or young adulthood (2). Behavioral problems include anxiety, mood disorders, psychosis, and autism that may correlate with specific PWS genetic subtypes or molecular classes (19).

Historically, PWS is divided into two clinical stages with failure to thrive during infancy representing the first clinical stage and hyperphagia with onset of obesity representing the second stage (2). Later, nutritional phases have been described for this obesity-related genetic disorder and include: Phase 0 with decreased fetal movement and growth retardation in utero, followed by Phase 1 related to hypotonia, failure to thrive with difficulty feeding, Phase 2 beginning at ∼2 years of age when weight gain is first noted and Phase 3 when lack of satiety is accompanied by food seeking and hyperphagia leading to obesity, if not externally controlled. Phase 3 begins at around 6–8 years of age (20).

Angelman syndrome is characterized by developmental delay often not apparent until about 6 months of age and subsequent onset of often difficult to control seizures, tremor, wide-based gait, and ataxia with a characteristic happy demeanor (3). There are four recognized molecular mechanisms of AS: *de novo* maternal deletions of chromosome 15q11–q13 (70–80%); mutations of the maternally inherited *UBE3A* gene (10–20%); paternal disomy 15 (3–5%); and imprinting defects (3–5%) within the 15q11–q13 region that alter the expression of the causative *UBE3A* gene (21).

Individuals with AS are often not noticed by medical professionals until ∼6 months of age when delays in development in particular delayed motor development are reported. By this time, parents may recognize the happy demeanor that includes frequent laughing, smiling, and excitability. A decreased need for sleep is reported in >80% of individuals with AS (22). They often develop seizures at ages 1–3 years (23). Epilepsy can be intractable and has a characteristic appearance on EEG described as an increased delta power with a characteristic triphasic wave. Individuals with AS are described as ataxic in their movements and walking (24, 25). Microcephaly may develop by ∼2 years of age. Stereotypic behaviors include a love of water and crinkly paper and individuals with AS are characteristically non-verbal and categorized as severely intellectual disabled. However, it is notable that individuals with AS have skills not well-captured on the currently available objective neuropsychological tests. They have strong abilities in manipulating electronics, but behaviors can be challenging and include anxiety with short attention spans.

As patients with PWS or AS may present with variable phenotypes depending on the molecular class and because potential treatment and surveillance approaches exist for each, a logical flowchart is needed for ordering genetic tests by the clinician evaluating these patients. The focus of our report is to describe the clinical and genetic findings of these two genomic imprinting disorders and illustrate genetic testing options available in the clinical setting and the order in which the different genetic tests can be obtained most productively.

LABORATORY GENETICS EXPERIENCE IN CHROMOSOME 15 IMPRINTING DISORDERS

Prader-Willi Syndrome

To serve as an example of the importance of high-resolution SNP microarray testing, a large multisite cohort of 510 participants with genetically confirmed PWS were recruited in the USA and grouped into three molecular classes. They were further characterized as 15q11–q13 deletion subtypes, maternal disomy 15 subclasses and imprinting center defects (16). In this largest reported PWS cohort, 303 individuals were found to have the 15q11–q13 deletion (60% of cases) composed of 118 individuals (38.9%) having the larger typical 15q11–q13 Type I deletion involving chromosome 15q11–q13 breakpoints BP1 and BP3 and 165 individuals (54.5%) had the smaller typical 15q11–q13 Type II deletion involving breakpoints BP2 and BP3 with 20 individuals having an atypical deletion which is larger or

smaller than the typical 15q11–q13 deletion (6.6%). In persons identified to have a deletion of chromosome 15, it is important to consider whether a balanced translocation could be present in the proband's father as this increases the recurrence risk of PWS in the father's offspring. For maternal disomy 15, 185 individuals (36%) had maternal uniparental disomy 15 (UPD15) with 13 individuals (12.5%) having total isodisomy of the entire chromosome 15 due to errors in maternal meiosis II; 60 (57.7%) showed segmental isodisomy from crossover events in maternal meiosis I and 31 showed heterodisomy (29.8%), while 81 individuals did not have SNP microarray analysis and maternal disomy 15 classification determined. Regarding PWS imprinting defects, 22 individuals (4%) were found with 13 (76.5%) having a non-deletion epimutation status, four individuals (23.5%) had a microdeletion of the imprinting center while the remaining five individuals did not have a type of imprinting defect established. In a related study, further analysis of imprinting defects in PWS was carried out by Hartin et al. (8) using droplet digital PCR and next-generation whole-exome sequencing in a separate PWS cohort of 15 unrelated patients and two individuals or 13% were found to have an imprinting center microdeletion defect. In the 60 individuals with segmental isodisomy 15 reported by Butler et al. (16), the total average size of the loss of heterozygosity (LOH) was 25.1 Mb with a range of 5–67.4 Mb and an average size of 16.4 Mb for individual LOHs. Thirty-two individuals had one LOH segment, 25 individuals had two segments and three individuals had three segments. The most common LOH sites were the proximal 15q11–q13 region and distal 15q26 region including the 15q12 and 15q26.1 bands as most commonly recorded.

The presence of maternal UPD15 and specific subclass (segmental or total isodisomy) determination may impact diagnosis and medical care surveillance as a second genetic condition may be present if the mother is a carrier of a recessive gene allele located in the LOH region leading to two identical copies. Hundreds of potentially disease-causing genes are found on chromosome 15 and these diseases should be checked or monitored closely in those with segmental or total isodisomy of chromosome 15. A proposed genetic testing flowchart to identify the different molecular classes for both PWS and AS patients can be seen in **Graphical Abstract**.

Angelman Syndrome

Four recognized molecular classes have been identified in AS which may be categorized by the impact on the methylation of the chromosome 15 region. The most common subtype is a deletion of the maternal 15q11.2–q13 region as similarly seen of paternal origin in PWS and found in ∼70% of individuals with AS (21). However, in AS the typical Class II deletion is more common. This typical smaller Class II deletion most commonly approximates 5 Mb in size from BP2–BP3 and is present in 50% of deletion AS cases. Class I deletions are 5–7 Mb in size and encompass BP1–BP3 (40% of deletion cases). Atypical deletions may extend from BP1 or BP2–BP4 or more distant breakpoints. In individuals with a deletion on the maternal copy of chromosome 15, one must consider whether there are signs on the chromossomal microarray showing disturbances that indicate there could be a maternal translocation. This increases the recurrence risk of AS in future maternal offspring. Uniparental paternal disomy 15 accounts for 5–7% of individuals with AS. Imprinting defects account for 3–5% of individuals with AS and are caused by defects in the imprinting control center summarized by Buiting et al. (26). In individuals with a defect in the imprint control center, epigenetic marking in the germline fails to properly switch from a paternal pattern with silenced *UBE3A* expression to allow a maternal pattern of expression at the *UBE3A* gene. In as high as 50% of reported cases, a mutation in the imprinting control center may be identified. Mosaic cases of imprinting center defects in which a percentage of cells lack expression of the 15q11.2–q13 region is reported and may be more common than previously thought (27). The final genetic defect in AS does not impact DNA methylation testing results but is caused by a mutation in the maternally inherited *UBE3A* gene. Mutations in this gene account for 11% of AS cases (28). A *UBE3A* mutation could be maternally inherited and therefore it is indicated to do targeted testing in the patient's mother to rule out a 50% recurrence risk in her future offspring. If the mutation is deemed to be inherited, we recommend consideration of testing the patient's maternal grandfather as this could have implications for the maternal aunt's future children.

DISCUSSION

Medical management of PWS and AS should be directed by a multi-disciplinary team during infancy. Both infants with PWS (more commonly) and AS may have failure to thrive. A dietitian plays an important role in care at first to address failure to thrive and later in childhood to avoid obesity with diet intervention with restriction and use of exercise programs (which is a concern noted more commonly for PWS, but now recognized in AS in some individuals). Clinical geneticists, orthopedic specialists, primary care physicians, specialized occupational (OT), physical (PT) and speech (SLP) therapists, mental health experts, sleep specialists, mental health experts, and endocrinologists are needed to address the multiple health issues in PWS that may occur. An AS team includes clinical geneticists, neurologists, specialized therapists for PT, OT, and SLP services, sleep specialists, gastroenterology, physical medicine and rehabilitation, orthopedics, and mental health experts. For PWS, appropriate medical care, management and counseling are goals to control weight gain and to monitor and treat associated comorbid conditions, behavior, and psychiatric problems. Growth and other hormone deficiencies common in this disorder require treatment. Rigorous control of the diet with food security and a managed routine environment with regular exercise are important strategies to control hyperphagia, obesity and related complications required throughout life. AS requires early intervention including knowledge of specialized therapeutic interventions such as augmentative and assistive communication devices and a strengthening program of intensive developmental exercises and activities for reaching maximal potential (e.g., SPIDER), early treatment with benzodiazepines for seizures and diet therapy such as use of a ketogenic diet. Maximizing all aspects of care including sleep disorders and constipation greatly influence seizure control. A specialized center familiar

with the intricacies and unique aspects of these disorders can affect outcome.

Early diagnosis is vital to ensure early intervention for both PWS and AS. For PWS, an early diagnosis should be made during infancy to initiate growth hormone treatment, manage feeding concerns, obesity, hormone deficiencies, developmental delays, and behavioral problems. Diagnosis in AS also ensures early therapies which impact developmental outcomes, as well as seizure prophylaxis including preparation with appropriate benzodiazepines. Other interventions that may prove beneficial include specialized diets for individuals with AS such as the ketogenic diet or low glycemic index therapy (LGIT). Early diagnosis may also lower the costs of medical care by preventing extended hospitalizations related to feeding problems in individuals with PWS and seizures for children with AS.

Identifying the PWS or AS molecular class with advanced genetic testing such as high-resolution SNP microarrays will allow more accurate diagnosis, leading to better indicators for prognosis, and more accurate genetic counseling of family members. High-resolution SNP microarrays, FISH analysis, methylation specific-multiplex ligation probe amplification (MS–MLPA), and/or chromosome 15 genotyping are all useful in determining 15q11–q13 deletions. High-resolution SNP microarrays can identify the deletion subtypes (typical and atypical) in both PWS and AS, and UPD15 subclasses (segmental isodisomy, and total isodisomy). The heterodisomy subtype of UPD and IC defects (microdeletion and epimutation) in both PWS and AS may require additional diagnostic work up as illustrated in **Graphical Abstract**. The subtype or classes impacts diagnosis, potential recurrence risk for family members, prognosis and monitoring for other genetic conditions and high-risk features related to the molecular class. For example, autistic features and psychosis are more common in those with PWS and maternal disomy 15 and may relate to the specific UPD15 subclasses. Those with the larger Class I deletions in AS are more likely to develop difficult to treat seizures and microcephaly.

A genetic testing flowchart incorporating testing options that are available including those used historically for both PWS and AS are listed in **Graphical Abstract**. Testing for PWS or AS often begins with DNA methylation and if abnormal then advances to other genetic testing methods including high-resolution SNP microarrays or MS-MLPA assays based on availability to the clinicians and families in their clinical setting. Preferably, a high-resolution SNP array would be ordered which is readily and commercially available in Westernized medical care. Next-generation sequencing (NGS) of the exome (or whole genome) is also available for clinicians but droplet digital PCR (ddPCR) is currently research-based (14). SNP arrays can identify specific molecular classes in the majority of patients presenting with features of PWS (about 85% of cases) or AS (about 80%) while the remaining patients will need additional testing as described in **Graphical Abstract**. Specific advanced genetic testing (e.g., ddPCR) may be appropriately sensitive to quantify mosaicism and may identify a diagnosis in a large subset of individuals with milder clinical features of PWS and AS, but more research is needed.

Early clinical differences were found when comparing those with PWS or AS having the deletion vs. non-deletion status (29) including hypopigmentation in those with PWS and AS having the 15q11–q13 deletion (30). Later, higher verbal IQ scores (31) or psychosis (32) were reported in those with maternal UPD15 compared to deletion in individuals with PWS. Furthermore, Butler et al. (19) reported lower adaptive scores and more obsessive-compulsive behaviors in PWS individuals with the 15q11–q13 Type I deletion compared with UPD15. Zarcone et al. (33) reported individuals with PWS and the 15q11–q13 Type I deletion had more compulsions with personal cleanliness and compulsive behavior that was difficult to interrupt and interfered with social activities more so than those with Type II deletions or UPD15. In a phenotype-genotype correlation study in Angelman syndrome, Moncla et al. (34) reported increased seizure activity in those with the larger Class I deletion compared with non-deletion. Microcephaly, ataxia, hypotonia, and feeding difficulties are also more likely in the deletion subtype (3). They may have more severe language impairment in particular receptive language and autistic traits (21, 35). Individuals with AS with paternal UPD may have improved receptive language, improved motor abilities, and a decreased prevalence of seizures. Mosaic individuals may also have a milder phenotype including improved language abilities, adaptive functioning, and fewer seizures (36).

Next-generation exome or whole-genome sequencing may also have a place in genetic evaluations in PWS or AS, particularly in those individuals presenting with unusual findings or delayed diagnosis (e.g., UPD15 segmental or total isodisomy) and in cases where parental DNA is not available (8). To address the use and type of genetic testing for PWS and AS, a new genetic testing flowchart was developed for the clinician as described and illustrated in **Graphical Abstract**. This flowchart can assist in ordering genetic testing based on clinical presentation to determine appropriate diagnosis, management, and treatment and to supply the most accurate genetic counseling information for other family members. We suggest the use of this algorithm to definitively complete the diagnostic work up for both PWS and AS. We argue the diagnosis is incomplete without knowledge of the patient's specific genetic subtype to guide counseling, anticipatory guidance, management and likely therapeutic options. The molecular class determination is important for medical care and treatment and helpful for the clinician engaged in genetic counseling of family members for PWS or AS.

AUTHOR CONTRIBUTIONS

MB and JD contributed to drafting the manuscript, review of literature, contributed their expertise and edited the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Parallel Tests of Whole Exome Sequencing and Copy Number Variant Sequencing Increase the Diagnosis Yields of Rare Pediatric Disorders

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Background: Both whole exome sequencing and copy number variants sequencing were applied to identify the genetic cause of rare pediatric disorders. In our study, we aimed to investigate the diagnostic yield of parallel tests of trio whole exome sequencing and copy number variants sequencing and its clinical utility.

Methods: After collecting detailed clinical information, a total of 60 patients were referred to parallel tests of whole exome sequencing and copy number variants sequencing, which used shared initial libraries.

Results: 26 pathogenic or likely pathogenic single nucleotide variants and 11 copy number variants were identified in 32 patients. 65.4% (17/26) of the SNVs were novel. The overall diagnosis rate was 53.3%. For the patients with positive results, 22 (36.7%) patients were diagnosed by whole exome sequencing and 10 (16.7%) patients were diagnosed by copy number variants sequencing. We also reviewed clinical impact on selected cases.

Conclusion: We adopted an approach by performing parallel tests of trio whole exome sequencing and copy number variants sequencing with shared initial libraries. This strategy is relatively efficient and cost-effective for the diagnosis of rare pediatric disorders with high heterogeneity.

Keywords: whole exome sequencing, copy number variants sequencing, pediatric disorders, cost-effective, clinical utility

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INTRODUCTION

High genetic heterogeneity in pediatric disorders has been an obstacle to phenotype-based diagnostic testing (Hu et al., 2018). Compared to conventional genetic tests on single-gene scale, tests on genomic scale are more likely to produce higher diagnostic yields and shorter turn-around times (Vissers et al., 2017). Generally, these genomic tests include whole exome sequencing (WES)

and chromosomal microarray analysis (CMA). WES has been ordered increasingly in clinical molecular diagnosis laboratories as a powerful tool for rare Mendelian disorders, especially for genetically heterogeneous disorders such as intellectual developmental disorders and multiple congenital anomalies (Bowling et al., 2017; Han and Lee, 2020). According to previous researches, the overall diagnostic yields among different cohorts were ∼25% (Yang et al., 2013, 2014; Lee et al., 2014). Recently, multiple researches on clinical utility and cost of WES have provided evidence endorsing it as a first-tier test for children with suspected monogenic disorders (Nguyen and Charlebois, 2015; Monroe et al., 2016; Stark et al., 2016; Hu et al., 2018). CMA has been used as a first-tier clinical diagnostic test in patients with developmental delay (DD)/intellectual disability (ID), autism spectrum disorders (ASD), and multiple congenital anomalies (MCA) since 2010 (Manning et al., 2010; Miller et al., 2010); and copy number variants (CNVs) detected by CMA explained the pathogenesis for over 10% of these cases (Sanmann et al., 2015; Homma et al., 2018; Jang et al., 2019).

Recently, WES or whole genome sequencing (WGS) data were also used to call CNVs through the development of various algorithms and software programs that utilize read-depth information as the main strategy (Wang et al., 2016; Yao et al., 2017). WES-data-based CNV calling is usually performed as a supplement of routine WES test and the accuracy is affected by capture area and amplification efficiency (Rajagopalan et al., 2020; Sun et al., 2020). On the other hand, low-coverage WGS or CNV-seq were widely used in spontaneous miscarriage and prenatal cases, but only a few pediatric CNV-seq testing studies were reported. Most of the studies focused only on neurological disorders (Gao et al., 2019; Jiao et al., 2019). In our study, we performed parallel tests of trio-WES and CNV-seq for 60 children from different clinical departments. Our results showed this parallel testing strategy significantly increased diagnostic yields and lightened the burden of physicians in selecting optimal test.

MATERIALS AND METHODS

Patients

Patients in this research were initially referred to the Beijing Children's Hospital from September 2018 to September 2019, with suspected Mendelian disorders. These patients had DD/ID, ASD, or MCA, and both SNVs and CNVs were highly suspected for their causes of disorders. Next-generation sequencing (NGS) was primarily ordered by the patient's physician and performed by the Laboratory for Genetics of Birth Defects at Beijing Children's Hospital. Written informed consent was obtained from the individuals and their legal guardians for the publication. This study was approved by the Beijing Children's Hospital institutional review board. After recruitment, a total of 60 patients were referred to parallel tests of WES and CNV-seq. The ages of the patients ranged from 1 month to 12 years, and the median age was 1 year. The male-to-female ratio in this study was 1.31:1 (34/26). For the referring reasons, 25 of the patients had multiple congenital anomalies. Ten had DD/ID. Six had the combination of DD/ID and multiple congenital anomalies. Four had autistic traits. The remaining 15 patients had other phenotype, such as congenital heart disease, short stature, recurrent infections, etc. (**Supplemental Table 1**).

Abbreviations: WES, whole exome sequencing; CNV-seq, copy number variants sequencing; SNVs, single nucleotide variants; CNVs, copy number variants; CMA, chromosomal microarray analysis; DD, developmental delay; ID, intellectual disability; ASD, autism spectrum disorders; MCA, multiple congenital anomalies; WGS, whole genome sequencing; NGS, next-generation sequencing; TAT, turn-around time; QC, quality control.

Whole Exome Sequencing

DNA was isolated from peripheral blood samples obtained from the proband and parents using the Gentra Puregene Blood Kit (QIAGEN, Hilden, Germany). 200-ng genomic DNA of each individual was sheared by Biorupter (Diagenode, Liège, Belgium) to acquire 150∼200-bp fragments. The ends of DNA fragment were repaired, and Illumina Adaptors were added (Fast Library Prep Kit, iGeneTech, Beijing, China). After sequencing library was constructed, the whole exome was captured with AIExome Enrichment Kit V1 (iGeneTech, Beijing, China) and sequenced on Illumina NovaSeq 6000 (Illumina, San Diego, USA) with 150 base paired-end reads. Raw reads were filtered to remove low-quality reads using FastQC. Clean reads were mapped to the reference genome GRCh37. Quality control (QC) information included: average read length of >100×, accurate mapping rate of >98%, bases capture rate of >55%, 20× mean depth coverage rate of >96%, duplication rate of <25%, and accurate mapping rate of <96%. Single nucleotide variants (SNVs) were annotated and filtered by TGex (tgex.genecards.org). Variants with a frequency over 1% in the databases of gnomAD, ESP or 1000G were excluded. Variants that lacked segregation in family members were also filtered. Variants were classified following the American College of Medical Genetics and Genomics and the Association for Molecular Pathology interpretation standards and guidelines (Richards et al., 2015).

Copy Number Variant Calling

To identify large copy number variants (CNVs), part of the library without capture was sequenced directly onto Illumina NovaSeq 6000, and each sample yielded one giga base (Gb) raw data (QC: average read length: >0.3× WGS). An in-house pipeline was applied to map and call CNVs based on the software CNV-seq (Xie and Tammi, 2009; Hu et al., 2019). Clean reads were mapped to the reference genome GRCh37. CNVs called from parental WGS data were used as controls. CNVs reported in multiple peer-reviewed publications or annotated in curated databases as benign or likely benign, CNVs observed frequently in the general population and CNVs containing no genes were filtered. Database of Genomic Variants, DECIPHER database, ClinVar, OMIM, and ClinGen were used for interpretation and classification of the clinical significance of candidate CNVs according to previously reported guidelines (Kearney et al., 2011; Riggs et al., 2019). WES data was also used for CNVs calling, and the samples of the same batch were used as controls. Putative pathogenic or likely pathogenic CNVs identified from low-pass WGS were validated using WES reads depth data.

TABLE 1 | Diagnosis rates of patients with different ordering phenotype categories.

Phenotype	All cases	WES	CNV-seq	All positive	Positive rate (%)
MCA	25	10	3	13	52.0
DD/ID	5	3	0	3	60.0
DD/ID+MCA	12	5	5	10	83.3
ASD	4	0	1	1	25.0
Others	14	4	1	5	42.9
Total	60	22 (36.73%)	10 (16.7%)	32	53.3

ASD, autism spectrum disorders; DD, developmental delay; ID, intellectual disability; MCA, multiple congenital anomalies.

RESULTS

Molecular Diagnosis Rates

We identified 37 pathogenic or likely pathogenic variants in 32 patients. The overall diagnosis rate was 53.3%. For the patients with positive results, 22 (36.7%) individuals were diagnosed by WES (**Table 1**) and 10 (16.7%) individuals were diagnosed by CNV-seq (**Table 2**). For patients with MCA and/or DD/ID, the diagnosis rates were all higher than 50%. For patients with autistic traits, only one individual was diagnosed (**Table 1**).

Characterization of Variants

For the 37 pathogenic or likely pathogenic variants, 26 were SNVs and 11 were CNVs (**Tables 2**, **3**). Twenty-six SNVs were located in 21 different genes, and most of them (17/26, 65.4%) were in autosomal dominant diseases genes. Six variants were located in three autosomal recessive diseases genes, and three variants were located in X-linked recessive diseases genes. 65.4% (17/26) SNVs were not reported in previous literature or public population databases. Thirteen variants were identified to be *de novo*, six variants were compound heterozygous inherited from the parents, five were inherited from parents with similar phenotypes, and two X-linked recessive genes variants inherited from maternal carriers. Mutation in only two genes, *ELN* and *FBN1*, occurred in more than one patient, indicating the high heterogeneity of pediatric disorders (**Table 2**). For 11 CNVs identified by CNV-seq, the sizes ranged from 1.41 to 43.68 Mb. All these variants were also called from WES data to eliminate false positive variants. All these variants were *de novo* (**Table 3**). Patient 23 had a distal deletion and distal duplication, which was potentially inherited from a parent with balanced translocation. Further karyotype test may help to verify this hypothesis.

Turn-Around Time (TAT) and Cost Analysis

We aimed to decrease the difficulty for physicians to select optimal test between WES and CNV-seq, and to shorten TAT, as well, by parallel testing. We tracked the TAT of our parallel test for these patients; it ranged from 38 days to 125 days. The median TAT is 72 days with 69.2% of patients received reports within 80 days. The raw data were usually obtained within 20 days. Initial test results were generated 10–20 days afterwards and were delivered to ordering physicians to further check potential phenotype. Typically, negative cases cost more days because multiple rounds of communication with ordering physicians would be guaranteed, and a second analysis by another geneticist was performed before the formal reports were issued.

The direct cost of running a parallel trio-WES and CNV-seq is about $600 US dollars ($200 per person), including library construction (∼$100 USD), WES (∼$80 USD, 100×) and low-coverage WGS (∼$10 USD, 0.3×), and Sanger validation (∼$10 USD). These results showed that our parallel test strategy was affordable and additional CNV-seq did not increase the prime cost since the generation of one Gb raw data usually costed <$20 USD.

Clinical Impact of Genetic Diagnoses

Over half of the patients ended their diagnosis odysseys after parallel tests. The positive diagnostic results affected clinical management including appropriate genetic counseling, other systemic evaluation, and change of treatment. We provided examples of impacts on patients' clinical managements, below.

Case 4 was a 12-month-old boy. He was referred to Clinic of Development & Behavioral Pediatrics for Short Stature and DD. A *de novo SMARCA2* mutation was identified and was therefore diagnosed as Nicolaides-Baraitser syndrome (Zhang P. et al., 2019). This syndrome was less recognizable and always misdiagnosed as Coffin-Siris syndrome, Williams syndrome, etc. Once molecular diagnosis was confirmed, this patient was referred to neurologist for seizure evaluation. Ophthalmological and audiological examinations were also ordered.

Case 40 was a 5-month-old boy who had developmental and growth delays. Mutations in two genes were identified. DD was caused by *SETD5* mutation inherited from his mother with intellectual disability and growth delay was caused by *ACAN* mutation inherited from his father with short stature. Referral to an early intervention program is recommended for access to occupational, physical, speech, and feeding therapy. Growth pattern should be monitored and growth hormone therapy can improve height increase (Hu et al., 2017).

Case 29 was a 15-year-old girl. She was first referred to Department of Respiration for Pulmonary Lesions. Further evaluation revealed short stature, ataxia, tooth agenesis, depigmentation/hyperpigmentation of skin and absence of secondary sex characteristics. Parallel tests identified a 3.1-Mb *de novo* interstitial deletion of the 14q13.2q21.1 region encompassing 17 OMIM genes (Hu et al., 2019). In these genes, *NKX2*-1 deletion is responsible for choreoathetosis, hypothyroidism, and neonatal respiratory distress and haploinsufficiency of *PAX9* causes oligodontia phenotype (Das et al., 2002; Santen et al., 2012; Hayashi et al., 2015). For this patient, thyroid function testing should be performed annually, and when hypothyroidism is discovered, thyroid hormone replacement therapy should be initiated. For choreoathetosis, Tetrabenazine and Levodopa therapy have been reported to effectively reduce chorea (Setter et al., 2009; Rosati et al., 2015).

Cases 1, 13, and 59 all had 22q11.2 deletion syndrome. This was the only recurrent CNV disorder in our cohort. Case 1 was initially considered as DiGeorge syndrome for the characteristic facial features. Case 13 was referred only for autistic behaviors, and Case 59 was referred for congenital heart

TABLE 2 | Patients with pathogenic and likely pathogenic variants identified by whole exome sequencing.

Case no.	Sex	Age (y)	Categories	Gene	Variant	Origin	Inheritance	Reported*/novel	Diagnosed disorders
4	Male	1	DD/ID+MCA	SMARCA2	c.3593T>G/ p.Val1198Gly	*de novo*	AD	Reported	Nicolaides-Baraitser syndrome
6	Female	0.33	MCA	RNU4ATAC	c.196-570C>T; c.196-605C>T	in-trans	AR	Reported; reported	Roifman syndrome
8	Male	6	Others	PPP2R5D	c.1492C>T/ p.Arg498*	*de novo*	AD	Novel	Mental retardation, autosomal dominant 35
10	Male	5	Others	ELN	c.1621C>T/ p.Arg541*	*de novo*	AD	Novel	Supravalvar aortic stenosis
14	Male	1	Others	DPH1	c.471delC/ p.Arg158fs; c.397T>C/ p.Tyr133His	in-trans	AR	Novel; novel	Developmental delay with short stature, dysmorphic features, and sparse hair
16	Male	0.67	DD/ID	SLC22A5	c.1130T>C/ p.Phe377Ser; c.1400C>G/p.Ser467Cys	in-trans	AR	Novel; reported	Carnitine deficiency, systemic primary
22	Female	3	MCA	MED12	c.6340C>T/ p.Gln2114*	*de novo*	XLR	Novel	Ohdo syndrome
24	Male	5	MCA	CHAF1A	c.1750C>T / p.Arg584*	*de novo*	AD	Novel	novel disorder
27	Male	0.08	MCA	COL1A1	c.4100C>A/ p.Thr1367Asn	Maternal	AD	Novel	Osteogenesis imperfecta
31	Female	3	DD/ID+MCA	NSD1	c.6367_6376delAGTTG TGGGG/ p.Ser2123fs	*de novo*	AD	Novel	Sotos syndrome 1
33	Female	6	DD/ID	KAT6B	c.3663_3664+2delAAGT /p.Asn1222fs	*de novo*	AD	Novel	SBBYSS/Genitopatellar syndrome
35	Male	4	MCA	MID1	c.1863_1879dupGAACTC CATCCACCTCT/ p.Tyr627fs	Maternal	XLR	Novel	Opitz GBBB syndrome, type I
38	Male	0.17	MCA	FGFR2	c.869G>T/ p.Trp290Leu	*de novo*	AD	Reported	Crouzon syndrome
40	Male	0.42	DD/ID	ACAN SETD5	c.7000C>T/ p.Gln 2334*; c.1357C>T/ p.Gln453*	Paternal maternal	AD AD	Novel; novel	Short stature and advanced bone age Mental retardation, autosomal dominant 23
41	Female	1	Others	ELN	c.800-1G>T	Maternal	AD	Reported	Supravalvar aortic stenosis
42	Female	1	MCA	FBN1	c.7180C>T/ p.Arg2394*	Paternal	AD	Reported	Marfan syndrome
46	Male	3	DD/ID+MCA	ATRX	c.6254G>A/ p.Arg2085His	Maternal	XLR	Reported	Alpha-thalassemia/ mental retardation syndrome
47	Male	1	DD/ID+MCA	ANKRD11	c.1681G>T/ p.Glu561*	*de novo*	AD	Novel	KBG syndrome
49	Male	0.42	MCA	PIK3CA	c.2740G>A/ p.Gly914Arg	*de novo*	AD	Reported	Megalencephaly-capillary malformation
50	Male	12	MCA	FBN1	c.1995C>A/ p.Tyr665*	*de novo*	AD	Novel	Marfan syndrome
54	Male	0.25	DD/ID+MCA	SOX9	c.340G>T/ p.Val114Leu	*de novo*	AD	Novel	Campomelic dysplasia
58	Male	0.92	MCA	CREBBP	c.3306T>A/ p.Tyr1102*	*de novo*	AD	Novel	Rubinstein-Taybi syndrome

*DD, developmental delay; ID, intellectual disability; MCA, multiple congenital anomalies; *Variants included in public databases or previously published literature.*

TABLE 3 | Patients with pathogenic and likely pathogenic variants identified by copy number variation sequencing.

Case no.	Sex	Age (y)	Categories	CNV	CNV length	Diagnosed disorders
1	Male	0.17	MCA	seq[GRCh37]del(22)(22q11.2)chr22:g.18963217_21541547del	2.58 Mb	22q11.2 deletion syndrome
3	Female	1	MCA	seq[GRCh37]del(5)(5p15.33p15.1)chr5:g.54215_16752126del	16.70 Mb	Cri-du-chat syndrome
11	Female	0.58	DD/ID+MCA	seq[GRCh37]del(9)(9p24.3p11.2)chr9:g.152081_43829455del	43.68 Mb	9p deletion syndrome
13	Male	8	ASD	seq[GRCh37]del(22)(22q11.21)chr22:g.19028192_21466206del	2.44 Mb	22q11.2 deletion syndrome
20	Male	4	DD/ID+MCA	seq[GRCh37]del(11)(11q24.2q25)chr11: g.124175265_134974797del	10.80 Mb	NA
23	female	0.17	DD/ID+MCA	seq[GRCh37]dup(8)(8q24.3)chr8: g.141319205_144918800dup; seq[GRCh37]del(18)(18q22.2q23)chr18: g.67971593_77882168del	3.60 Mb; 9.91 Mb	NA
28	Female	8	DD/ID+MCA	seq[GRCh37]del(7)(7q11.23)chr7:g.72645343_74161920del	1.52 Mb	Williams-Beuren syndrome
29	Female	15	MCA	seq[GRCh37]del(14)(14q13.1q21.1)chr14: g.35268524_38367321del	3.10 Mb	NA
36	Male	3	DD/ID+MCA	seq[GRCh37]del(4)(4q25q26)chr4:g.111697561_116176319del	4.48 Mb	NA
59	Female	0.67	MCA	seq[GRCh37]del(22)(22q11.2)chr22:g.18905953_20318038del	1.41 Mb	22q11.2 deletion syndrome

ASD, autism spectrum disorders; DD, developmental delay; ID, intellectual disability; MCA, multiple congenital anomalies.

disease and laryngomalacia. After diagnosis, these patients took further evaluation and treatment following the clinical practice guidelines for individuals with 22q11.2 deletion syndrome (Bassett et al., 2011).

DISCUSSION

In this study, we performed parallel tests of WES and CNV-seq for 60 patients. Thirty-seven pathogenic or likely pathogenic variants in 32 patients were identified, and the overall diagnosis rate was 53.3%. Our study provided preliminary results of clinical utility of parallel tests of CNVs and SNVs at the same time in pediatric patients with developmental delay/intellectual disability, autism spectrum disorders, and multiple congenital anomalies.

Over 6500 phenotypes were included in the OMIM by the end of 2019 and most of them were onset during childhood. Genetic pediatric disorders are highly heterogeneous and relatively rare, which, for investigating them, necessitates a process of serial testing for specific conditions (Han and Lee, 2020). In consequence, this strategy may be expensive and time-consuming. The molecular diagnosis of these rare diseases is based on the appropriate choice among tests of karyotype, Sanger sequencing, multiplex ligation dependent probe amplification, CMA, NGS, etc., which requires a physician with medical genetics training and experience. However, most children's hospitals do not have independent genetic clinic for patients with genetic disorders (Hu et al., 2018). Meanwhile, a more comprehensive strategy of genomic-first approach should be applied to achieve higher positive rate and reduce diagnostic odyssey (Johnson, 2015).

Previous WES studies revealed a diagnosis rate of ∼25–30% in nonselective patients (Yang et al., 2013, 2014; Lee et al., 2014; Daoud et al., 2016; Retterer et al., 2016). Moreover, additional pathogenic and likely pathogenic CNVs were also identified in over 10% of patients (Sanmann et al., 2015; Homma et al., 2018; Jang et al., 2019). The development of NGS data based CNV calling algorithm makes it possible to identify SNVs and CNVs at same time. In this study, we performed trio-WES and low-coverage WGS (0.3×) simultaneously and uncovered pathological causes in over half of the patients. The rate of patients with neurological features was 67% (14/21), which was higher than previous reports (Gao et al., 2019; Jiao et al., 2019). However, it was notable that our sample size was relatively small, and more researches were needed to better the clinical efficacy.

The application of NGS to detect genome-wide CNVs is a recently developed method (Xie and Tammi, 2009). Comparing to pediatric disorders, CNV-seq was more widely used in prenatal screening and diagnosis, which required lower resolution (Liang et al., 2014; Zhang R. et al., 2019; Zhao and Fu, 2019). The resolution of CNV-seq can be adjusted by increasing or decreasing the sequencing data volume of raw data. For pediatric diagnosis, the resolution was usually >100 Kb (Gao et al., 2019; Jiao et al., 2019). By comparing differences of aligned reads number between case and control samples, losses or gains of chromosomal regions can be identified and quantitated.

Therefore, it has been reported that it is easier to identify low-level mosaicism by CNV-seq, rather than CMA (Grotta et al., 2015). Furthermore, identifying CNVs by sequencing has other advantages, including lower starting input of the DNA, cheaper cost, shorter TAT and higher throughput.

In the present study, our parallel tests of WES and low-pass WGS (0.3×)-based CNV-seq decreased TAT and human labor without a significantly increased cost. Compared to WES, the extra cost was used for WGS of each sample to yield one Gb raw data directly, using the same sample library constructed during WES before capture, and the sequencing cost for 1 Gb raw data was about $10 USD with Illumina NovaSeq, in this research. We identified pathogenic or likely pathogenic variants with WES and CNV-seq in 36.7 and 16.7% of patients, respectively, which was comparable with previous study, indicating that our strategy is efficient to simultaneously identify SNVs and CNVs. Therefore, the ordering physicians do not need to choose between CMA and NGS, a choice which is dependent on clinical expertise, and they are more likely to identify clinically relevant variants in patients with atypical presentation or novel phenotypes. The mean age of positive cases at diagnosis was 3 years (95% CI: 1.73, 4.26). Early diagnosis avoided long diagnostic odyssey once symptoms appear, but also provided opportunities for early intervention, helping to improve outcomes and prolonging life.

In conclusion, our study provided preliminary experience of parallel tests of WES and CNV-seq with same initial library and evaluated the clinical utility. Compared to traditional trio-WES test, our strategy increased the diagnosis rate from 36.7 to 53.3% in our relatively small cohort. With less depending on physicians' selection between SNVs and CNVs tests, we propose this strategy is efficient and cost-effective for the diagnosis of genetic pediatric disorders with high heterogeneity.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

Written informed consent was obtained from the individuals and their legal guardians for the publication.

AUTHOR CONTRIBUTIONS

XH, RG, JG, and ZQ collected the clinical data. CH and WL designed the study. XH, RG, and JG performed data analysis. XH and CH wrote the manuscript. All authors contributed to data acquisition and data interpretation, critically revised the manuscript for important intellectual content, approved the final, submitted version of the manuscript, and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00473/full#supplementary-material

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CASE REPORT
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Clinical and Genetic Analysis of CHD7 Expands the Genotype and Phenotype of CHARGE Syndrome

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CHARGE syndrome is a life-threatening disease caused by mutations of chromodomain helicase DNA-binding protein 7 gene (CHD7). The disease is characterized by a pattern of congenital anomalies that involve multiple organs. In this study, five patients were diagnosed as CHARGE syndrome with CHD7 mutations by whole exome sequencing. Although the clinical phenotypes of probands are highly variable and typical symptoms such as coloboma and choanal atresia are not commonly manifested in this cohort, they all presented congenital heart defects. Of note, dyspnea is the most prominent symptom in all five neonatal patients, suggesting that dyspnea might be a phenotypic clue of CHARGE syndrome.

Keywords: CHD7, CHARGE syndrome, dyspnea, neonate, mutation

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INTRODUCTION

CHARGE syndrome (OMIM#214800), a rare congenital disorder, occurs in approximately 1/8,5000 to 1/15,000 livebirths. It previously described by Hall and Hittner et al. as association of coloboma, choanal atresia, and congenital heart defects and then summarized by Pagon et al. as the acronym of multiple anomalies, including **C**oloboma of eye, **H**eart defects, **A**tresia of choanae, **R**etardation of growth and/or development, **G**enitourinary defects and/or hypogonadism, and **E**ar anomalies with or without deafness (Hall, 1979; Hittner et al., 1979). The clinical diagnostic criteria for CHARGE syndrome were proposed by Blake et al. and Verloes that generally divided into (i) major criteria, including coloboma, choanal atresia/cleft, and hypoplastic semi-circular canals and/or abnormal ears; and (ii) minor criteria, including heart/or esophagus malformation, ear anomalies, rhombencephalic dysfunction, hypothalamic-pituitary deficiency, and intellectual disability (Blake et al., 1998; Verloes, 2005). Moreover, Verloes further classified the CHARGE syndrome into typical CHARGE, partial CHARGE, and atypical CHARGE based on how many characteristics the patient meets (Verloes, 2005). Until 2004, mutations of chromodomain helicase DNA-binding protein 7 (CHD7) was found to be major cause for CHARGE syndrome and appear autosomal dominant inheritance pattern (Vissers et al., 2004). So far, more than 1,000 variants of CHD7 have been identified with next-generation sequencing (NGS), and 90–95% of patients carried a CHD7 variant meet Blake or Verloes' diagnostic criteria (Jongmans et al., 2006; Bergman et al., 2011). Moreover,

most of variants are *de novo*. Therefore, the latest clinical criteria have incorporated pathogenic CHD7 mutations into a major criterion in 2016 (Hale et al., 2016).

CHARGE syndrome causes patients to suffer multiple life-threatening symptoms after birth and brings a lot of burden to their family. However, molecular diagnosis is only made after termination of pregnancy. An efficient strategy for prenatal diagnosis of CHARGE syndrome is urgently needed (Colin et al., 2012).

In our cohort, we have reported five patients diagnosed with CHARGE syndrome and the phenotypes were highly variable. Despite the typical phenotypes sparsely presented, dyspnea was found to be the most prominent symptom in all five patients. Molecular analysis identified five variants of CHD7 including two types of mutations and three of them are novel. Genotype analysis of CHD7 based on ClinVar and CHD7 database demonstrated the mutation spectrum and phenotypes of CHARGE syndrome which might be contributed to the study of CHD7 mutation-associated CHARGE syndrome. Therefore, both reported phenotypes and the mutation spectrum in this study can facilitate the development of prenatal screening for CHARGE syndrome. Our findings may provide new molecular evidence and expand the phenotypes for CHARGE syndrome which would be helpful for clinical diagnosis.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Genomic DNA was isolated from peripheral blood lymphocytes using Lab-Aid DNA kit (Zeesan Biotech Co., Ltd, Xiamen, China).

Whole Exome Sequencing and Data Analysis

Genomic DNA was extracted and captured to create the library for whole exome sequencing by Agilent SureSelect Human Exon V6 kit or Agilent SureSelect Clinical Research Exome V2 kit (Agilent Technologies, Santa Clara, CA, United States) according to the manufacturer's protocol. The libraries were sequenced by HiSeq X Ten or Nova Seq 6000 with PE150 strategy (Illumina, San Diego, CA, United States) with a read depth over 120X and more than 95% of the targeted regions covered over 20X. The sequencing reads were mapped to the Genome Reference Consortium Human genome build 37 (GRCh37). The Genome Analysis Toolkit (GATK) was used for variant calling. TGex software (LifeMap Sciences, Alameda, CA, United States) was used to annotate the variants. Transcript NM_017780.3 was used as the reference sequence. The CHD7 variants and their origins were verified by Sanger sequencing. All the variants were classified according to ACMG/AMP guidelines.

Editorial Policies and Ethical Considerations

The study was approved by the ethics committees of Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region. Written informed consent for participation in this study was collected from the family.

PATIENTS AND CLINICAL INFORMATION

In this cohort, five patients including four males and one female were diagnosed by whole exome sequencing. All infants presented dyspnea after birth. Four of them presented different extent of congenital heart defects with symptoms of patent ductus arteriosus (PDA, patients 3, 4, and 5), atrial septal defect (ASD, patients 1, and 5), patent foramen ovale (PFO, patients 4, and 5), and right aortic arch (RAA, patient 1). Three of them presented malformations of either the middle or external ear (patients 1, 2, and 4). Apart from that, macrocephaly (patient 1), microcephaly (patient 2), and ocular coloboma (patients 3, 4, and 5) were also presented.

Specifically, patient 1 was a premature infant with macrocephaly, low-set ears, short neck, ASD, RAA, and micropenis. Both patients 2 and 4 were born with stridor, microtia, and dyspnea. Additionally, patient 2 also had microcephaly with 34 cm (-3 SD) of head circumference and patient 4 suffered PFO, PDA, and retinal coloboma. Patient 3 was a male neonate with severe clinical manifestations who was born by cesarean section due to fetal distress on 37 + 2 w of pregnancy. After his birth, he had no cry and was diagnosed with neonatal pneumonia which lead to a suspicion of congenital airway deformities. Chest CT scan of mediastinal cystic image revealed that he had hiatus hernia. The blood test detected neonatal hypoglycaemia, hypocalcemia, low thyroid stimulating hormone, and lymphopenia. Microcephaly, PDA, abnormal thorax, micropenis, and dysphagia were also presented. Moreover, MRI found anomalies in his bilateral frontal gyrus, sulcus, and middle lobe of cerebellum, as well as widened posterior fossa and cerebellar vermis hypoplasia (CVH) in his brain. Patient 5 was hospitalized for dyspnea and fever and then admitted to neonatal pneumonia. He presented microphthalmia, ASD, PDA, PFO, cardiac insufficiency, optic discs coloboma (ODC), and chorioretinal coloboma, as well as neonatal hyperbilirubinemia and subependymal hemorrhage. The clinical information of patients can be found in **Table 1**.

MOLECULAR ANALYSIS

Data of whole exome sequencing identified five heterozygous null variants of CHD7 in these patients and they were classified to be pathogenic according to ACMG/AMP guidelines. They include (i) two frameshift: c.2828_2829delAG and c.4667dupC; and (ii) three nonsense: c.1480C > *T*, c.6079C > *T*, and c.7873C > *T*. Moreover, except c.1480C > *T* and c.6079C > *T* were reported mutations, the other three were identified as novel mutations in CHD7 (NM_017780.3). Sanger sequencing of parental DNA confirmed that all these variants were *de novo*.

As two types of mutations occurred in five different exons and different domains of CHD7 (**Figure 1A**), we further raised two questions: (i) Did the observed mutations match with the

TABLE 1 | Clinical features of patients with CHD7 mutation.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Variants	c.1480C > *T* p.Arg494*	c.2828_2829delAG p.Glu943fs	c.4667dupC p.Arg1557fs	c.6079C > *T* p.Arg2027*	c.7873C > *T* p.Gln2625*
Inheritance	*De novo* Known	*De novo* Novel	*De novo* Novel	*De novo* Known	*De novo* Novel
Age	13 days	2 months	4 months	12 days	15 days
Gender	Male	Male	Male	Female	Male
Perinatal	Premature birth, Dyspnea	Stridor, Dyspnea	Fetal distress, Dyspnea, Hypoglycemia, Hypocalcemia, Low TSH, Dysphagia	Stridor, Microtia, Dyspnea	Dyspnea, Hyperbilirubinemia, Subependymal hemorrhages
Head & Neck	Low-set ears, Macrocephaly, Short neck	Microtia, Microcephaly	Microcephaly, ODC, Chorioretinal coloboma, Hiatus hernia, CVH, Widened posterior fossa	Retinal coloboma, Microtia	Microphthalmia, ODC, Chorioretinal coloboma
Cardiovascular	ASD, RAA	Normal	PDA	PDA, PFO	ASD, PDA, PFO
Genitourinary	Micropenis	Normal	Micropenis	Normal	Normal
Classification	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic

*TSH, thyroid stimulating hormone; ODC, Optic disk coloboma; CVH, Cerebellar vermis hypoplasia; PDA, Patent ductus arteriosus; ASD, Atrial septal defect; PFO, Patent foramen ovale; RAA, Right aortic arch; and *, nonsense.*

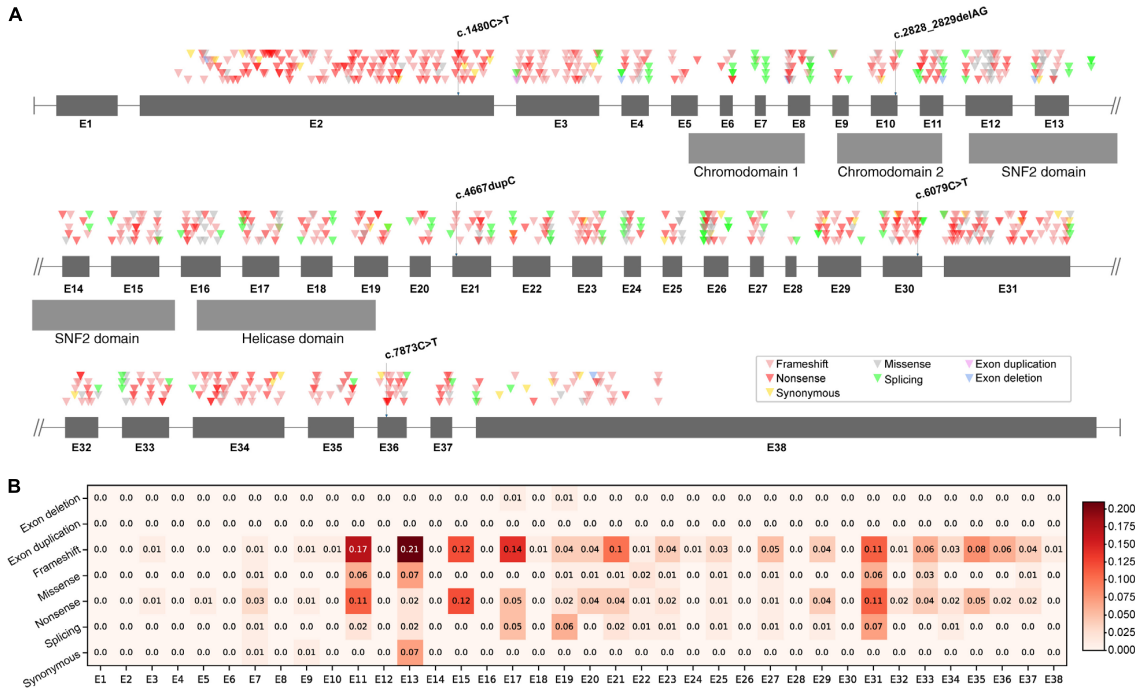


FIGURE 1 | Mutations distribution of CHD7. **(A)** Pathogenic and likely pathogenic variants were collected from ClinVar and CHD7 database and then shown in the schematic according to their chromosomal location. **(B)** The mutation rate of each exon was computed as the quotient between the number of mutations and the number of bases of the exon. Darker color stands for higher mutation rate.

expected mutations rate of CHD7? (ii) Did the CHD7 genotypes correlate with the CHARGE phenotypes? In order to clarify the mutation spectrum and phenotypes of CHARGE syndrome, we summarized all the pathogenic and likely pathogenic variants from ClinVar and CHD7 database[1]. A total of 925 variants including 375 frameshift (40.54%), 299 nonsense (32.3%), 101 missense (10.92%), 109 splicing (11.78%), 34 synonymous (3.68%), 6 exon deletions (0.65%), and 1 exon duplication (0.11%) were shown in CHD7 exons based on their chromosomal location (**Supplementary Table 1**). In our cohort, the observed mutation rate of frameshift (2/5, 40%) matched with the expected frameshift mutation rate, the nonsense (3/5, 60%) were more than

[1]www.CHD7.org

the expected. To locate the expected mutation type on each exon, frameshift is most frequently detected in E2. Noticed that there are no pathogenic or likely pathogenic variants in E1 and 3′-terminal of E38. Correlation between genotype and phenotype is negative. The exonic mutation rate for each mutation type can be found in **Figure 1B**. Comparing the incidence of certain type of variants to the exonic mutation rate, there is no significant relevance between the incidence and the type of mutations of CHD7 for our cohort.

DISCUSSION

CHARGE syndrome is a genetic disorder with highly variable phenotypes even within a family and results from mutations of CHD7 gene. In this study, we report five patients diagnosed as CHARGE syndrome by whole exome sequencing. The phenotypes of patients in this cohort were quite variable. Specifically, the typical features of CHARGE syndrome were dispersedly manifested in patients: (i) **C**oloboma were found in patients 3, 4, and 5, (ii) **H**eart defects were presented in patients 1, 3, 4, and 5, (iii) **A**tresia was not found, (iv) **R**etardation of growth or development needed follow-up examination, (v) **G**enital malformation (micropenis) was only found in patients 1 and 3, and (vi) **E**ar anomalies including microtia and low-set ears were found in patients 1, 2, and 4. Apart from that, premature birth that occurred in patient 1 was also considered to be one of clinical features of CHARGE syndrome (Sanchez et al., 2019). Fetal distress was presented in patient 3 and lead to cesarean section. In addition, the malformations of head and neck were commonly among probands in this cohort. Although the phenotypes are variable in patients with CHARGE syndrome, it is worth noting that dyspnea presented in all five infants after birth which is consistent with previous reports (Daniel, 2008; Verloes, 2005; Xu et al., 2018), suggesting that dyspnea might be considered to be one of clinical diagnosis clues of CHARGE syndrome.

Genetic analysis of CHD7 variants in these patients found that all five variants were *de novo* and were null mutations including two frameshift and three nonsense, leading to loss of function. In addition, three of them were novel and two have been reported in CHARGE patients previously (Janssen et al., 2012; Busa et al., 2016; Moccia et al., 2018). Analysis of mutation spectrum and exon mutation rate in CHD7 (NM_017780.3) revealed that the most common type of mutations is frameshift (375/925, 40.54%), followed by nonsense (299/925, 32.32%). In addition, these frequently detected mutation types are enriched in E2. Surprisingly, there is no record of pathogenic or likely pathogenic variant in exon 1, suggesting that mutations occurring in E1 are probably lethal *in utero*, and the mutations in 3′-terminal of E38 may have no effect on CHD7 protein function due to nonsense mediated decay (Nguyen et al., 2014). Our cohort found two frameshifts (2/5, 40%) occurred in E10 and E21 which matched with the expected mutation rate; three nonsense (3/5, 60%) were found in E2, E30, and E36 which were more than the expected. However, the sample size is very limited and needs to be further explored. Furthermore, to investigate the correlation between certain type of mutation and exonic mutation rate, we calculated the exonic mutation rate for each mutation type (**Figure 1B**). However, there was no significant correlation between them.

Using genetic testing to predict the risk or confirm a diagnosis of inheritable disease has been extensively implicated. We tried to correlate the genetic mutations of CHD7 to the clinical symptoms of CHARGE patients in this cohort and data from CHD7 database. However, clinical and genetic analysis of CHD7 variants revealed that there are no significant correlations between phenotype and genotype (**Supplementary Material**). It is consistent with previous report published by Lalani et al. which have analyzed and confirmed that there was no correlation of severity of phenotype with mutations in specific domains of the CHD7 protein (Lalani et al., 2006). And the genotype-phenotype correlations were also confirmed to be negative in three other cohorts (Jongmans et al., 2006; Sohn et al., 2016; Legendre et al., 2017).

Prenatal diagnosis of CHARGE syndrome is still a challenge and mostly depends on the clinical diagnosis with prenatal ultrasound screening. Heart defects, malformations of head and neck such as microcephaly, microtia, and microphthalmia found by ultrasound can be the clues for prenatal diagnosis of CHARGE syndrome. Moreover, fetal *de novo* mutations screening by non-invasive prenatal test (NIPT) with maternal plasma is highly efficient for diagnosis. Detection of mutations in E1 and E38 may also provide clues for predicting severity of CHARGE syndrome by NIPT with maternal plasma.

Despite increasing number of CHD7 variants and associated features of CHARGE syndrome have been reported, the incidence of certain mutations and the effects on the phenotype remain to be illusive. Our study identified three novel variants (c.2828_2829delAG, c.4667dupC, and c.7873C > *T*) and two reported variants (c.4667dupC and c.1480C > *T*) that contributed to CHARGE syndrome. In summary of clinical phenotypes of five cases, our study suggested that postnatal dyspnea can be considered as one of clinical diagnosis clues for CHARGE syndrome, especially when it presents along with typical structural malformations. In conclusion, although there is no significant correlation between genotype and phenotype in this cohort, our study can provide a fundamental understanding and enhance the importance of studying molecular pathology of CHARGE syndrome.

DATA AVAILABILITY STATEMENT

The BAM files of whole exome sequencing have deposited in BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/604965).

ETHICS STATEMENT

The study was approved by the Ethics Committees of Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region. Written informed consent to

participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JS and ZQ conceived and designed the experiments, and wrote the manuscript. QZ, FC, SY, WLu, WLi, and LH performed the experiments. ML, QY, SY, HZ, JX, and YS were involved in data analysis. JL helped to revise the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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A *de novo DDX3X* Variant Is Associated With Syndromic Intellectual Disability: Case Report and Literature Review

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De novo DDX3X variants account for 1%–3% of intellectual disability (ID) in females and have been occasionally reported in males. Here, we report a female patient with severe ID and various other features, including epilepsy, movement disorders, behavior problems, sleep disturbance, precocious puberty, dysmorphic features, and hippocampus atrophy. With the use of family-based exome sequencing, we identified a *de novo* pathogenic variant (c.1745dupG/p.S583*) in the *DDX3X* gene. However, our patient did not present hypotonia, which is considered a frequent clinical manifestation associated with *DDX3X* variants. While hand stereotypies and sleep disturbance have been occasionally associated with the *DDX3X* spectrum, hippocampus atrophy has not been reported in patients with *DDX3X*-related ID. The investigation further expands the phenotype spectrum for *DDX3X* variants with syndromic intellectual disability, which might help to improve the understanding of *DDX3X*-related intellectual disability or developmental delay.

Keywords: intellectual disability, exome sequencing, variants, *DDX3X* gene, female

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INTRODUCTION

Intellectual disability (ID) is characterized by serious impairment in intellectual functioning and adaptive behavior (1) and affects ∼1%–3% of humans, with a gender bias toward males (1, 2). ID is clinically heterogeneous, accompanied frequently by one or more comorbidities, including seizures, autism spectrum disorder (ASD), and structural and/or functional abnormalities in systems other than the central nervous system (3, 4). Variants in more than 500 candidate genes have been reported in children with intellectual disability, and most of them are associated with neurodevelopment and function of the central nervous system (5, 6). To date, variants of more than 100 genes have been reported to cause monogenic X-linked intellectual disability (XLID) in males (7); however, relatively little is known about X-linked ID genes in females. Recently, an increasing number of X-linked genes, including *PHF6*, *USP9X* and *DDX3X*, have been identified as carrying *de novo* variants in females with an overlapping but often distinct phenotype in males (8–10).

DDX3X is an X-linked gene, encodes a key regulator of the Wnt signaling pathway, and is vital for many cellular processes (11). Recently, *DDX3X* variants have been associated with ID and are considered one of the most common causes of ID in females, accounting for 1%–3% of unexplained ID in females (10). However, patients with ID carrying *DDX3X* variants have been reported in a few studies, and there is no evidence for an obvious correlation between the type of variants and the degree of ID. In the present study, we performed exome sequencing in a proband and

her healthy parents to investigate the genetic cause of severe intellectual disability. As a result, we identified a *de novo* frameshift variant in *DDX3X*. Furthermore, we reviewed previously reported patients with *DDX3X* variants and summarized their characteristics, hoping to improve the clinical understanding of the genotype-phenotype correlation.

CLINICAL FEATURES AND FAMILY HISTORY

The patient, 7 years and 5 months of age, who was second-born to healthy non-consanguineous Chinese parents, was referred to the pediatric department due to severe intellectual disability (ID) and epilepsy. She was born at 38 weeks of gestation after an uncomplicated pregnancy and delivery, with a birth weight of 3,100 g (approximately, 50th centile). There were no complications during the perinatal or neonatal periods. Family history is negative for intellectual disability and seizures.

The patient first presented for clinical evaluation at 11 months of age when she was unable to sit without support and did not control her head nor speak any words; the weight and occipito-frontal circumference (OFC) were 8 kg (10th centile) and 44 cm (25th centile), respectively. Her first electroencephalogram (EEG) at 19 months showed increasing theta activity during the awake period but without any seizure. However, at the age of 4 years and 6 months, seizures were captured during video-EEG monitoring and were classified as atypical absence seizures. Subsequently, the patient became seizure free with levetiracetam, and treatment was withdrawn at ∼5 years old. However, the patient developed focal seizure with five similar attacks altogether at 7 years and 2 months of age, without motor or language functional regression. Then, she received levetiracetam again after abnormal interictal video-EEG presentations (**Supplementary Figure 1**, EEG), and was seizure free following daily administration of 5 mg/kg levetiracetam for 3 months.

The patient has shown severe motor and speech delay since infancy, but overall, there has been slow but forward developmental progress. She did not show a social smile until 8 months. Her motor milestones were delayed profoundly, as she developed head control at 12 months, sitting at 18 months, learning to walk at 3 years and 2 months, walking without support at 5 years and running at 6 years slowly with persistent clumsiness. Language acquisition was also obviously delayed but without regression. She said her first words at ∼2 years and 4 months, and at 7 years and 5 months, the last visit, she was able to inarticulately express short, three-word sentences. The patient did not present typical autistic-like features, but she was easily agitated and hyperactive, screaming and crying gratuitously, with temper outbursts, intermittent aggressiveness, and exaggerated movements or gestures at times. Moreover, sleep disturbances that consisted of night awaking with screaming spells were observed. However, she did not receive special education services or speech/language therapy.

When on admission at 7 years and 5 months, the patient's weight was 25 kg (75th centile), with microcephaly (OFC 49 cm, <3rd centile), dysmorphic features (brachycephaly, a flat face, a thin upper lip, low-set ears; **Figure 1A**), high-arched palate, wide-based gait, precocious puberty, and mild lower extremity hyperreflexia. Hand stereotypies were frequently observed, presenting as slow fine finger and rapid alternating movements and hand wringing.

We performed electrocardiogram (ECG), cardiac ultrasound (CUS), and metabolic testing, including plasma amino acids and acylcarnitines, urinary organic acid analysis, serum creatine kinase, biotinidase activity, folate, homocysteine, copper, blood lactate, and ammonia, all without abnormalities. Her auditory evoked potential was normal. At 7 years and 2 months, testing using the Chinese Revision of the Wechsler Intelligence Scale for Children (WISC)-III showed verbal, performance and full-scale IQ scores of 51, 59, and 51, respectively. Her brain magnetic resonance imaging (MRI) scan at the age of 7 years showed a reduced volume of the bilateral hippocampus, suggesting hippocampus atrophy, with enlargement of the lateral ventricles and peculiar temporal horn dilatation (**Figure 2**).

METHODOLOGY

Genetic Findings

Her karyotype analysis was normal. Blood samples were obtained from the patient and her parents for genetic analysis, and informed consent was obtained from her parents. Exome sequencing was performed in the patient and her unaffected parents. Finally, we identified one distinct *de novo* frameshift variant in *DDX3X*: c.1745dupG/p.S583* [ChrX (GRCh37), NM_001193416.1; **Figure 1B**]; no likely pathogenic copy number variant was identified through an analysis of the exome data. The variant identified in our patient has not been previously reported in the literature or in any public database. The variant pathogenicity was then determined according to the American College of Medical Genetics and Genomics (ACMG) guidelines (12): This variant showed very strong evidence of pathogenicity since it was a null variant (variant leading to a premature stop codon) in *DDX3X* (PVS1), was a *de novo* variant (PS2), was absent in population databases (PM2), and further met the supporting criteria of PP4. In conclusion, we regarded c.1745dupG/p.S583* as a pathogenic variant (PVS1+PS2+PM2+PP4).

Literature Search

To characterize *DDX3X* variants in intellectual disability and to explore the likely association between the phenotype and *DDX3X* gene variants, we performed a systematic literature search in the PubMed database, the Human Gene Mutation Database (HGMD), the Online Mendelian Inheritance in Man (OMIM) and the China National Knowledge Infrastructure (CNKI) databases for publications using "intellectual disability" or "developmental delay" and "*DDX3X*" as the keywords (until January 9th, 2020). In addition to our patient, we identified 96 patients with *DDX3X* variants reported previously (10, 13–19). We excluded four patients reported in a single publication and one fetus from further analysis since all comprehensive clinical presentations were unavailable (17, 18). Complete nomenclature of variants was derived from the Mutalyzer (https://mutalyzer.

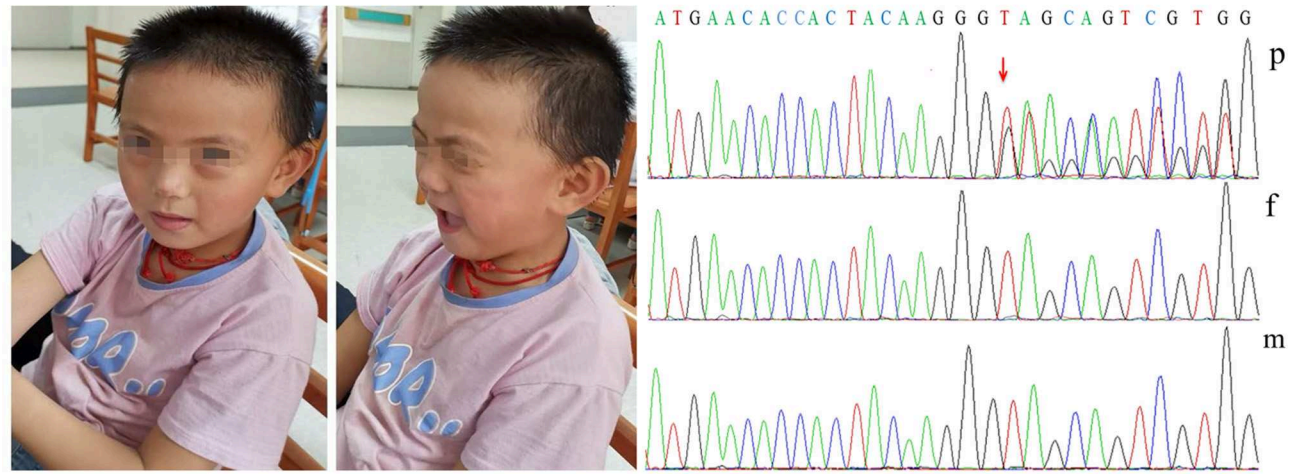


FIGURE 1 | Dysmorphic features and variant analysis. **(A)** Photographs of our patient at 7 years and 5 months showing brachycephaly, a flat face, a thin upper lip, and low-set ears. **(B)** The proband had the c.1745dupG variant of *DDX3X* (p), leading to a premature stop codon; however, her parents had no detectable variants (f and m).

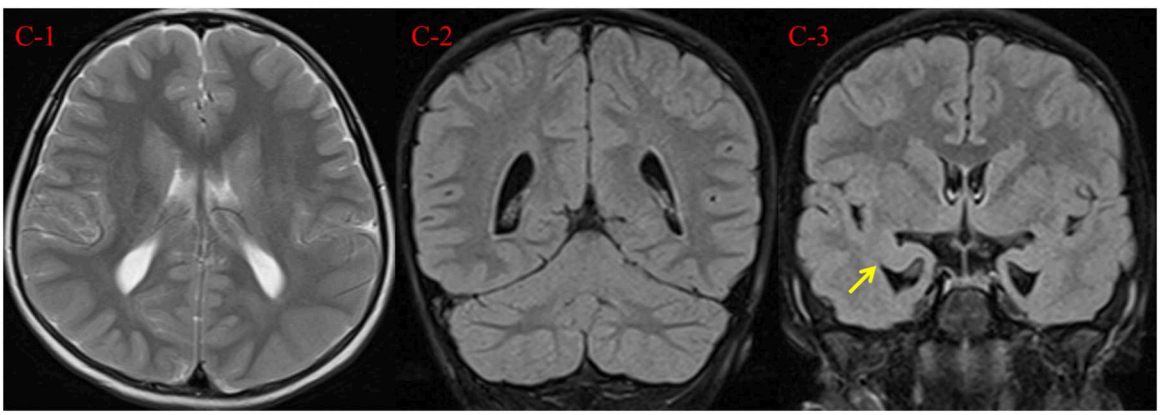


FIGURE 2 | Brain MRI findings at 7 years and 2 months. (C-1, C-2) Axial T2 weighted image and coronal T2 FLAIR demonstrate mild enlargement of the lateral ventricles. (C-3) Coronal T2 FLAIR demonstrates reduced volume of the bilateral hippocampus (arrow), with peculiar temporal horn dilatation.

nl/name-checker). Finally, we summarized the clinical and genetic characteristics of the remaining 91 patients reported in 10 publications (10, 13–16, 18–22); the proportion of females was ∼89% (81/91), and only 10 male patients have been described. The findings are presented in **Table 1** and **Supplementary Table 1**.

DISCUSSION

In this study, we performed trio-exome sequencing for a female patient with severe intellectual disability (ID) and identified a novel *de novo* pathogenic variant (c.1745dupG/p.S583*) of *DDX3X*. *DDX3X* encodes a 662-amino-acid protein and contains a helicase core composed of two RecA-like domains (D1D2) and N- and C-terminal extensions (NTE and CTE, respectively) beyond the two RecA-like domains. D1D2 harbors 12 highly conserved sequence motifs involved in nucleoside triphosphate binding, RNA binding, helicase activity and hydrolysis (23). Both the N- and C-terminal domains are involved in the specific biological role of the protein (24, 25). Therefore, the functional core of DDX3X was recently redefined to contain the NTE-D1D2-CTE fragment [residues 132-607; **Figure 3**; (23, 26)].

DDX3X has been associated with many cellular processes, such as cell cycle control, apoptosis, and tumorigenesis (27, 28). In 2014, the Deciphering Developmental Disorders Study identified *de novo* variants of *DDX3X* in 4 female patients with ID and proposed *DDX3X* as a candidate ID gene (17). In a large cohort of 6,072 patients with unexplained ID (2,659 females), *de novo* variants of *DDX3X* were identified in 38 females (10). According to previous reports, *de novo* variants of *DDX3X* account for 1%-3% of unexplained ID cases in females (10, 18).

TABLE 1 | Clinical features of affected females and males with *DDX3X* variants.

Reference		F									M			Total (%)
		(10)	(18)	(19)	(16)	(14)	(20)	(21)	(22)	Present case	(10)	(15)	(13)	
ID/DD		38/38	28/28	3/3	2/2	2/2	1/1	6/6	1/1	+	5/5	3/3	2/2	92/92 (100.0)
Growth	Microcephaly	12/38	7/28	2/3	0/2	2/2	0/1	1/6	0/1	+	2/5	0/3	0/2	27/92 (29.3)
	Low weight	12/38	3/13	0/3	0/2	0/2	NA	1/6	1/1	–	NA	1/3	NA	18/69 (26.1)
	Macrocephaly	0/38	0/28	0/3	0/2	0/2	0/1	0/6	0/1	–	0/5	0/3	2/2	2/92 (2.2)
Craniofacial Abnormalities	Dysmorphic features	30/38	19/28	3/3	2/2	2/2	1/1	6/6	1/1	+	2/5	3/3	0/2	70/92 (76.1)
	Plagiocephaly	0/38	1/13	1/3	0/2	0/2	0/1	0/6	0/1	–	0/5	2/3	0/2	4/77 (5.2)
	Brachycephaly	0/38	0/13	0/3	1/2	0/2	0/1	1/6	0/1	+	3/5	1/3	0/2	7/77 (9.1)
Neurology	Hypotonia	29/38	19/28	2/3	1/2	2/2	0/1	0/6	0/1	–	0/5	2/3	0/2	55/92 (59.8)
	Movement disorder	17/38	17/28	3/3	1/2	2/2	0/1	2/6	0/1	+	3/5	3/3	2/2	51/92 (55.4)
	Behavior problems	20/38	6/28	0/3	0/2	0/2	0/1	2/6	1/1	+	1/5	0/3	1/2	32/92 (34.8)
	Epilepsy	6/38	1/13	1/3	0/2	2/2	0/1	1/6	0/1	+	0/5	1/3	0/2	13/77 (16.9)
Brain MRI	Abnormal	13/37	18/20	3/3	0/2	2/2	0/1	1/4	1/1	**1/1**	NA	2/3	2/2	52/76 (68.4)
	CCH	13/37	NA	3/3	NA	2/2	0/1	1/4	1/1	–	NA	1/3	0/2	
	VE	13/37	NA	3/3	NA	2/2	0/1	0/4	1/1	+	NA	0/3	2/2	
	CM	4/37	NA	2/3	NA	1/2	0/1	0/4	0/1	–	NA	0/3	0/2	
	CCA	0/37	NA	0/3	NA	0/2	0/1	0/4	0/1	–	NA	0/3	2/2	
	HA	0/37	NA	0/3	NA	0/2	0/1	0/4	0/1	+	NA	0/3	0/2	
	Other*	0/37	NA	0/3	NA	0/2	1/1	0/4	0/1	–	NA	2/3	0/2	
Sleep disturbance		0/38	0/28	0/3	0/2	0/2	NA	2/6	0/1	+	NA	0/3	0/2	3/86 (3.5)
Ophthalmological abnormalities		13/38	9/28	2/3	1/2	1/2	0/1	3/6	1/1	–	2/5	2/3	0/2	34/92 (37.0)
Skin abnormalities		14/38	5/28	NA	1/2	0/2	NA	2/6	1/1	–	0/5	0/3	0/2	23/88 (26.1)
Hyperlaxity		14/38	2/13	0/3	0/2	0/2	NA	0/6	1/1	–	0/5	0/3	0/2	17/76 (22.4)
Scoliosis		4/38	0/28	2/3	0/2	2/2	NA	2/6	1/1	–	0/5	0/3	0/2	11/91 (12.1)
CHD		NA	5/7	NA	NA	2/2	NA	1/6	NA	–	NA	3/3	NA	11/18 (61.1)
Dyspnea		NA	5/28	NA	0/2	2/2	NA	0/6	0/1	–	NA	1/3	0/2	8/45 (17.8)
Audiological abnormalities		3/38	0/28	1/3	0/2	2/2	1/1	0/6	0/1	–	0/5	1/3	0/2	8/92 (8.7)
Precocious puberty		5/38	NA	NA	NA	0/2	NA	0/3	0/1	+	NA	0/3	0/2	6/50 (12.0)

CCA, corpus callosum atrophy; CCH, corpus callosum hypoplasia; CHD, congenital heart disease; CM, cortical malformation; DD, developmental delay; F, female; HA, hippocampus atrophy; ID, intellectual disability; M, male; NA, not available; Other including watershed infarcts, Dandy–Walker variant and arachnoid cyst; VE, ventricular enlargement.*

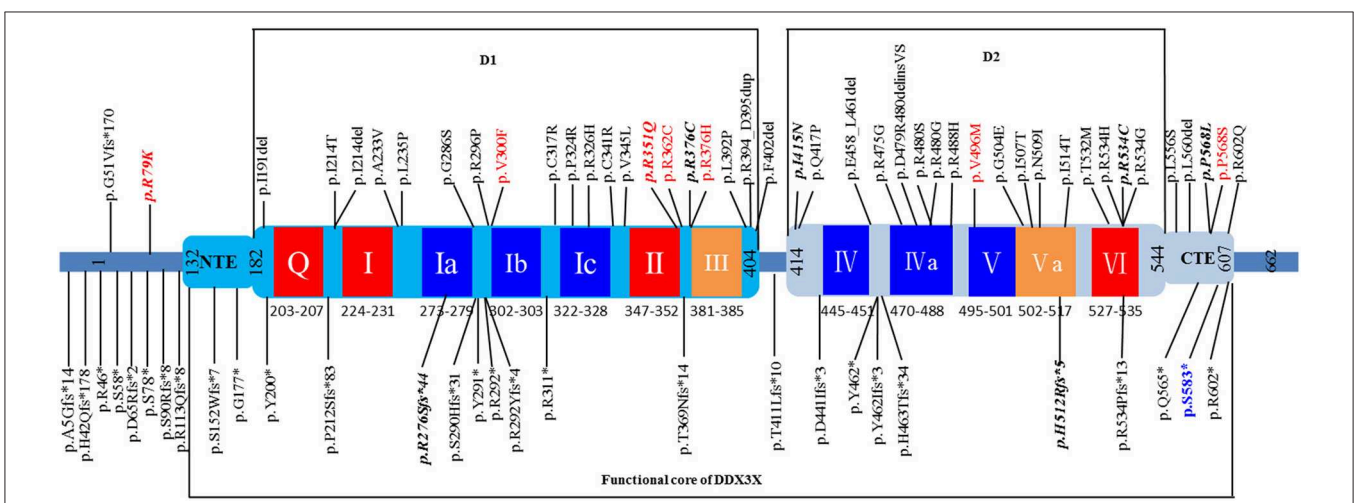


FIGURE 3 | Schematic view of the DDX3X protein structure based on Song et al. (26) showing D1D2, NTE, and CTE fragments. The 12 highly conserved sequence motifs are color coded: red, ATP binding; blue, RNA binding; orange, coordination between ATP and RNA binding. Unique variants (total: 73) are listed in the schematic view, showing the location of the variant identified in our patient (p.S583* in blue). The variants found in affected males are shown in red, and recurrent variants are indicated with bold italics. The 8 splice site variants identified in published cohorts are not shown in this figure.

To date, a total of 80 unique *DDX3X* variants have been described as causing intellectual disability or developmental delay, including 36 missense variants, 18 frameshift variants, 11 nonsense variants, eight splice site variants and seven small in-frame deletion/duplication variants, and most of the variants are located within the NTE-D1D2-CTE fragment of DDX3X (**Figure 3** and **Supplementary Table 1**). *De novo* patterns occurred in all female patients, and two of the *de novo* variants (c.573_575delCAT/p.I191del and c.1805G>A/p.R602Q) are mosaic in the proband, with allele fractions of 21% and 14%, respectively (18). Eight unique variants were identified in 10 male patients, including three *de novo* variants (15, 18), four maternally inherited variants (10, 13, 18), and one variant with unknown parental status (c.1702C>T/p.P568S) (15). The pathogenicity of these maternally inherited variants was determined by functional testing, which suggested partial loss of function of DDX3X as the most likely pathogenic mechanism (10, 13). Pathogenicity of the variant with unknown parental status was predicted to be damaging on the basis of similar phenotypes, family history and *in silico* prediction algorithms, including the consensus predictor, PredictSNP2 (15). Moreover, eight recurrent variants were reported, including c.236G>A/p.R79K, c.828_831delAGAG/p.R276Sfs*44, c.1052G>A/p.R351Q, c.1126C>T/p.R376C, c.1244T>A/p.I415N, c.1535_1536delAT/p.H512Rfs*5,c.1600C>T/p.R534C, and c.1703C>T/p.P568L (**Supplementary Table 2**).

The frequent clinical presentations reported include ID and/or developmental delay (DD) (92/92), dysmorphic facial features (70/92), hypotonia (55/92), movement disorders (51/92, e.g., dyskinesia, spasticity, stiff-legged or wide-based gait, ataxia, and choreoathetosis), structural brain abnormalities (52/76), ophthalmological abnormalities (34/92), behavior problems (32/92, e.g., agitation and hyperactivity, aggression and autism spectrum disorder), and microcephaly (27/92). The most frequent structural brain abnormalities reported include corpus callosum hypoplasia and ventricular enlargement. Thirteen patients experienced seizures, and five patients individually presented with general akinetic seizures, absence seizures, tonic-clonic seizures, spasms and febrile seizures; however, other clinical data, including EEG and treatment, were not available [**Supplementary Table 3**; (10, 14, 15, 18, 19, 21)]. Though all reported female and male patients had ID and/or DD and a majority suffered from movement disorders, compared to female patients, male patients rarely showed hypotonia, skin abnormalities, hyperlaxity, or precocious puberty, and often showed brachycephaly and plagiocephaly.

There is substantial clinical overlap between the phenotype in our patient and those of the females described to date, including severe ID, microcephaly, movement disorder, behavior problems, epilepsy, dysmorphic features, and ventricular enlargement. However, our patient also presented features that are distinct from those of the patients reported previously. In comparison to published data, our patient did not present hypotonia, which was a common feature in previous reports. Furthermore, hand stereotypies and temporal horn dilatation have been occasionally associated with the *DDX3X* spectrum (15, 19), while sleep disturbance has been reported in only two female patients (21), and hippocampus atrophy has not been reported in patients with *DDX3X*-related ID to date.

There is no evidence for an obvious correlation between the location of variants and the severity of phenotype in previous reports (10, 18). However, variants located in the D1D2 and CTE fragments seemed more likely to be associated with moderate-severe or severe intellectual disability in our literature review (**Supplementary Table 1**). In addition, review of clinical data revealed that the same recurrent variants may result in very similar phenotypes (**Supplementary Table 2**). For example, two of three patients with the *de novo* c.1703C>T/p.P568L variant exhibited hypotonia, microcephaly, visual problems, scoliosis, seizures, ventricular enlargement, and corpus callosum hypoplasia; two patients with the c.236G>A/p.R79K variant had progressive spastic paraparesis, tremor, behavioral problems, decreased lower extremity strength, ventricular enlargement and corpus callosum hypoplasia. Conversely, the two patients with c.828_831delAGAG/p.R276Sfs*44 had no abnormal neurological or brain imaging findings, corresponding with only mild to moderate ID.

To assess the correlation between the type of variants and the severity of clinical phenotype, we investigated the frequency of severe ID in patients who carry missense and frameshift or nonsense variants [predicted loss of function (LoF)]. These missense and LoF variants were found in 81.3% (65/80) of the cohort, and most of these variants fall within the NTE-D1D2-CTE fragments. Surprisingly, patients with missense variants (45.2%, 19/42) were more likely to have severe ID than patients with frameshift or nonsense variants (13.0%, 3/23) ($p < 0.01$). We consider these LoF variants likely to undergo nonsense-mediated RNA decay (NMD), which may have different effects on phenotype than missense variants. NMD is thought to serve as an mRNA surveillance mechanism to prevent the synthesis of truncated proteins that would have the potential to have toxic effects, such as dominant negative interactions (29, 30). The association between severe ID and the type of *DDX3X* variants likely suggests the possibility of a dominant negative impact of *DDX3X* missense variants in severely affected individuals; in contrast, patients with frameshift or nonsense LoF variants may show milder phenotypes because of the NMD mechanism, which decreases the levels of potentially deleterious proteins (30). However, the definite phenotype-genotype correlations need to be studied with a larger, more fully phenotyped patient cohort in the future.

Somatic variants of the *DDX3X* gene have also been identified in a variety of malignancies (19). In two studies, five variants (c.1600C>T/p.R534C, c.1703C>T/p.P568L, c.641T>C/p.I214T, c.931C>T/p.R311*, c.1084C>T/p.R362C) were reported to occur somatically in association with malignant melanoma, medulloblastoma, and esophageal squamous cell carcinoma (10, 18). However, malignancy was not reported in any patients in a previous study except one patient diagnosed with global DD harboring a *DDX3X* missense variant (c.1511G>A/p.G504E) (19). A larger study needs to be performed to elucidate whether patients with ID/DD harboring *DDX3X* germline variants are at greater risk of developing malignancies.

CONCLUSION

We identified a novel pathogenic nonsense variant of *DDX3X* in a female patient suffering from severe ID and various other features. This is the first study to report a Chinese case of ID with a *DDX3X* variant. According to our systematic literature review, most of the variants are located within the two RecA-like domains of *DDX3X*. Patients with missense variants were more likely to have severe ID compared to the patients with a frameshift or nonsense variants. This investigation further expanded the number of *DDX3X* variants and their associated phenotypic spectrum, which might help to improve the understanding of *DDX3X*-related intellectual disability or developmental delay. However, the phenotype-genotype correlations need to be studied with a larger, fully phenotyped patient cohort in the future.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association (WMA), and written informed consent was obtained from the parents for the research study, presentation of photographs, clinical details, and publication of this case report.

AUTHOR CONTRIBUTIONS

YC drafted the manuscript. YC, K-YL, Z-LY, X-HL, and RX contributed to the clinical data acquisition. YC and HZ contributed to the analysis and genetic evaluation. HZ critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00303/full#supplementary-material

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REVIEW
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Genomic Diagnosis for Pediatric Disorders: Revolution and Evolution

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Powerful, recent advances in technologies to analyze the genome have had a profound impact on the practice of medical genetics, both in the laboratory and in the clinic. Increasing utilization of genome-wide testing such as chromosomal microarray analysis and exome sequencing have lead a shift toward a "genotype-first" approach. Numerous techniques are now available to diagnose a particular syndrome or phenotype, and while traditional techniques remain efficient tools in certain situations, higher-throughput technologies have become the *de facto* laboratory tool for diagnosis of most conditions. However, selecting the right assay or technology is challenging, and the wrong choice may lead to prolonged time to diagnosis, or even a missed diagnosis. In this review, we will discuss current core technologies for the diagnosis of classic genetic disorders to shed light on the benefits and disadvantages of these strategies, including diagnostic efficiency, variant interpretation, and secondary findings. Finally, we review upcoming technologies posed to impart further changes in the field of genetic diagnostics as we move toward "genome-first" practice.

Keywords: genetic syndromes, genomic diagnostics, genetics, pediatrics, sequencing, copy number variants, next-generation sequencing

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INTRODUCTION

Tools for genomic diagnosis have evolved rapidly over the past two decades, resulting in remarkably improved diagnostic rates as well as a significant increase in the number of disease genes. The core technologies for genetic testing laboratories have evolved from Sanger sequencing and karyotype analysis to Next Generation Sequencing (NGS)-based tests (targeted panels, exomes, and genomes) and chromosomal microarrays (CMA) (**Table 1**). As a result, the scope of identifiable mutations now ranges from changes in the *amount* of a particular genomic locus, such as loss or gain of entire chromosomes (i.e., aneuploidy) or smaller regions of DNA (i.e., copy number variants, CNVs), to changes in the *structure* of the genome (i.e., translocations, inversions, insertions), and to changes in the *sequence* of the genome (i.e., single nucleotide variants and short insertions/deletions). These advances have had a profound impact on how we diagnose patients who present with clinical features of known genetic disorders. In the realm of cytogenetics, traditional techniques such as cytogenetic analysis of banded chromosomes are still warranted for some conditions (e.g., trisomies, Turner syndrome). However, classic cytogenetic deletion syndromes (such as Wolf-Hirschhorn and Cri-du-Chat syndromes) are now better tested by CMA, with the opportunity to identify smaller deletions or duplications including precise breakpoints.

Advances in molecular technologies have had an even more transformative effect on diagnostics. The shift to NGS-based sequencing methods has made evaluation of larger portions of the genome

TABLE 1 | Genomic technologies for chromosomal and molecular syndromes.

	Types of aberrations	Resolution	Clinical indication examples
Karyotyping	Large structural changes: aneuploidies, translocations, isochromosomes, rings, CNVs >5–10 Mb, etc. Balanced changes (translocations, insertions, inversions, rings)	5–10 Mb* *depends on region and banding level	- Suspicion of chromosome syndrome - Infertility or recurrent miscarriage - Rule out structural variant after microarray finding
FISH	Aneuploidies, CNVs, translocations, inversions, insertions Probes must be designed for specific aberration	50 kb−1 Mb; most 200–400 kb	- Prenatal aneuploidy - Parental studies for proband with structural rearrangement (balanced or imbalanced) or CNV - Follow-up studies after abnormal karyotype (e.g., SRY FISH on abnormal Y)
SNP array	Copy number changes associated with *unbalanced* structural changes; Regions of homozygosity/Uniparental disomy; Mosaicism	10–100 kb *depends on probe density and reporting criteria may be significantly larger	- Congenital anomalies - Intellectual disability
aCGH	Gene or exon level copy number changes associated with *unbalanced* structural changes	Based on designed, clinical grade typically single-exon resolution for genes of interest	- As part of a phenotype-specific panel test - A complement to exome sequencing
MLPA, real-time PCR	Deletions or duplications	Exon-level	- SMA - Thalassemia - Imprinting disorders
NGS panel or exome	SNVs, indels, copy number changes Mitochondrial DNA if long-range PCR used first	SNVs: single-nucleotide CNVs: exon-level unless breakpoint within exon, then nucleotide-level as most panels only cover exonic regions	- Phenotype-specific gene panel
NIPS	Chromosomal aneuploidies and recurrent deletion/duplication syndromes	Variable depending on methodology. Some designed to detect recurrent CNVs	- Prenatal aneuploidy screening
Sanger sequencing	Sequence variants including SNVs, small indels; CNVs smaller than the amplicon size can also be detected but not typical usage	1 bp	- Specific phenotype known to be caused by sequence variants in a single gene - Targeted testing for familial variant
Repeat-primed PCR	Repeat expansions	Quantify 1–220 repeats; Detect up to 1,000 repeats	Repeat expansion disorders
MS-MLPA and MS-qPCR	Deletion, UPD and imprinting center defect in the imprinted regions	Exon level	Imprinting disorders such as Prader-Willi Syndrome

possible simultaneously, resulting in targeted panels including only a few or up to hundreds of genes, as well as examination of the entire exome [i.e., the protein coding regions of the genome, see section Exome Sequencing (ES)] or genome. These improvements allow a streamlined approach to diagnosis of genetic disorders, by avoiding unnecessary evaluations and diagnostic studies such as Ophthalmology and Cardiology evaluations for hearing loss patients or functional studies for patients with Fanconi anemia or Osteogenesis Imperfecta. This has allowed faster and improved diagnostic rates of heterogeneous disorders (e.g., Osteogenesis Imperfecta, Noonan syndrome, and Cornelia de Lange syndromes). Highly heterogeneous phenotypes like hearing loss, intellectual disability, and/or seizures are now amenable to diagnosis using large, NGS-based panels. The ability to test a large number of genes (if not all) simultaneously has had a significant effect on the practice of Clinical Genetics, as it becomes possible to take a "genotype-first" approach to diagnostics.

In this review, we will discuss current core technologies for the identification of cytogenetic (section Chromosomal and Copy Number Variations), sequence (section Sequencing Variants), and other (section Disorders Requiring Special Testing) alterations causing a variety of genetic disorders (**Figure 1A**), and we will illustrate how these technologies have impacted our ability to make genomic diagnoses, identify new genetic causes of disease and expand the phenotypic spectrum for many classic genetic disorders (section Impact on Clinical Care). Finally, we will review several new and emerging technologies currently or predicted to further impact the field of pediatric genetic diagnostics (section Emerging Technologies).

CHROMOSOMAL AND COPY NUMBER VARIATIONS

Brief Description and Historical Perspective

Cytogenetic analysis has traditionally involved detection of the number and composition of metaphase chromosomes based on examination in the light microscope. However, in recent

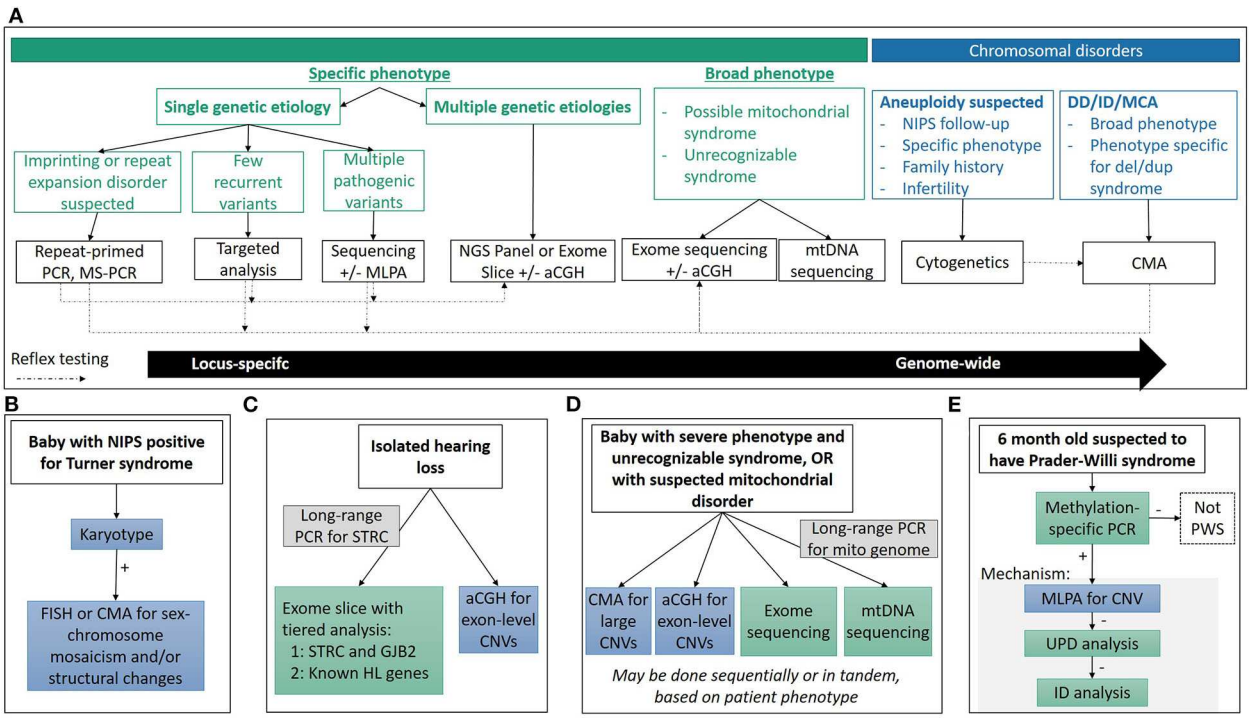


FIGURE 1 | Diagnostic technologies and applications. **(A)** General considerations used in determining the appropriate technology used for diagnosis. Choosing the appropriate technique is a multi-factorial process, depending on reason for study, clinical presentation and associated genetic heterogeneity, molecular mechanisms, time and cost considerations, among others, and there is often no single "right" approach. **(B–E)** Common clinical diagnostic workflows for various genetic syndromes. See clinical examples in text for more detail.

years with the development of molecular techniques capable of identifying chromosome aneuploidies and smaller copy number deletions or duplications, that is copy number variants (CNVs), the line between cytogenetic and molecular (DNA-based) genetic testing has become blurred. There now exist multiple techniques to identify changes chromosomal aneuploidies, CNVs, structural variants, or even to evaluate for absence of heterozygosity which could be related to uniparental disomy or identity by descent (**Table 2**).

Cytogenetic discoveries have been driven by technical innovation. The characterization of human cells having 46 chromosomes followed the discovery that chromosomes could be visualized by treating cells with colchicine (a microtubule inhibitor) and hypotonic solution (to swell the cells) (1–4). Three years later, the first chromosomal disorder was described by Lejeune in 1959, associating Down Syndrome with trisomy of "the smallest chromosome" (5), and within 1 year the genetic basis of Turner, Klinefelter, XXX, trisomy 13 and trisomy 18 were elucidated (6–10). These findings are quite remarkable given that the first *banded* human karyotype was still 10 years from being described. The development of G-banding in 1971, which is still the most commonly used banding technique today in North America, resulted in widespread adoption of cytogenetics, and numerous subsequent discoveries of chromosomal abnormalities in a constitutional and neoplastic setting (11, 12).

"Molecular cytogenetics" emerged in the 1980s with the development of fluorescence *in situ* hybridization (FISH), which determines the presence or absence of discrete segments of DNA, thus bridging molecular and cytogenetic analysis. FISH allows targeted identification of deletions and duplications associated with known disorders, such as Cri-du-Chat or Wolf-Hirschhorn syndromes, as well as characterization of translocations or marker chromosomes identified in karyotypes. The revolutionary development of PCR which, along with many other applications, allowed for targeted CNV detection of even smaller regions, and ultimately led to the development of numerous other CNV assays which rely on PCR, such as multiplex ligation-dependent probe amplification (MLPA) and chromosomal microarray analysis (CMA). Indeed, modern CMA, which enable unbiased scanning of the genome to detect CNVs, are a result of decades of work in molecular biology, bio-engineering and robotics, and have resulted in the discovery of numerous disorders associated with submicroscopic CNVs and elucidation of the underlying mechanism (**Table 3**) (13–18).

Today, chromosome analysis using conventional cytogenetic techniques remains a first tier test for patients with a suspected aneuploidy (trisomies or sex chromosome aneuploidy), family history of a rearrangement (translocation or inversion), or infertility (19). However, for more subtle changes, such as deletions or duplications, CMA has proven to have increased sensitivity, and is the first tier test for patients with developmental

TABLE 2 | Technical comparison of technologies available for detection of chromosomal changes and/or CNVs.

Abnormality	G-banding	FISH	SNP array	aCGH	MLPA	NGS panel or exome	NIPS	WGS
Aneuploidy	+	+	+	+	+	+	+	+
Balanced rearrangement (translocation, inversion, insertion)	+	+	–	–	–	–	–	+
CNVs >5–10 Mb	+	+	+	+	+/–**	+	+	+
CNVs <5–10 Mb	–	+	+	+	+	+	+/–	+
Single exon deletion	–	–	–	+	+	+/–	–	+
Polyploidy	+	+	+	–	–	+	+	+
Min. mosaicism	10% with 30-count	2–5% depending on probe characteristics	5–10%*	20%*	40% for dup; 20–30% del;	Depends on coverage	Depends on methodology	Unclear
Clonal relationships	Y	Y	N	N	N	N	N	N

*Depends on probe coverage and size of CNVs.
**MLPA is a targeted CNV detection strategy, and if the region of interest is involved in a much larger CNV, a CNV would be detected but additional technologies are required to delineate the size and breakpoints.

TABLE 3 | Examples of syndromes associated with recurrent and non-recurrent CNVs. See Spinner et. al. (13) for further details including clinical descriptions.

Syndrome	CNV	Recurrent breakpoints?	Notes
1p36	1p36 deletion	No	Variable size from 0.5 to 10 Mb; ∼50% due to terminal deletion, ∼30% interstitial deletion, and remainder due unbalanced to rearrangements
Wolf-Hirschhorn	4p partial deletion (4p-)	No	Critical region is 4p16.3 (165 kb); ∼45–50% due to unbalanced translocation
Cri-du-chat	5p partial deletion (5p-)	No	Critical regions: - Cat-like cry: 1.5 Mb region of 5p15.31 - Speech delay: 3 Mb region of 5p15.33-5p15.32. - Dysmorphic facial features: 2.4 Mb region of 5p15.31-p15.2 ∼90% associated with *de novo* deletions
Williams	7q11.23 deletion	Yes	1.5 Mb deletion involving 25 genes in >90% patients Critical genes: - *ELN*: cardiovascular and connective tissue phenotypes - *LIMK1*: impaired visual motor integration - *BAZ1B*: hypercalcemia
Miller-Dieker	17p13.3 deletion	No	*LIS1* is responsible for lissencephaly, and mutations in *LIS1* result in isolated lissencephaly
Hereditary neuropathy and pressure palsies (HNPP)	17p12 deletion	Yes	1.5 Mb deletion in 80% of patients *PMP22* is critical gene
Charcot Marie Tooth Type 1	17p12 duplication	Yes	1.5 Mb duplication, reciprocal to HNPP deletion *PMP22* is critical gene
Smith-Magenis	17p11.2 deletion	Yes	3.7 Mb deletion in >90% patients *RAI1* associated with sleep disturbances
Potocki-Lupski	17p11.2 duplication	Yes	3.7 Mb duplication, reciprocal to Smith-Magenis deletion
22q11.2	22q11.2 deletion	Yes	3.0 Mb deletion in 85%, rest have deletions associated with two of four recurrent breakpoints >90 genes involved, *TBX1* may be partially responsible for cardiac abnormalities

delay, intellectual disability, autism spectrum disorder, or multiple congenital anomalies of unknown etiology (19). Furthermore, development of next-generation sequencing (NGS) has provided the ability to simultaneously (i.e., with a single assay) detect chromosomal aneuploidies, CNVs and sequence variants. There is thus another shift in the field as laboratories are starting to rely more on NGS for CNV detection, and less on CMA or targeted approaches.

This shift in technologies toward NGS, coupled with the ability to identify fetal DNA in maternal serum, has greatly impacted prenatal aneuploidy screening with the implementation of non-invasive prenatal screening (NIPS) (20–22). As NIPS providers are starting to offer screening for microdeletion syndromes, genome-wide aneuploidy detection (23) and even single gene disorders (24, 25), we anticipate a shift in diagnosis from the

phenotypic-driven postnatal setting to screening-based prenatal detection.

Technologies

Multiple assays can detect chromosomal aberrations (**Table 2**), and thus understanding the differences in their clinical utility and limitations is important (**Table 1**). The following sections describe and contrast chromosomal and CNV technologies as they are used today.

Cytogenetics (Karyotype and FISH)

In general, karyotype analysis is indicated if one suspects a chromosomal aneuploidy or a large (>5–10 Mb) structural change, especially if balanced. Only karyotype analysis, whole genome sequencing, and FISH (if the abnormality is known a priori) can detect balanced rearrangements but whole genome sequencing is currently cost-prohibitive for most indications. Molecular cytogenetics using FISH tends to be used in familial studies, cytogenetic follow-up studies, or to test for recurrent changes, such as William's syndrome (see caveats in clinical examples below). Because karyotype and FISH analysis involve visualization of individual cells, clonal relationships can be resolved when multiple cell lines are present (26). For example, a patient with a mosaic gain found by CMA requires cytogenetic studies to determine the exact number of copies (i.e., duplication, triplication, etc.), the location of the additional material (i.e., tandem duplication or inserted elsewhere), and the orientation of the additional material (i.e., inverted or not).

Chromosomal Microarray Analysis (CMA)

There are two major type of arrays to detect CNVs, genome-wide single-nucleotide polymorphisms (SNP) arrays which assay SNPs across the genome, and array comparative genomic hybridization (aCGH) which use larger oligonucleotide probes tiled across the genome, often with a "backbone" of SNP probes. SNPs are generally benign, single-base DNA changes that are present at an appreciable frequency in normal populations. SNP probes can detect deviations in the allele ratio which can help identify low-level mosaicism, and are required to identify regions of homozygosity due to identity by descent or uniparental isodisomy with CMA. Both arrays are widely used and have overlapping clinical utility but SNP arrays are best used to identify multi-gene or larger CNVs, identify regions of homozygosity, and can better detect low level mosaicism, while aCGH is particularly useful for gene-level CNVs, with some clinical aCGH platforms designed to identify CNVs as small as one exon (27–29). This distinction is due to the underlying technology with aCGH oligonucleotide probes capable of capturing any unique sequence in the genome while SNP array probes are limited to specific positions in the genome known to be polymorphic, which tend to fall outside coding regions. The exact resolution of both types of arrays depends on the designed probe density.

The resolution of SNP arrays is typically in the range of 10–100 kb for a clinical platform. This drastic increase in resolution compared to karyotype analysis allows for the detection of small CNVs including those found at translocation breakpoints, and elucidation of gene content of CNVs. In practice, clinical laboratories have larger size thresholds for reporting CNVs, typically between 200 kb and 1 Mb, but may choose to report smaller CNVs with clear clinical significance (19). Balanced changes such as inversions, insertions or balanced translocations cannot be detected since there is no net loss in material and no visualization of chromosomes. Similarly, when a duplication is detected, its orientation or placement within the genome cannot be deduced. Identification of CNVs of unknown significance or CNVs associated with disorders outside of the clinical indication pose significant challenges in CMA interpretation. These issues are discussed in relation to sequence variants below (section Next Generation Sequencing).

Multiple Ligation-Dependent Probe Amplification (MLPA)

MLPA is a semi-quantitative technique to assess copy number via multiplexed fragment size analysis (30, 31). Sequence-specific probe pairs are hybridized to the DNA of interest, ligated and amplified, and each target region has a "stuffer sequence" of a specific length allowing for identification of the multiplexed targets. MLPA is best suited for detection of smaller CNVs, such as single gene deletions or recurrent microdeletion/duplication syndromes. Commercial kits from MRC-Holland (www.mlpa.com/) are available for loci commonly involved in CNVs, such as *DMD* associated with Duchene Muscular Dystrophy (see below) and *HBB* associated with Beta-thalassemia, among many others.

NGS Panels and Exomes

In recent years, NGS has been widely adopted in clinical diagnostic laboratories due to its ability to efficiently interrogate multiple genes at a time. Additionally, NGS offers the ability to detect both sequence variants and structural variants (including deletions, duplications, inversions, translocations, and other alterations). For targeted panels evaluating coding regions only, structural variant detection is limited to exonic CNVs, and breakpoints typically cannot be identified. Standardized methods are not yet available and laboratories need to develop and validate internal bioinformatics pipelines, and characterize the pipeline's performance metrics in terms of minimum size of detection and mosaicism. While clinical laboratories have reported very high analytical sensitivity of NGS-based CNV detection, analytical specificity is lower particularly for smaller CNVs (1–3 exons) which typically require orthogonal confirmation (32–35). However, the major advantage with calling CNVs using NGS data is the decrease in cost compared to running two different assays (i.e., sequencing and MLPA or aCGH). Additional details of NGS are described in section Next Generation Sequencing.

Non-invasive Prenatal Screening (NIPS)

The ability to assay fetal cells in maternal serum has greatly altered the practice of prenatal diagnostics (20–22). It is beyond the scope of this article to fully review detailed methodological and clinical characteristics of NIPS, but readers are encouraged to refer to existing reviews [e.g., (36, 37)]. Prenatal screening for aneuploidies such as Trisomies 13, 18, and 21, which have a combined incidence of 4 in 1,000 newborns

and are associated with advanced maternal age, is offered to all pregnant women using various strategies (38). In the traditional approach, a woman is offered a maternal serum screening test which measures specific maternal hormones and fetal metabolites, and women flagged as "high-risk" for fetal aneuploidy are subsequently offered a diagnostic test, either CVS or amniocentesis to collect placental tissue or fetal cells, respectively, which is sent for karyotype or FISH analysis. Now with NIPS, maternal plasma, which contains circulating fetal and maternal DNA, is analyzed with NGS or SNP arrays, as early as 10 weeks gestation. The major advantage of NIPS compared to previous serum screening is the decrease in false positive rate (or increase in specificity) without compromising the sensitivity, thus reduction of unnecessary invasive procedures (CVS or amniocentesis).

However, it must be stressed that NIPS is a *screening* test and findings from NIPS must be confirmed by a diagnostic method using cytogenetic techniques. Several reports of misuse and misunderstanding of NIPS include mis-interpretation of findings as diagnostic, or using NIPS to screen for genetic conditions other than those indicated such as sequencing changes (21, 39). False positives and false negatives are rare but do occur at a clinically significant rate due to both biological and technical reasons, including confined placental mosaicism, maternal aneuploidies or CNVs, occult maternal malignancy, vanished twin and true fetal mosaicism (40, 41). The positive predictive value for NIPS in women of advanced maternal age is highest for trisomy 21 (>90%), but decreases below 90% for trisomy 13 and 18, and as low as ∼50% for sex chromosomal aneuploidies (42, 43). Because the risk of aneuploidy increases with maternal age, the positive predictive value is even lower for younger women.

Clinical Examples

Turner Syndrome: Multiple Roads to Diagnosis—Karyotype, FISH, CMA, and NIPS

Turner syndrome (45,X) occurs in ∼1 in every 2,500 live births, and accounts for 6–8% of spontaneous abortions (11). If Turner syndrome is suspected, karyotype analysis is the recommended technique, with some important subtleties (**Figure 1B**). Only 45% of Turner syndrome cases are due to a 45,X karyotype, while the rest have structurally abnormal X or Y chromosomes, and/or mosaicism for another cell line with a different sex complement (XX, XXX, XY, etc.) which may have phenotypic and/or management consequences including predisposition to gonadoblastoma if Y chromosome material is present (11, 44). Therefore, when a karyotype consistent with Turner syndrome is identified, an appropriate work up must rule out the presence of Y chromosome material, as well as characterization of any identified abnormal sex or marker chromosomes by FISH or CMA. While it may be tempting to order a CMA as the first-tier test, karyotype analysis is needed to identify structural rearrangements involving the sex chromosomes.

Sex chromosomal aneuploidies are commonly screened for in NIPS, but as discussed above, the positive predictive value is low, especially for Turner syndrome. This is at least partially due to maternal mosaic aneuploidies, such as age-related (benign) loss of X or undetected XXX syndrome, which can result both in false positive and false negatives in relation to the fetus (45). Thus, one should be wary of NIPS results that have not been confirmed by a diagnostic method, and if clinically indicated, order cytogenetic confirmatory studies. This situation may become more common as women are declining prenatal diagnostic/confirmation studies and there is ambiguity in responsibility and possible lack of communication between prenatal obstetrical care and postnatal pediatric care (42).

Potocki-Lupski and Smith-Magenis: CMA vs. FISH

The development of CMA led to the discovery of multiple syndromes caused by submicroscopic deletions or duplications, including so-called genomic syndromes which are caused by misalignment of repetitive sequences during recombination (16). The resulting deletions and duplications are recurrent with the same breakpoints identified in most patients, and deletions and duplications result in different, and in some cases, mirror phenotypes (**Table 3**).

One example of reciprocal deletion/duplication syndromes is the Smith-Magenis syndrome, which is most commonly caused by a 3.6 Mb deletion of 17p11.2 including the *RAI1* gene, and Potocki-Lupski syndrome caused by a duplication of the same region (46–49). While both syndromes are characterized by intellectual disability and sleep and behavior disturbances, they differ in many other respects and are clearly two distinct pathological entities (50). FISH studies can be used to detect these CNVs, and indeed were once the preferred test, but CMA is now the most appropriate assay due to the ability to define the deletion size and gene content, to detect atypical CNVs, and to interrogate CNVs at other loci which cover a broader differential diagnosis (48, 49, 51). FISH studies remain useful for follow-up parental studies in patients with typical CNVs.

Duchenne Muscular Dystrophy: Detection of Exon-Level CNVs With MLPA

For some conditions with very specific phenotypes and a high rate of deletion or duplications, MLPA is considered a first-tier test as it offers a fast and sensitive approach to molecular diagnosis. For instance, in Duchenne muscular dystrophy 65–80% of patients have a deletion or duplication within the *DMD* gene located on the X chromosome (52). High-resolution characterization of these CNVs is critical for patient management, as individuals with out-of-frame CNVs tend to present with the more aggressive phenotype of Duchenne muscular dystrophy, while those with in-frame CNVs will present with the milder Becker muscular dystrophy. Together with the characteristic presentation of a boy with progressive proximal weakness and high serum creatine phosphokinase values, a targeted MLPA panel is appropriate for molecular diagnosis in that it is rapid, cost-efficient and most importantly can determine the frame of CNVs. While aCGH platforms *may* have the resolution to detect single exon changes, SNP arrays most likely do not, and overall CMA is not recommended for molecular diagnosis of muscular dystrophy (52).

SEQUENCING VARIANTS

Brief Description and Historical Perspective

DNA-based molecular diagnostics originated following the introduction of techniques for manipulation and analysis of DNA including the discovery and utilization of restriction enzymes ("molecular scissors") in the late 1960s and early 1970s (53) and development of DNA-based hybridization methods, including the Southern blot in 1975 (54). The earliest applications of these methods were used for those few disorders whose molecular basis was known during the 1970s. Early molecular diagnostic tests included prenatal diagnosis focused on the well-studied disorders of hemoglobin including α and β thalassemia and sickle cell anemia (55–57).

Methods for DNA sequencing were also introduced during the 1970s (58, 59) with increasing automation and continuous improvements over the next four decades. The introduction of PCR in the mid 1980s, with its ability to target and amplify specific segments of DNA, revolutionized molecular biology as well as medical genetics, paving the way for analysis of the increasing number of known disease genes. The early explosion of tools for DNA analysis presented tantalizing possibilities to understand the genetic basis of disease, and then in 1991 The Human Genome Project was officially launched, with the goal of "Understanding Our Genetic Inheritance" by sequencing all of the bases in the human genome and creating maps of each chromosome. The first draft of the human genome was published in Nature in 2001 (60) and the "final" draft followed 3 years later in 2004 (61). As the human genome project was progressing, several technical advances in genetic analysis were also introduced that have had wide impact on genomic diagnostics including CMA and NGS, paving the way for clinical exome sequencing (ES) and genome sequencing (GS) (19, 62–64).

A consequence of the enormous resources and the explosion in technology to analyze the human genome, has been a parallel revolution in the identification of human disease genes. In 1986 as sequencing was becoming mature and PCR was introduced, there were <10 human genetic disorders for which the disease gene was known. This number ballooned to close to 950 by 2000 as the first draft of the human genome was being finalized, to over 4,000 by 2016 driven by the utilization of genome-wide tests such as ES and GS (65). Currently, there are over 9,600 conditions with a known genetic cause that can be tested using genomic technologies (https://www.ncbi.nlm.nih.gov/gtr/).

Methods for Genetic Testing

Genetic tests analyze chromosomes, DNA, RNA, proteins, or metabolites in order to identify alterations that are associated with a clinical disease phenotype. For purposes of this section of this review, it is assumed that we are discussing changes in DNA that lead to altered gene products including single nucleotide variations, nucleotide level insertions or deletions, and generally intragenic variants. The techniques discussed here are those most commonly used for genetic testing, and are not meant to provide an exhaustive discussion. In the early years of clinical molecular genetic diagnostics, there were a number of techniques for the identification of altered sequence that are primarily hybridization based, which have almost completely been supplanted by DNA sequencing. In this review, we will limit our discussion to techniques that are in current usage, primarily sequencing.

Sanger Sequencing

Sanger sequencing (chain termination method), first introduced in 1977 works using the selective incorporation of nucleotides that are modified such that they "terminate" the sequencing reaction, thereby identifying the position of the base last incorporated (59). Sanger sequencing is a mature technology with excellent accuracy and is considered the "gold standard" for sequence determination, although it does not capture mosaicism below 15–20%. Sanger sequencing is most useful for disorders caused by a single, shorter gene where PCR amplicons are sequenced with highly focused accuracy and relatively long reads (∼600–800 base pairs). Note deletions, duplications or structural genome rearrangements will not be identified by this technology, and it is often necessary to include a secondary method to identify these alterations. While Sanger sequencing is being replaced in many assays by NGS-based approaches, it retains an important role in the clinical laboratory for diagnosis of single-gene disorders, as an orthogonal method to confirm sequence variants identified by NGS, and as a means to provide coverage for genomic regions that are not well-covered by NGS testing due to poor capture or amplification.

Next Generation Sequencing

Several papers published in 2005 described advances that became known as "next generation" or "massively parallel" sequencing. The critical advance was the ability to multiplex, such that a complex library of DNA templates could be presented to the sequencing reagents simultaneously, followed by *in vitro* amplification of each template. With Illumina sequencing chemistry, for example, sequencing then proceeds by analysis of labeled nucleotides and subsequent imaging, known as "sequencing by synthesis." Between 2005 and 2012, improvements in sequencing were fast and furious, with increasing accuracy and throughput and decreasing costs, such that beginning around 2011, sequencing of large portions of the genome became possible in the clinical diagnostic laboratory (66, 67).

Targeted gene panels

The first clinical application of NGS was to capture and analyze a small groups of genes to look for disease predisposing or disease-causing variants. In 2010, this method was applied to analysis of 21 genes involved in breast and ovarian cancer, when Walsh et al., simultaneously analyzed the sequence for these genes following hybridization capture in a cohort of 20 women who had a known mutation in one of the breast and ovarian cancer predisposition genes (68). This successful proof of principle study laid the foundation for a wide variety of targeted panels that are currently in use in diagnostic laboratories around the world. Targeted panels generally analyze between 2 and 500 genes, with different diagnostic strategies depending on details and specificity of the

clinical phenotype, the differential diagnosis and the genetic heterogeneity of the phenotype. The major advantages of targeted gene panels compared to ES are (1) higher depth sequencing, and (2) guaranteed 100% coverage of genes which may require Sanger fill-in.

Exome sequencing (ES)

Most disease-causing variants (∼85%) are concentrated in the 1–2% of the genome that is protein coding (exonic regions), and the collection of all of the exons is known as the exome. These exonic regions can be selectively "captured," allowing sequencing of these high yield genomic regions, reducing the amount of sequence significantly in comparison to the genome, allowing for a relatively efficient test for both new causes of genomic disease or identification of pathogenic variants in known disease genes (69, 70). The diagnostic yield of ES varies between 10 and over 50%, depending on the clinical features of the population tested, year of testing, and analytical strategy (66, 67, 71–73). For example, diagnostic yield of exome sequencing for chronic kidney disease is 9% based on one recent study (74), compared to 29–55% for neurodevelopmental disorders, and 26–58% in unselected cohorts with a suspected genetic etiology (75). It has greatly impacted the practice of clinical genetics, since the requirement for a precise clinical phenotype is less stringent, in contrast to the ordering of specific single-gene tests, where a decision regarding which gene to test has to be made up front. Indeed, a recent meta-analysis on the diagnostic yield of ES for neurodevelopmental disorders concluded that ES should be considered as a first line test for these patients (76) (see section Genotype-First Approach for further discussion).

Challenges in Interpretation of NGS Data

The techniques for exome capture and sequencing have matured greatly and are relatively stable in most laboratories, but the primary challenges today are in the complexity of data analysis and interpretation of the clinical significance of variants identified. The large amount of sequence data generated by NGS platforms has fueled the addition of bioinformatics teams into clinical diagnostic laboratories to develop data handling and analysis pipelines for these complex tests. Once sequencing is completed, the sequence is aligned and compared to the reference genome to identify sequence variants and filters are applied to retain only high quality variants. Variants that are rare in the population and are predicted to have a functional impact on the gene, mostly commonly by altering the gene's protein coding sequence (non-sense, missense, frameshift mutation, etc.), are typically prioritized for analysis and human interpretation.

Analytical Sensitivity and Specificity

Short read sequencing by NGS is prone to both false positives and false negatives, with analytical sensitivity and specificity depending on assay design (amplification strategy, target coverage, instrumentation, etc.). False positives can occur due to PCR artifacts, sequencing errors, low coverage, and errors in alignment, among other reasons (77). False positives tend to have characteristic features which enables clinical laboratories to either filter them away, or to devise analytical standards to defining which variants require orthogonal confirmation (78). Similarly, false negatives can be due to one of several technical reasons. First, like all amplification-based technologies, variants present in the primer or probe binding sets can result in allele-drop out, where only one strand of DNA is amplified and analyzed. Next, sequence context such as high GC-content (e.g., in promoter regions) and repetitive and complex genomic regions (e.g., pseudogenes) can result in lower coverage and misalignment, respectively. To help laboratories handle these and other related challenges, several professional groups have developed standards and guidelines for the implementation, bioinformatics and analysis of next-generation sequencing assays, including strategies for handling repetitive regions and low-coverage regions (79, 80). Finally, because laboratories restrict analysis to variants most likely to impact the protein sequence, non-coding, intronic and synonymous variants which could impact the transcription of genes may be overlooked. Indeed, coupling genome and transcriptome assays (i.e., DNA and RNA analysis) has shown promise in increasing the clinical sensitivity of diagnostic testing (see section RNA-Sequencing and Methylation Pattern Analysis) (81).

Variant Classification

Beyond analytical considerations, a significant remaining challenge is the interpretation of the clinical significance of the variants identified. A variant is assessed to determine the likelihood that the it results in a non-functional or poorly functioning protein. This requires consideration of the inheritance of the variant, the literature detailing the genes functions, the expression profile in human tissues, the mechanism by which mutations are known to cause specific phenotypes (haploinsufficiency, dominant negative), frequency of the particular variant in the general population using databases such as gnomAD (82) and frequency of the variant in patients with clinical abnormalities using the general literature and databases such as ClinVar (83). These latter steps are straightforward in principle but difficult in practice, and variant interpretation has been the subject of a number of Guideline and Recommendation papers from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (79, 84, 85). Indeed, even among highly experienced laboratories, there is often disagreement between the classification of a variant as Pathogenic, Likely Pathogenic, Uncertain [Variant of Uncertain Significance (VUS)], Likely Benign or Benign. Further, high-throughput sequencing can reveal potentially pathogenic variants in genes with limited clinical validity, that is, genes with insufficient information regarding their association with a genetic syndrome or phenotype. As a result, such variants are also classified as VUS. Reporting of variants of uncertain significance can be difficult for clinicians to interpret, and this is an area of active work to develop methods to lessen the uncertainty in interpretation.

Incidental and Secondary Findings

The ability to look for alterations in a large percentage of human genes in one test has many obvious advantages over the tedious

TABLE 4 | Common disorders related to the *HBB* gene.

HBB disorder	Genotype	Phenotype	% HbA
β-thalassemia minor	$\beta\beta^0$, $\beta\beta^+$ (i.e., carriers)	Asymptomatic or mild microcytic hypochromic anemia may be present.	92–95%
β-thalassemia intermedia	$\beta^+\beta^+$, $\beta^+\beta^0$ (typically with alpha gene deletion)	Later onset, microcytic hypochromic anemia, jaundice, hepatosplenomegaly, risk of iron overload.	10–30%
β-thalassemia major	$\beta^0\beta^0$, $\beta^+\beta^+$, $\beta^+\beta^0$	Onset within 2 years of life, severe microcytic hypochromic anemia, hepatosplenomegaly, failure to thrive.	0%
Sickle cell disease	HbS/HbS [homozygous for c.20A>T (p.Glu7Val)]	Onset in infancy, severe anemia, splenomegaly, jaundice, episodes of severe pain including swelling of hands and feet, stroke in childhood	Low to absent

β, wildtype HBB locus; β^0, pathogenic variant resulting in no production of HBB protein (e.g., deletion); β^+, pathogenic variant resulting in reduced production of HBB protein (e.g., promoter variant).

process of examining genes one at a time. However, it is now well-understood that in addition to finding pathogenic variants that might explain a patient's clinical problems, there is the possibility of finding a wide variety of variants unrelated to this phenotype. Identification of medically actionable findings has become a critical part of genome wide tests such as ES. The medical genetics community has worked diligently to develop guidelines for providing medically actionable incidental findings to patients undergoing NGS based testing. The ACMG has adopted a new term, "secondary findings," to refer to pathogenic variants in a list of 59 genes that are deemed "medically actionable." Clinical laboratories are recommended to specifically look for pathogenic variants in these 59 genes (therefore not incidental), and report them if patients would like to have this information provided (86). Significantly, it is recommended that VUS's in these genes not be reported, as it is felt that these would cause more confusion than benefit (87). It is important to contrast secondary findings from incidental findings, which are pathogenic variants in genes unrelated to a patients' clinical indication and which are not curated as a secondary finding gene by the ACMG; reporting of these variants are laboratory-specific. For further discussion of how secondary findings impact pediatric care, see section Counseling and Ethical Considerations.

Clinical Examples

B-Globin: Sanger Sequencing

The beta-globin gene (*HBB*) encodes a major subunit of hemoglobin and pathogenic variants in beta-globin, are associated with multiple phenotypes depending on whether one or both copies are altered, and on the type of variant (**Table 4**) (88). Disorders of hemoglobin, or hemoglobinopathies, are the most common monogenic disease with a combined carrier rate of 5%. In hemoglobinopathies, there is inadequate hemoglobin due to a reduction in synthesis (i.e., thalassemias) or due to structural changes (e.g., Sickle cell disease) in the encoded protein (89, 90).

While there are more than 300 unique pathogenic variants in *HBB* (89), >90% of patients have one of 15 variants (90, 91). Loss-of-function variants, referred to as β^0 alleles, result in no protein production, while other variants such as missense or promoter variants result in reduced production of the HBB protein (β^+). Large deletions in the promoter region of *HBB* are observed in certain populations and result in loss of HBB expression. The *HBB* gene is relatively small, and is composed of 3 exons which can be amplified and sequenced with only two amplicons. Due to its small size and the specific phenotype associated with *HBB* diseases, analysis of *HBB* alone is appropriate and is almost universally performed with Sanger sequencing to detect sequence variants, and MLPA to detect deletions (89).

Cystic Fibrosis: Targeted Mutation Analysis to NGS Panels

Cystic fibrosis (CF) is an autosomal recessive multi-systemic condition affecting the epithelia of the respiratory tract, exocrine pancreas, intestine, hepatobiliary system, and exocrine sweat glands (92). Clinical features include progressive obstruction of the lungs with worsening pulmonary disease, pancreatic insufficiency and malnutrition, recurrent sinusitis ad bronchitis and male infertility. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*), which codes for a protein that functions as a chloride ion transporter. The 27 exon *CFTR* gene was identified as the CF disease gene by positional cloning based on linkage analysis in 1989, and was the first successful utilization of linkage analysis for disease gene identification (93).

Currently, over 1,000 distinct mutations have been identified, with a subset of relatively common mutations enriched in specific populations, and many rare mutations. There are several options for molecular diagnosis including (1) targeted genotyping analysis for common pathogenic variants, (2) Sanger sequencing of *CFTR* followed by deletion/duplication analysis if no sequence variants are found, or (3) sequencing of a multigene panel that includes *CFTR*, as well as other genes that may cause disorders on the differential diagnostic list for a patient with an uncertain diagnosis (92). Targeted analysis, which has been the first-line test for many years, must account for the individual's ethnicity, and provide a residual risk after a negative test [e.g., American College of Medical Genetics and Genomics recommended panel (94)]. On the other hand, sequencing of the entire gene with NGS, which has now become the most common technique for screening or diagnosis, can scan for all mutations in a single assay, reducing the need for reflex testing and improving the clinical sensitivity (92, 95, 96). When patients present with a broader differential, NGS panels including additional genes associated with primary ciliary dyskinesia, cholestasis, bronchiectasis, and/or congenital diarrhea, depending on the presenting features, are the most efficient route to a molecular diagnosis.

Noonan Spectrum: NGS Panel for a Distinctive but Genetically Heterogeneous Syndrome

Noonan spectrum is a group of disorders characterized by variable dysmorphic features, cardiac disease, short stature, developmental delay, and hematologic/oncologic disorders. Cardiac disease can manifest as structural heart disease, cardiac conduction defects, and cardiomyopathy. Patients have an ∼10 times increased risk for childhood cancers, especially juvenile myelomonocytic leukemia and embryonal rhabdomyosarcoma, but risk varies by gene (97). Noonan syndrome is caused by activating mutations in a number of RAS pathway genes (most commonly *PTPN11*, *SOS1*, *RAF1*), and together with other similar, so-called RASopathy syndromes, over 20 genes have been implicated in this group of disorders which include Noonan syndrome with multiple lentigines, Cardiofacio cutaneous syndrome (associated with *BRAF*, *MAP2K1*, and *MAP2K2*), Costello syndrome (associated with *HRAS*), among others (98). Most forms are autosomal dominant, with *PTPN11* accounting for over half of Noonan syndrome patients, and many genes are associated with multiple RASopathies (98, 99).

While some genotype-phenotype associations exist, there is considerable phenotypic and genotypic overlap between RASopathies which can complicate diagnosis. Therefore, NGS gene panels are the most cost- and time-efficient path to diagnosis, and identifies an underlying genetic cause in 80% of cases (98, 100). Furthermore, these syndrome have important difference in terms of co-morbidities, and a genetic diagnosis aids in optimizing patient care (98, 99). For example, patients with Costello syndrome have a 40 times increased risk of malignancy, and require additional screening compared to other RASopathies (see section Genotype-First Approach for further discussion) (97, 101).

Hearing Loss: A Highly Heterogeneous Disorder Requiring Large Panel Testing

Hearing loss occurs in nearly 1/500 individuals and can have congenital, childhood, or adulthood onset (102–104). Greater than 50% of early onset hearing loss is caused by a single gene defect with the remainder due to other factors such as infection (103–106). Hearing loss can be isolated (non-syndromic) or associated with additional phenotypic features (e.g., Usher syndrome causing hearing loss, blindness and vestibular dysfunction and Jervell and Lange-Nielsen syndrome causing hearing loss and long QT syndrome). Hearing loss genetics is complicated by hundreds of associated genes that have autosomal recessive, autosomal dominant, X-linked and mitochondrial patterns of inheritance (107). Expert panel review of hearing loss genes reported in the literature has shown strong clinical validity for 142 genes, including 105 causing non-syndromic and 59 causing syndromic hearing loss (108). Thus, diagnostic testing requires comprehensive gene panels or ES to capture the diversity of genetic drivers giving rise to this phenotype (**Figure 1C**).

Despite this genetic heterogeneity, the majority of non-syndromic hearing loss is caused by two genes: gap junction protein β2 (*GJB2*) and stereocilin (*STRC*). Mutations in *GJB2* is the most frequent cause of severe-to-profound autosomal recessive non-syndromic hearing loss, occurring in up to 50% of these cases (109). Founder mutations c.35delG (European), c.167delT (Ashkenazi Jewish), and c.235delC (East Asian) have higher minor allele frequencies in their respective populations (1.6–0.6%), however the majority of pathogenic variants in *GJB2* are extremely rare and up to 70% of all *de novo* mutations in this gene are predicted to be pathogenic (110). Additionally, large deletions upstream of *GJB2* which includes the neighboring *GJB6* gene and impair expression of *GJB2* are prevalent in European populations (111). Thus, comprehensive testing for this gene requires gene sequencing (Sanger or NGS) and the ability to detect large CNVs upstream of the gene (e.g., SNP array). Mutations in *STRC* is the most common cause of mild-to-moderate autosomal recessive, non-syndromic hearing loss, causing up to 20% of these cases (112). *STRC* testing is complicated by the presence of a nearby pseudogene which is non-functional but contains nearly identical sequence information. To overcome this challenge, the *STRC* locus needs to be PCR amplified before sequencing analysis can be performed (113). Thus, a comprehensive diagnostic testing strategy is required for *GJB2*, *STRC*, and the >140 additional genes causing this phenotype (112, 114, 115). Using a comprehensive strategy that incorporates multiple laboratory methods ultimately yields a genetic diagnosis in 33–48% of hearing loss cases (112, 114, 115).

DISORDERS REQUIRING SPECIAL TESTING

Repeat-Expansion Disease

Some disorders have long been known not to follow the rules of Mendelian inheritance, including Fragile X syndrome (FXS) and Spinal and bulbar muscular atrophy. The puzzle of their inheritance was solved in 1991, when cloning of the disease genes showed that these disorders were linked to repeat expansions in the corresponding genes (74, 75). Short tandem repeats (STRs), also known as microsatellites or simple sequence repeats, are tandemly repeated nucleotide motifs scattered throughout the human genome. Repeat expansion within STRs can happen due to genetic instability, and in a small subset of cases, can directly lead to human diseases, known as repeat-expansion disorders (**Table 5**).

The repeat motifs vary in length, including trinucleotide repeats, such as CAG in Huntington's disease (HD), tetramer, pentamer, hexamer, and even dodeca-nucleotide repeats. The repeats can be located in the 5′ untranslated region (FXS), exon (HD), intron (Friedreich ataxia), or 3′ untranslated region (Myotonic dystrophy 1) of a gene (116–119). The length of pathogenic repeats vary between disorders, and are associated with multiple well-known mechanisms of pathogenicity, including loss of function (e.g., by hypermethylation of promoter, transcription interference or disruption of splicing); protein gain of function due to a polyglutamine/polyalanine tract expansion; and RNA gain of function to sequester the critical RNA splicing factors (119, 120). Despite the diversity of repeats and molecular mechanisms, the length of repeat expansion is usually positively correlated to the disease severity, but negatively

TABLE 5 | Examples of Repeat-expansion disorders.

Disease	Inheritance	Gene	Type	Repeat motif	Normal range	Disease range
Huntington disease	AD	*HTT*	Coding exon	CAG	≤26	>40
Spinal and bulbar muscular atrophy	X-linked	*AR*	Coding exon	CAG	≤34	≥38
Spinocerebellar ataxia 1	AD	*ATXN1*	Coding exon	CAG	6–35	≥39
Spinocerebellar ataxia 2	AD	*ATXN2*	Coding exon	CAG	≤31	≥33
Spinocerebellar ataxia 3	AD	*ATXN3*	Coding exon	CAG	12-44	∼60–87
Spinocerebellar ataxia 6	AD	*CACNA1A*	Coding exon	CAG	≤18	20–33
Spinocerebellar ataxia 7	AD	*ATXN7*	Coding exon	CAG	4–35	37–460
Spinocerebellar ataxia 17	AD	*TBP*	Coding exon	CAG	25–40	≥49
Dentatorubral-pallidoluysian atrophy	AD	*ATN1*	Coding exon	CAG	6-35	48–93
Huntington disease-like 2	AD	*JPH3*	3′UTR, coding exon	CTG	6–28	40–60
Fragile X syndrome	X-linked	*FMR1*	5′UTR	CGG	6–54	200–1,000+
Fragile X-associated tremor/ataxia syndrome	X-linked	*FMR1*	5′UTR	CGG	6–54	55-200
Myotonic dystrophy 1	AD	*DMPK*	3′UTR	CTG	5–34	50–10,000
Myotonic dystrophy 2	AD	*CNBP*	Intron	CCTG	11–26	75–11,000
Friedreich ataxia	AR	*FXN*	Intron	GAA	5–33	66–1,300
Frontotemporal dementia and/or lateral sclerosis 1	AD	*C9orf72*	Intron	GGGGCC	<25	>60
Unverricht-Lundborg disease	AR	*CSTB*	Promoter	CCCCGCCCCGCG	2–3	≥30
Oculopharyngeal muscular dystrophy	AD	*PABPN1*	Coding exon	GCG	≤10	12–17

correlated to the age of onset (121–125). One striking feature of these diseases is clinical anticipation, in which the disease tends to worsen in successive generations of a family as the repeat expansion extends. Indeed in repeat-expansion disorders, milder, or even distinct symptoms may be seen in individuals with so-called "permutation" alleles, that is, expanded alleles that are shorter than "full" mutations, but longer than normal alleles. Therefore, pediatric onset is usually associated with longer repeat expansions.

Molecular Genetic Testing

Diagnostic testing determines the number of repeats within the locus of interest. Additionally, in some cases such as FXS, methylation secondary to the aberrant repeat expansion is clinically important and must therefore be detected, for example with methylation-sensitive MLPA (see below). While traditional PCR and Southern blot analysis were once the standard diagnostic tests, they have now been replaced by repeat-primed PCR which is more efficient at identification of expanded repeats and at determination of the precise number of repeats. Of note, most disease-causing expansion alleles are longer than the read length of current NGS technologies, and repetitive sequences prevent the assembly of multiple reads into a consensus contig (126). As such, current NGS technologies are unable to accurately detect or size most tandem repeats; however, long-read sequencing is emerging to solve this problem (see section Long-Read Sequencing).

Repeat-primed PCR (RP PCR)

RP PCR uses three primers in a single PCR reaction, with two primers anchored outside the repeat to amplify the whole region, similar to a standard PCR amplification strategy. The third primer is a chimeric primer that anneals along the repetitive region to form multiple amplicons, which results in PCR products that differ in length by one repeat motif. Therefore, RP PCR increases the amount of full-length product for large repeat alleles, and allows the accurate detection of repeat numbers (127–129). However, there is a maximum number of repeats that can be quantified with RP PCR, and alleles greater than this can be detected but not quantified (e.g., 200 repeats for FXS).

Clinical Example

FXS, FXTAS, and FXPOF

FXS and related syndromes, Fragile X Tremor Ataxia Syndrome (FXTAS) and Fragile X Premature Ovarian Failure (FXPOF), are X-linked disorders caused by expansion of a CGG triplet in the 5′ untranslated region (the promoter) of the *FMR1* gene on the X chromosome. The *FMR1* gene encodes the FMRP protein, which is important for mRNA transport, translational regulation and synaptic plasticity (130). Fragile X is characterized by moderate intellectual disability, characteristic physical features (long face, large ears, prominent jaw and forehead, and enlarged testes in males after puberty) and behavioral abnormalities such as autism spectrum disorder, while FXTAS and FXPOF manifest later in life with progressive cerebellar ataxia and intention tremor, and ovarian insufficiency, respectively (131, 132).

Healthy individuals possess 5-44 CGG repeats (normal alleles) or 45–54 repeats (intermediate alleles) within the *FMR1* gene. Neither normal nor intermediate alleles cause disease, but a small percentage of intermediate alleles are unstable and may expand into the premutation range (55–200 repeats), which will lead to FXTAS (130, 131, 133). Both male and female carriers of a premutation can be affected by FXTAS with higher penetrance in males, while female have a 20% risk of FXPOF (127, 134). The premutation enhances *FMR1* transcription by shifting the transcription start site, and increases sequestration

of RNA splicing proteins within the nucleus resulting in RNA-mediated toxicity (135, 136). A definite diagnosis of FXTAS requires the identification of a *FMR1* premutation as well as the neuroradiologic and clinical findings (132).

Premutation alleles can further expand to full-mutation alleles (>200 to over 1,000 repeats) which are often associated with aberrant hyper-methylation of the *FMR1* promoter and inhibition of *FMR1* transcription (137). Since it is the absence of FMR1 protein that leads to FXS, full-mutation alleles without hyper-methylation may not result in transcriptional silencing nor FXS (138). Female heterozygotes for the full-mutation may manifest with milder phenotypes of FXS. Less than 1% of FXS can be caused by *FMR1* deletion or single-nucleotide variants, which requires additional testing modalities such as those discussed in the previous sections (132, 139).

Mitochondrial Diseases

Mitochondrial diseases are a rare group of disorders resulting from dysfunction of the mitochondria and are highly heterogeneous, both genetically and phenotypically. Mitochondria, the energy producing cellular organelles, are essential organelles present in almost all eukaryotic cells, and are involved in multiple cellular processes including calcium homeostasis, apoptosis, and most importantly oxidative phosphorylation for ATP production (140–143). Mitochondria are so important that mitochondrial defects often impact multiple organ systems, and the disorders can manifest in the neonatal phase, childhood or adulthood. Clinical variability is very common and the phenotypes do not always fit into a discrete syndrome. Therefore, diagnosis often relies on genetic testing (144).

The dual genetic control of mitochondrial function further complicates the mechanisms of mitochondrial diseases, as the 37 genes in the mitochondrial genome (mtDNA) and over 1,500 nuclear genes control mitochondrial function together (145–147). Consequently, mitochondrial diseases can have autosomal or X-linked inheritance for nuclear gene mutations, or maternal inheritance for mtDNA mutations. Furthermore, there are many mitochondria present in each cell and the clinical presentation of mitochondrial diseases is, in part, a function of the frequency of the mutant mtDNA sequence in each cell (i.e., heteroplasmy). Disease manifestation generally requires heteroplasmy levels above a certain threshold, and increasing levels of heteroplasmy are often associated with increasing clinical severity (144, 148).

Molecular Genetic Testing

Testing for mitochondrial disease is complicated not only by the need to assay both nuclear and mitochondrial genomes, but also because there are hundreds of genes implicated with mitochondrial disease, and for mtDNA mutations, tissue-specific heteroplasmy complicates interpretation.

Historically, targeted mutation analysis for common, known pathogenic variants was performed by one of several hybridization-based techniques which were limited in the scale and scope of testing. Sanger sequencing of the entire mitochondrial genome allowed for an unbiased assessment of the mtDNA, but required ancillary testing to assess heteroplasmy of an identified variant. Large deletions were typically identified through Southern blot analysis of DNA extracted from muscle samples, but sensitivity of lower heteroplasmy levels is limited. The emergence of NGS has enabled simultaneous interrogation of both point mutations and deletions in nuclear genes, and in mitochondrial genes when combined with long range PCR amplification of mtDNA (**Figure 1D**). The additional advantage of NGS is the quantitative nature of the sequencing, providing an estimate of heteroplasmy for a given variant.

Leigh syndrome

Leigh syndrome, the most common mitochondrial disease in childhood, is a progressive neurodegenerative disorder with typical onset before 2 years of age. Patients show brainstem and/or basal ganglia dysfunction, elevated serum or cerebrospinal fluid lactate, and neurological features including developmental delay and regression, hypotonia, spasticity and peripheral neuropathy (149). Episodic progression of the disease typically results in death before 3 years of age due to respiratory or cardiac failure. Over 75 mitochondrial or nuclear genes are linked to LS, and almost one-third of these genes are related to complex I function. Mutations in mitochondrial genes contribute to ∼20% of LS, with the mtDNA mutations m.8993T>G and m.8993T>C in *MT-ATP6* underlying ∼10%. Most of the mutations in mitochondrial genes require more than 90% heteroplasmy to cause the LS symptoms. Rarely, LS can also result from a large-scale mtDNA deletion (149). Currently, the molecular diagnosis of LS is a two-pronged NGS approach with large panels or ES for nuclear genes and long-range PCR amplification of mtDNA followed by NGS (**Figure 1D**).

Mitochondrial DNA deletion syndromes

Large mitochondrial DNA deletions cause a spectrum of diseases including three major groups according the distribution of disease manifestation (150). Kearns-Sayre syndrome (KSS) with mtDNA deletion present in all tissues, is a progressive multisystem disorder with onset before the age of 20. KSS patients have progressive external ophthalmoplegia and pigmentary retinopathy, as well as cardiac conduction defects or cerebellar ataxia. Pearson syndrome with mtDNA deletions that are abundant in blood leukocytes, is characterized by sideroblastic anemia. Progressive external ophthalmoplegia (PEO) with mtDNA deletion confined to skeletal muscle, can manifest with ptosis, impaired eye movements, oropharyngeal weakness and variably severe proximal limb weakness. Almost all mitochondrial DNA deletion syndromes occur *de novo*. Approximately 90% of KSS cases arise from a *de novo* large scale 1.1–10 Kb deletion of mtDNA that includes ∼12 genes. The most common deletion known as the "common 4,977 bp deletion" accounts for over one third of total cases. NGS on the products of long-range PCR targeting mtDNA can reveal one or more mtDNA deletions with the exact breakpoints, and also estimate the heteroplasmy of the deletion.

Imprinting Disorders

A small fraction of genes are marked during gametogenesis as being transmitted through the maternal or paternal gamete,

and these genes are only expressed if transmitted either maternally or paternally. In other words, they are subject to monoallelic, parent-of origin specific expression (151). Imprinting is established during gamete formation and many imprinted genes are important for embryonic development and early postnatal growth, and therefore, imprinting disorders tend to share common features including aberrant fetal or postnatal growth, abnormalities in glucose regulation, abnormal neurologic behaviors or mental deficits and abnormal gonadal maturation. At least 150 human imprinted genes were identified by 2015, and most exist in clusters (152, 153). The imprinted expression pattern of these gene clusters are epigenetically controlled by methylation at *cis*-acting discrete elements known as imprinting control regions (154).

Perturbed expression of imprinted genes leads to congenital disorders of imprinting, and there are four major categories of mutations that will cause disease. These four categories are (1) chromosomal rearrangements such as deletions, which can result in lack of expression of the corresponding imprinted genes; (2) uniparental disomy (UPD) in which both copies of a chromosome, or chromosomal segment, are inherited from one parent; (3) imprinting defects, which are caused by aberrant methylation of the imprinting control regions or small deletions involving the imprinting center; and (4) intragenic mutations in imprinted genes that disrupt the normal expression or function of those genes. Intragenic mutations are rare overall, but relatively common when imprinting disorders are inherited (155).

Molecular Genetic Testing

Multiple techniques have been developed to interrogate one or multiple disease mechanisms, but methylation-specific PCR (MS-PCR) and methylation-specific MLPA (MS-MLPA) are the gold-standard techniques for imprinting disorders due to their ability to identify abnormal DNA methylation patterns irrespective of the underlying disease mechanism (**Figure 1E**). However, the underlying mechanism is important for genetic counseling issues such as recurrence risk, and additional techniques are needed delineate it. FISH, CMA, or MLPA can be performed to detect a deletion; SNP arrays or STR analysis can be used to evaluate UPD; MLPA can be used to detect deletions of imprinting control regions; while NGS or Sanger sequencing can detect intragenic mutations in the imprinted genes. Depending on the disease, each mechanisms accounts for varying proportions of patients, and the order of reflex testing is adjusted accordingly.

MS-PCR

The critical component of MS-PCR is bisulfite treatment of DNA prior to amplification. Sodium bisulfite treatment of DNA converts all unmethylated cytosines to uracil, which can reveal the methylation differences between paternal and maternal alleles. Two pairs of primers are designed based on the converted maternal and paternal sequences, respectively. The PCR amplification products from maternal and paternal DNA can be separated in electrophoresis because of their different sizes (156). Therefore, MS-PCR can detect loss of maternal or paternal alleles caused by deletion, UPD or imprinting defect, but additional testing is required to reveal the underlying mechanism of the imprinting defect.

MS-MLPA

MS-MLPA uses methylation-sensitive endonucleases which digest unmethylated DNA and prevent probe ligation and amplification. Therefore, only methylated alleles are visualized and parent-of-origin can be determined based on test design. MS-MLPA is typically performed in parallel with regular MLPA to detect any underlying CNVs in the region of interest. Therefore, the combination of MS-MLPA and standard MLPA can identify the loss of a maternal or paternal allele and an underlying deletion or ID, but additional testing is required to detect UPD or sequence variants (157).

Clinical Examples

Prader-Willi syndrome (PWS) and Angelman syndrome (AS)

PWS and AS result from imprinting abnormalities at 15q11.2-q13 and illustrate how disruption of imprinting patterns on either the maternal or paternal allele result in distinct diseases. The imprinting control regions within 15q11.2-q13 is normally unmethylated on the paternal allele, and methylated on the maternal allele. Therefore, in MS-PCR and MS-MLPA, loss of the maternal PCR product suggests AS, while loss of paternal PCR product indicates PWS. Overall MS-PCR and MS-MLPA can diagnose ∼99% of PWS and ∼80% of AS, and 11% of AS patients are diagnosed by sequencing of *UBE3A* (158, 159).

PWS is clinically characterized by severe hypotonia and feeding difficulty in early infancy, obesity caused by hyperphagia in later infancy or early childhood, developmental delay, intellectual disability, distinctive behavioral abnormalities, hypogonadism, and characteristic facial features (159, 160). PWS is caused by genomic or epigenetic changes that result in loss of expression of at least 13 paternally expressed genes on chromosome 15q11.2-q13, and deficiency of *SNORD116* is thought to be responsible for the key characteristics of PWS (160). There are three major mechanisms leading to PWS. The most common, accounting for 65–75% of patients, is a paternal deletion of 15q11.2-q13, most commonly one of two recurrent deletions (type I: ∼6 Mb and type II: ∼5.3 Mb). Maternal UPD of chromosome 15 is identified in 20–30% of PWS patients, in which ICRs of both alleles are methylated so that normally paternally expressed genes are silenced on both alleles. An imprinting defect accounts for 2.5% of PWS patients (159).

AS is characterized by severe developmental delay or intellectual disability, severe speech impairment, movement, or balance problems, and a unique behavioral phenotype with a happy (although sometimes inappropriate) demeanor (158, 161–163). Clinical manifestations are usually apparent after 1 year of age. In contrast to PWS, AS is caused by the genomic or epigenetic changes that lead to loss of expression of the maternally expressed genes within the chromosome 15q11.2q13 imprinted region. The fundamental features of AS are believed to result from deficiency of *UBE3A* expression in the neurons, where the paternally expressed *SNURF-SNRPN* transcript serves as an antisense RNA for the *UBE3A* gene (158, 162, 163). AS can be caused by deletion of the 15q11.2-q13 critical

region on the maternal allele (65–75%), paternal UPD (3–7%), imprinting defect (∼3%), and maternal *UBE3A* pathogenic variants (11%).

IMPACT ON CLINICAL CARE

The Importance of Genetic Testing

The evolution of genetic testing has had a tremendous impact on the clinical care of patients. As our diagnostic capabilities continue to expand, our ability to provide a diagnosis for our patients is rapidly increasing. For many families, simply having an answer for the cause of their child's medical issues can be very powerful. At times, answering the "why" is a family's primary motivation for pursing genetic testing. Having a name for the condition allows them to find a peer group with parents of similarly affected children with whom they find shared experiences. Additionally, many families unnecessarily harbor a great deal of personal guilt about their child's condition, having the belief that they somehow caused their child's medical issues. Being able to provide a concrete genetic answer can alleviate that guilt and allows the family some closure.

A specific genetic diagnosis provides a general prognostication for a patient's future. This can be particularly impactful for infants and small children who may have yet to manifest some of the comorbidities of the condition. It allows for realistic expectations to be set with the parents and allows for directed care with condition-specific recommendations. The intention is to not set up a self-fulfilling prophecy, but rather ensure that all of the necessary support is in place to allow the individual to achieve to their maximum potential.

Providing a genetic diagnosis can also significantly impact family planning. Many couples are hesitant to expand their family without knowing the underlying cause of their child's condition. Once the genetic etiology is known, that information can be used to make an educated decision about if and how they decide to have more children. If they so choose, a specific gene mutation can be used for pregnancy screening and/or *in vitro* fertilization.

Genotype-First Approach

As genetic testing has become more readily available and expansive, the practice of clinical genetics has advanced as well. Recently, there has been a trend toward the early genotyping of patients. This has multiple benefits in regards to patient care. For conditions that are highly heterogenous, NGS panels and ES allow for the simultaneous analysis of hundreds or thousands of genes, resulting in higher diagnostic rate in those groups of disorders. Epilepsy is one such example in which there is an ever-expanding list of genes that are known to be causative in both syndrome and non-syndromic phenotypes. Helbig et al. (164) showed that ES was not only an efficient means to the identification of known disease-causing genes in patients with epilepsy, but also allowed for the identification of novel candidate genes. In their cohort, the diagnostic rate varied by phenotype, with epileptic encephalopathy patients having the highest yield.

In the recent past, CMA have been recommended as the first line test for individuals with neurodevelopmental disorders with a diagnostic yield of 15–20% (19, 165, 166). In a recent meta-analysis by Srivastava et al. (76) a review of 30 articles with data on the yield of ES in neurodevelopmental disorders showed that the test was diagnostic in 36% percent overall, with 31% in isolated neurodevelopmental disorders and 53% in neurodevelopmental disorders with additional features. Based on that data, given the higher diagnostic rate in ES when compared to CMA, they proposed that ES be considered as a first line test in that group of disorders. With time, it is anticipated that ES will become a much more widely used first line tool for the evaluation of many varied phenotypes.

Utilizing a genotype-first approach allows for the avoidance of unnecessary evaluations and diagnostic studies, and permits condition-specific care. For example, sensorineural hearing loss is a highly heterogeneous condition (see section Hearing Loss: A Highly Heterogeneous Disorder Requiring Large Panel Testing) in which ∼30% of individuals have a syndromic form of hearing loss that have a variety of known comorbidities (167). Due to the variability in presentation and age of onset, it has been proposed that individuals with sensorineural hearing loss should undergo screening evaluations for comorbidities in order to guide genetic testing (168). Recommended evaluations include an ophthalmology evaluation to assess for vision differences seen in Usher syndrome, renal ultrasound to look for structural differences seen in Branchio-oto-renal syndrome, cardiac evaluation to evaluate for cardiac conduction defects associated with Jervell and Lange-Nielson syndrome, and temporal bone MRI to assess for the inner ear anomalies seen in Pendred syndrome. However, as the understanding of the genetic etiology of sensorineural hearing loss improves and as testing becomes more available, many of these evaluations become unnecessary if the genotype is able to be established soon after the hearing loss is detected via genetic testing (104).

At times, evaluation of a suspected genetic diagnosis may require painful or invasive procedures for confirmation of a suspected diagnosis. Osteogenesis imperfecta is a group of disorders characterized by bone fragility due to defects in type 1 collagen resulting in fracturing with minimal trauma. Prior to the expansion of our knowledge of the underlying genetic etiologies for the condition, the diagnosis often relied on clinical criteria and biochemical analysis via skin fibroblasts (169, 170). Now, however, biochemical analysis is much more rarely utilized as we rely on molecular studies for diagnosis. Likewise, neuromuscular conditions are a highly heterogeneous group of disorders that include neuropathies, myopathies, motor neuron disease, and neuromuscular junction disorders. Given the expansive differential for these conditions, clinicians have traditionally utilized such procedures as muscle biopsies and electromyography (EMG) as a diagnostic means in order to narrow their differential prior to proceeding with targeted testing (171, 172). As large scale genomic testing becomes available, however, it is predicted that those painful procedures may be able to be avoided as first line evaluations.

Perhaps most importantly, establishing a genetic diagnosis allows for condition-directed care. Identifying an underlying genetic etiology for an individual allows for appropriate specialty referrals to assess for associated comorbidities. For some conditions, a particular diagnosis may necessitate dietary

or medication changes, alter screening recommendations, or impact an individual's ability to qualify or benefit from a transplant procedure.

For example, in Noonan spectrum disorders (see section Noonan Spectrum: NGS Panel for a Distinctive But Genetically Heterogeneous Syndrome), the type of cardiac risk varies depending on the underlying genetic etiology (101, 173), with pulmonary stenosis being more common in *PTPN11* disease and hypertrophic cardiomyopathy present in 95% of individuals with *RAF1* mutation. Additionally, individuals with mutations in *PTPN11*, particularly those with germline pathogenic variants in codons 61, 71, 72, and 76, are at risk for juvenile myelomonocytic leukemia (JMML), and patients with HRAS mutations have a significantly increased risk of malignancy even compared to other RASopathies (101, 174). Therefore, knowing the genotype of the patient ensures that proper screenings are in place. Given the phenotypic variability of Noonan spectrum disorder, and the importance of genotype identification, targeted panel testing is recommended.

Counseling and Ethical Considerations

Along with genetic testing comes a myriad of ethical considerations. Vital to the practice of clinical genetics is maintaining an individual's autonomy, particularly in regards to the use of genetic testing. Prior to proceeding with any testing, it is important that informed consent be obtained, ideally by an individual with a background in clinical genetics. As part of the informed consent process, pretest counseling should include a discussion about potential test results or expected outcomes, possible benefits and limitations of testing, and the possibility of incidental or secondary findings (175).

Secondary findings are a particularly important and challenging point of discussion as individuals are typically asymptomatic in that regard. In the context of ES and GS, currently, the American College of Medical Genetics recommends that an individual should be offered analysis of a minimum of 59 genes that are considered medically actionable in that they would convey a change in medical management if identified (see also section Variant Classification) (86). Included in that list are cancer predisposition genes such as *BRCA1* and *BRCA2* as well as genes that are associated with risk of cardiomyopathy. Given the impact that these findings have on an individual's care, it is important that this testing be thoroughly discussed prior to proceeding.

As part of the informed consent process for genomic testing, it is important for individuals to understand the implications that genetic test results could potentially have on employment and insurance eligibility. In 2008, the Genetic Information Non-discrimination Act (GINA) was passed into law (176). GINA prohibits health insurers form using genetic information to discriminate against individuals in regards to their eligibility and coverage. It also prevents employers from using that information to make employment decisions including hiring, pay, and firing. However, there are caveats to the policy and some people are not protected by this law including, individuals who work for employers with fewer than 15 employees, individuals with Federal or military insurance, and people with additional insurance policies including long-term and life insurance. It is important that these protections and limitations are discussed and understood prior to proceeding with testing. This is particularly important when discussing pre-symptomatic testing.

A particularly challenging ethical area is genetic testing in children. Genetic testing for children who are symptomatic of a genetic condition is typically considered acceptable with appropriate pre and post-test counseling (177). When applicable, it is important to involve the child or adolescent in the discussion and obtained their assent prior to proceeding with testing (178). However, broad carrier screening and testing for adult onset conditions are not recommended in the pediatric population as to maintain autonomy for that individual to make that decision for themselves in adulthood (177, 179).

As large-scale genomic testing becomes more commonplace, we will undoubtedly continue to encounter an ever-expanding list of ethical concerns that will need to be discussed. It will be important to readdress these issues as the field evolves.

EMERGING TECHNOLOGIES

The diversity of genetic diagnostic testing methods reflects the diversity of genomic alterations that cause Mendelian disorders. As NGS-based assays have transitioned from being used primarily in research settings to becoming established clinical assays, it remains to be seen whether newer techniques can become standard in clinical practice. The technologies listed below represented techniques recently adopted or on the cusp of adoption in clinical laboratories. One promising techniques which continues to be evaluated is optical mapping which is a non-sequencing genome assembly assay. Optical mapping is promising for the resolution of structural variants (180), repeat expansion (181), and may dramatically impact cancer cytogenetics by offering one assay to replace karyotype, FISH and CMA (182). Indeed, the trajectory of pediatric genetic testing appears focused on streamlining approaches to limit the number of tests required to identify any disease causing mutation to save both time and reduce costs. Whether a "one test fits all" approach is practical or even desirable has yet to be clearly demonstrated from a technical, patient care, or cost-benefit perspective. Nonetheless, leading the way for a single, comprehensive genomic diagnostic test for rare disorders is clinical GS.

Clinical Genome Sequencing

In the next 5–10 years, GS is poised to supplant ES as the most comprehensive clinical genomic diagnostic test. Technical advantages of GS over ES include (1) more even coverage across exons, (2) coverage of non-coding regions of the genome (e.g., intergenic, promoter, UTR, and intron), (3) comparable CNV detection to CMA (including mapping breakpoints), (4) the ability to detect structural rearrangements such as balanced translocations and inversions, and (5) faster and cheaper library preparation (183–186). The major limitations of GS compared to ES is the bottleneck created after libraries are made since (1) fewer patient samples can be sequenced per run and (2) the bioinformatic processing of the increased amount of data is more

computationally intensive and requires increased storage space. The additional work and storage needs contribute to GS being at least twice as expensive to perform (187). Despite the current logistical challenges of implementing GS for all genetics patients, numerous studies have shown that GS can be done rapidly in the setting of acutely ill, neonatal and pediatric intensive care unit patients (72, 73, 188–191). These studies performed in a research and clinical laboratory setting has provided diagnoses primarily for patients with multiple congenital anomalies and neurological phenotypes (75, 189). Guidelines for a "rapid genome" are somewhat ambiguous as some studies show a turnaround time of 28 days (189) while others have results closer to 24 h (73). Diagnostic yield also varies between studies and suffers from case-selection bias; nonetheless, most studies found diagnoses in 28–43% of patients (72, 73, 189). These successful efforts show GS is gaining significant traction for immediate testing of critically ill children with suspected Mendelian disorders and we anticipate that more studies will demonstrate the efficacy of this approach in the clinical laboratory.

The wider implementation of GS for non-critically ill patients is likely to evolve more slowly as early adopters of this technology will need to demonstrate its increased diagnostic utility compared to ES and/or evidence that advancements in sequencing and computational technology has made it cost effective. Meta-analysis of the median diagnostic yield from 37 studies, comprising 20,068 children found a diagnostic rate of 41% (95% CI 34–48) for GS and 36% (95% CI 33–40) for ES, which are both significantly better than CMA (diagnostic yield of 10%, 95% CI 8–12), but not significantly different from each other (75). Comparable diagnostic yield between GS and ES was further replicated in two additional studies not included in this meta-analysis (192, 193). The inability of GS to show a marked increase in diagnoses is likely due to restricting variant analysis to coding regions. Analyzing non-coding variants is one possible way to gain new diagnoses that would otherwise be missed by ES, however analysis of these variants poses multiple challenges. For instance, a proband with a *de novo*, deep-intronic or promoter variant that has not been previously reported as disease causing in a gene of interest, while interesting, is unlikely to be diagnostic since the consequence of this mutation needs functional assessment of the impacted transcript (84).

Studies on the genetics of autism and related neurodevelopmental disorders could push GS into new territory with recent work suggesting an excess of *de novo* mutations in enhancer elements of neurodevelopmental genes in affected individuals (194–198) and may account for 1.0–2.8% of negative exomes (197). However, interpreting the additive role of *de novo* and common population variants across multiple genes and non-coding elements poses significant analytical challenges. Although oligogenic inheritance models have been hypothesized for autism and other disorders (199–201), this is not uniformly supported (202) and the data does not meet standards for clinical validity for diagnostics at this time. Lastly, while it is enticing to think that genome sequencing can act as a catch-all diagnostic test, certain mutation types such as repeat expansions and regions of high homology (e.g., segmental duplications and pseudogenes), remain very difficult to assay by short-read sequencing (190). Altogether, clinical GS is emerging as a frontline diagnostic test but is likely a few years away from usurping ES as the premiere testing method for rare disorders.

Long-Read Sequencing

Most next-generation sequencing protocols used in clinical laboratories start with the controlled fragmentation of the human genome to create 200–500 bp DNA fragments. Following library preparation these fragments are sequenced with short-read lengths of 100–250 bp by Illumina-based platforms. Although short-read lengths enable highly accurate identification of SNVs and small indels, this approach has difficulty with other types of genetic variants like nucleotide repeat expansions, distinguishing regions of high homology, structural variants (including copy neutral) and phasing pathogenic alleles. To overcome these challenges, long-read sequencing technologies have been developed to create read lengths of >10,000 bp (203, 204). Having long reads allows for sequencing across challenging genomic regions and may even help unmask novel damaging variants and discover new disease genes (205, 206). Two emerging technologies leading the way in this field include single molecule real-time (SMRT) sequencing (Pacific Biosciences) and measuring electric currents as nucleic acids pass through a protein nanopore (Oxford Nanopore Technologies) (203, 204). Clinical laboratories have been reluctant to adopt these technologies in part because they initially had high costs, high error rates for base pair calling (PacBio ∼15%, Nanopore 5–25%), and unestablished clinical validity (207–209). However, newer versions of these sequencing platforms have seen improvements in cost and performance such that a case can now be made for laboratories to explore their utility for diagnostic testing. In a research setting this technology can identify clinically relevant structural variants (210–213), sequence entire nucleotide repeat expansions found in genes like *HTT*, *FMR1*, and *ATXN10* (214–216), bypass pseudogenes affecting *IKBKG* and *CYP2D6* (217, 218), and phase disease alleles (219). Although the studies outlined above provide evidence that challenging genomic regions can be reliably analyzed with long-read technology, an initial long-range PCR to enrich for the target region was often performed limiting the scalability and clinical utility of these methods. A potential future clinical application built upon continued improvement in technology and sequencing throughput would be performing long-range sequencing across the whole genome and doing *de novo* assembly which would yield fully phased patient specific genomes generated independent of alignment to a reference sequence (220, 221). *De novo* assembly of long-read patient genomes represents the future of this emerging technology allowing detection of all normal and pathogenic genomic variation.

RNA-Sequencing and Methylation Pattern Analysis

Sequencing of messenger RNA (mRNA) can provide unique information compared to DNA sequencing as it (1) allows interrogation of the results of splicing, (2) detects allele specific expression, and (3) allows examination of mRNA abundance, in addition to identifying germline variants found in coding regions

of expressed transcripts (222–225). Thus, RNA-seq can be seen as a hybrid test that assesses abnormal expression and splicing and capable of detecting pathogenic coding variants. Improving methods to detect abnormal splice events is important to note since it is estimated that nearly 10% of all disease causing variants disrupt splicing (226) and variants impacting canonical splice sites at intron-exon boundaries are categorically defined as null variants by ACMG guidelines (84, 227). Detection of abnormal splicing by RNA-seq could therefore emerge as a critical reflexive diagnostic test for negative exomes and genomes (222).

An ongoing challenge associated with implementing clinical RNA-seq is deciding which patient tissue to assay, since tissue restricted transcript expression and alternative splicing limit the total number of genes that can be reliably tested for abnormalities. Fortunately, databases such as the Genotype-Tissue Expression (GTEx) project, which provides the expression level and transcriptional diversity of 53 non-diseased tissues from nearly 1,000 adults (228), can be used as a reference for the expected transcriptional state of biopsied tissue. Tissues from patients that are accessible for RNA-seq testing include fibroblasts derived from skin biopsy (229), muscle biopsy (222, 223), whole blood (224), and B lymphoblastoid cell lines made from Epstein-Barr virus transduced whole blood. RNA-seq has so far been performed on muscle biopsies and fibroblasts for a restricted set of neuromuscular and mitochondrial diseases, respectively (222, 223, 229). RNA-seq on a cohort of 25 neuromuscular disorder patients with negative exome and/or panel testing had a diagnostic rate of 36% (222) and in 48 mitochondriopathy patients a diagnosis was reached in 10% of cases (229). RNA-seq testing of whole blood in 94 patients with a broad set of phenotypes resulted in a diagnostic rate of 7.5% (224). These studies detected multiple pathogenic mRNA mechanisms causing premature termination, including exon skipping, activation of cryptic donor and acceptor splice sites, and incorporation of *de novo* exons. From these studies, RNA-seq appears to have the greatest benefit when affected tissue is assayed (e.g., muscle for patients with neuromuscular phenotypes) in patients that have exhausted all current standard of care DNA sequencing tests (222, 223). Another interesting challenge that emerges with clinical RNA-seq of mature mRNA is the possibility of detecting an abnormal transcript (e.g., defective splice product) but not identifying the underlying genomic variant causing the event (e.g., deep intronic mutation). Thus, genetics professionals will need to establish appropriate guidelines for how to interpret pathogenicity of abnormal splice products without knowing the causative genomic variant. Perhaps a future of GS paired with RNA-seq will provide the greatest benefit to patients since suspected pathogenic non-coding variants detected by GS can be interpreted concurrently for their functional impact by RNA-seq.

An interesting new approach to determine whether or not someone is affected with a Mendelian disorder is to assay changes in DNA methylation from whole blood. This approach is based on recent studies showing mutations in genes functioning as epigenetic and transcriptional regulators frequently result in neurodevelopmental disorders (230), and as a result of their molecular functions drive unique DNA methylation signatures that mirror the gene containing the primary mutation (231–233). Thus, an individual with a mutation in the histone methyltransferase *KMT2D* causing Kabuki syndrome results in a unique DNA methylation profile compared to someone with a mutation in the SWI/SNF complex gene *ARID1B* causing Coffin-Siris syndrome (231, 232). This diagnostic testing approach has made the jump to clinical testing and will be especially useful when variants of uncertain significance are detected in chromatin remodeling genes or for reflex testing from negative exomes. Limitations of this approach are mainly the restricted number disorders that transmit unique and reproducible methylation signatures in blood. Additionally, similar to RNA-seq, this method reveals functional consequences instead of causative genomic variants, thus scenarios where novel signatures are detected would likely be non-diagnostic until the culprit genomic variant is found.

TABLE 6 | Key points from review.

- Technical advances have driven changes in genomic diagnostics
- CMA enables genome-wide detection of CNVs, including more efficient detection of atypical CNVs associated with recurrent CNV syndromes
- NGS enables cost-efficient, high-throughput sequencing
- Diagnosis often requires multiple technologies, either to detect different types of genetic variants, or to narrow the differential diagnosis
- Panel-based testing has largely supplanted individual gene tests
- Certain conditions require specialized testing and are not covered in exome sequencing, such as repeat-expansion disorders and imprinting disorders
- Clinical care is moving toward a genotype-first approach
- Interpretation of genetic data is imperfect; variants of unknown significance are common and pose challenges for genetic counseling
- High-throughput evaluation can identify incidental and secondary variants outside the indication for testing
- Pre- and post-test genetic counseling is critical
- Clinical genome sequencing is emerging and is expected to further change genetic diagnostics as sequencing costs continue to decrease

DISCUSSION

Genomic diagnostic testing for pediatric disorders has transformed from low resolution (e.g., karyotype) and single locus (e.g., Sanger, FISH, PCR) analyses to high-throughput and high resolution genome-wide testing (e.g., CMA, ES, GS), with an-ever diminishing distinction between cytogenetic and molecular genetics (**Table 6**). These technical advances have transformed how rare diseases are diagnosed and treated, and has massively broadened the spectrum of genes causing rare Mendelian disorders. However, the widespread adoption of these methods has led to challenges in how to rapidly and accurately interpret the staggering number of single nucleotide and copy number variants that exist in the human genome. The interpretation of and counseling for the many variants of uncertain significance remains an enormous challenge. These uncertain diagnoses can become especially problematic in disorders such as hearing loss which can have mild phenotypes indistinguishable from environmental insults and emerge progressively and later in life. Aside from variants that are

strictly rare and therefore of unknown impact, there is also the problem of the limited information available for many genes. Some disease-causing genes have only been reported in a few patients and thus an incomplete phenotypic spectrum. The challenge on this front is therefore whether there is convincing overlap between what is known about the gene and the patient's phenotypes, highlighting the importance of the partnership between clinical and laboratory geneticists. Education and counseling for both non-genetics clinicians and patients on the significance of secondary findings is also a current challenge, and highlights the need for appropriate informed consent prior to undertaking genetic studies. Currently, the genomics field is moving toward improved diagnostic rates through utilization of new technologies and the increasing use of genome sequencing, to limit the number of tests required for diagnosis and decrease turn-around times. As genomic knowledge increases, we anticipate that tests will become more definitive and will provide answers for an increasing number of patients. The education of non-geneticists and patients remains a key challenge and we look forward to the increased utilization of genomic information in medical management.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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16q24.3 Microduplication in a Patient With Developmental Delay, Intellectual Disability, Short Stature, and Nonspecific Dysmorphic Features: Case Report and Review of the Literature

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We describe the case of a seven-year-old female patient who presented in our service with severe developmental delay, intellectual disability, facial dysmorphism, and femur fracture, observed in the context of very low bone mineral density. Array-based single nucleotide polymorphism (SNP array) analysis identified a 113 kb duplication involving the morbid OMIM genes: *ANKRD11* (exon1), *RPL13,* and *PGN* genes. ANKRD11 deletions are frequently described in association with KBG syndrome, the duplications being less frequent (one case described before). The exome sequencing was negative for pathogenic variants or of uncertain significance in genes possibly associated with this phenotype. The patient presented subtle signs of KBG syndrome. It is known that the phenotype of KBG syndrome has a wide clinical spectrum, this syndrome being often underdiagnosed due to overlapping features with other conditions, also characterized by multiple congenital anomalies and intellectual disability. The particularity of this case is represented by the very low bone mineral density in a patient with 16q24.3 duplication. ANKRD11 haploinsufficiency is known to be associated with skeletal involvement, such as short stature, or delayed bone age. An effect on bone density has been observed only in experimental studies on mice with induced missense mutations in the *ANKRD11* gene. This CNV also involved the duplication of the very conserved *RPL13* gene, which could have a role for the skeletal phenotype of this patient, knowing the high level of gene expression in bone tissue and also the association with spondyloepimetaphyseal dysplasia Isidor Toutain type, in case of splicing mutations.

Keywords: developmental delay, short stature, 16q24.3 duplication, osteoporosis, dysmorphic features

BACKGROUND

The copy number variants (CNVs) involving 16q24.3 region are often associated with KBG syndrome (OMIM 148050), which is a rare genetic condition with autosomal dominant inheritance, firstly described by Hermann in 1975 (1). Initially, this disorder was considered to be very rare, now we know that it is probably underdiagnosed due to milder form of disease, the

studies on patients with developmental disability demonstrating that this genetic condition is a frequent etiology for these disorders (2, 3).

KBG syndrome is often caused by heterozygous mutations in the *ANKRD11* gene (ankyrin repeat domain–containing protein 11) or chromosomal microdeletion of 16q24.3 (3).

The *ANKRD11* gene codes for a chromatin regulator and controls histone acetylation. The gene product is an inhibitor of ligand-dependent transcriptional activation that plays an important role in the proliferation and development of cortical neural precursors and neural plasticity and may be also involved in normal bone development (4). *ANKRD11* appears to be a major gene associated with neurodevelopmental delay (2, 3).

Clinical manifestations of KBG syndrome include distinctive craniofacial features (triangular face, brachycephaly, hypertelorism, wide eyebrows, synophrys, prominent nasal bridge, elongated philtrum, thin upper lip, and macrodontia), short stature, skeletal abnormalities, and neurological involvement, with developmental delay, intellectual disability, and seizures (5). There are several recommendations to use clinical diagnostic criteria for KBG syndrome to facilitate accurate recognition of this condition (6–8). However, the clinical picture is not always very evocative for this disorder, Low et al. showing in a study on 32 patients with KBG syndrome that less than a half of these patients presented suggestive clinical features, the others having a more subtle clinical features, the diagnosis being established only after exome testing (8). They observed that the most common findings in KBG syndrome were not specific features, such as: developmental delay, speech delay, learning difficulties, or behavioral problems (8). Taking into account these data, Low et al. recommended using the major criteria (macrodontia or gestalt of KBG syndrome; height below 10th centile; recurrent otitis media or hearing loss; 1st degree relative with KBG syndrome) and the minor criteria (hand anomaly; seizures; cryptorchidism; feeding problems; palate abnormalities; formal diagnosis of autism; large anterior fontanelle, or delayed closures), the diagnosis being established if the patient present developmental delay/learning difficulties, speech delay, or significative behavioral issues with at least two major criteria or one major criteria and two minor criteria (8).

CASE PRESENTATION

Clinical Report

The patient, a seven-year-old girl, presented to the Department of Genetic Diseases of the Emergency Hospital for Children in Cluj-Napoca, Romania, due to a severe developmental delay, craniofacial abnormalities and femur fracture caused by a minor trauma.

The patient was born at 35 weeks of gestation by spontaneous vaginal delivery, after a pregnancy complicated by a double nuchal cord, birth asphyxia and postnatal intraventricular hemorrhage. At birth, the weight was 1,960 g (−0.56 *SD*), length 44 cm (−0.32 *SD*), and Apgar score 7/7 (1′/5′). During the pregnancy, the mother denied the possible exposal to toxic substances (i.e., alcohol or drugs), known infectious pathogens or other harmful environmental factors.

The patient presented with significant global developmental delay, first noticed at 6–12 months of age in the following areas: gross and fine motor skills (delay for all motor skills, with walking achieved at two years and four months), cognitive and social skills (diagnosed as autism spectrum disorder), speech and language (at the age of seven, she had a limited vocabulary of 10–15 words, could not build simple sentences and also to imitate familiar sounds and to understand simple instructions).

The patient had a first epileptic episode (generalized seizures), without a fever context, at 2½ years and has been treated with valproic acid, with no further seizures to date. No significant family history of congenital anomalies, intellectual disability, or other neuropsychiatric abnormalities were mentioned.

During the first admission to our department, we noticed microcephaly (head circumference 46.2 cm, −2.82 *SD*), short stature (height 101.7 cm, −3.97 *SD*), weight deficit (body mass index 12.1 kg/m^2, −2.61 *SD*). Auxologic parameters were calculated according to the growth charts published by the Romanian Society of Endocrinology for the Romanian population (birth parameters and height) and WHO Charts (head circumference and body mass index) (9, 10).

The patient presented with facial dysmorphism, featuring low frontal, and posterior hairlines, prominent anteverted ears, convergent strabismus, blue sclerae, telecanthus, broad nasal bridge with bulbous nasal tip, macrodontia of the upper central incisors, long philtrum, and thin upper lip, as well as short neck and bilateral clinodactyly of the fifth finger.

The patient also presented intellectual disability (IQ of 30), global developmental delay, autism spectrum disorder, and muscular hypotonia.

The diagnosis of intellectual deficiency was based on an IQ (for children older than six years) evaluated by the WISC-IV test (Wechsler Intelligence Scale for Children).

Bone age estimated using the Greulich–Pyle method, was concordant with chronological age. Hand radiography evidenced an osteoporotic aspect of this region. Echocardiography showed a bicuspid aortic valve. Abdominal ultrasounds did not reveal pathologic changes. MRI investigation did not show malformations or sequels indicating possible effects of perinatal asphyxia.

Laboratory tests showed moderate increased thyroid-stimulating hormone levels of 9.58 μIU/mL (normal range 0.58–5.2), associated with normal free thyroxine 1.09 ng/dL (normal range 0.8 – 1.7), interpreted as subclinical hypothyroidism. The patient was receiving treatment with L-thyroxin, 25 mcg/kg per day.

One month before admission to our department, the patient presented a transverse fracture across the proximal left femur that occurred due to a minor trauma (after falling on the same level).

Considering the clinical features, bone densitometry was performed, to exclude a pathological skeletal background. Bone mineral density values were significantly lower than the normal limits for her age and sex: left femur, with Z-score of −8.4; right femur with Z-score of −5.2 and L1–L4 region, with Z-score of −2.0. Before treatment, serum calcium, phosphorus, parathyroid hormone and vitamin D levels were in normal ranges (Ca = 10.2 mg/dL, P = 4.9mg/dL,



FIGURE 1 | Dysmorphic features of the patient with 16q24.3 duplication (prominent anteverted ears, convergent strabismus, telecanthus, broad nasal bridge with bulbous nasal tip, long philtrum, thin upper lip, macrodontia of the upper central incisors and bilateral clinodactyly of the fifth finger). Written informed consent was obtained from the parents for publication of this case report and accompanying images.

PTH = 11.1 pg/mL, and 25hydroxy D Vitamin = 43.2 ng/mL). For the osteoporosis treatment, intravenous pamidronate therapy was initiated at 1 mg/kg/dose, three doses every four months.

The clinical features of the index case are presented in **Figure 1**.

Genetic Testing

A G-banding karyotype of the proband was performed, which showed a double satellite in chromosome 21. Parental chromosome analysis indicated that the genetic polymorphism was paternally derived, with no associated phenotype and no significant family history of neurodevelopmental disorders or multiple congenital anomalies. The mother's karyotype was normal.

Array-based single nucleotide polymorphism (SNP array) analysis was performed using Infinium OmniExpress-24 v1.2 Kit (Illumina), Human Genome Build 37 (hg19). The interpretation was based on Genome Studio 2.0 software. All genetic investigations performed on this patient and her family members were done after informed consent was obtained following local Institutional Review Board policies and procedures.

SNP array analysis identified arr[GRCh37]16q24.3(89542695_89656251)x3, a 113.556 bp duplication including four OMIM genes, three of them morbid OMIM genes: *ANKRD11*, *RPL11* and *PGN* (**Figure 2**). This duplication event involves the ANKRD11 gene, including the promoter, 5'UTR region and the first exon, the breaking point being in intron 1.

It was also performed exome analysis, to assess the genes possibly associated with osteoporosis, mainly osteogenesis imperfecta and, also the genes associated with neurodevelopmental disorders, no pathogenic variants, or VUS (variants of uncertain significance) were detected.



FIGURE 2 | Image of the 16q24.3 duplication identified by SNP array analysis.

DISCUSSION

We presented a female patient with developmental delay, craniofacial dysmorphism, short stature, and very low bone mineral density with 16q24.3 duplication of 113 kb, involving three morbid OMIM genes, *ANKRD11, RPL13,* and *PGN*. The exome testing did not reveal pathogenic or VUS variants in genes possibly associated with this clinical picture.

ANKRD11 gene haploinsufficiency has been reported in patients with KBG syndrome. Sporadic and familial cases have been described with an autosomal dominant inheritance pattern. The pathogenic variants reported in the literature were mainly represented by: heterozygous splice site variants, nonsense and frameshift variants, intragenic deletion, or duplication in the *ANKRD11* gene (3, 7, 11–22).

The duplication observed in our patient has not been reported until now, larger CNVs including this one has been mentioned in the ClinGen and Decipher databases, in association with developmental delay, intellectual disability or autistic spectrum disorders.

Crippa et al. reported the only cases described in literature with KBG syndrome and *ANKRD11* microduplication (of the region between exon 4 and exon 8) and these authors demonstrated the presence of two mutant *ANKRD11* transcripts containing a premature stop codon, allowing to predict a truncated non-functional protein (20). The patients described by these authors presented a moderate clinical presentation with facial dysmorphism, minor skeletal anomaly (brachymetacarpia), kidney anomalies, and mild cognitive impairment (20). In our case the duplication involved *ANKRD11* gene in his first region, including promoter, 5′UTR region and the first exon, which could have an influence on gene expression, functional studies were not performed to validate specific pathogenic relevance of this CNV.

ANKRD11 gene code for a protein with 2663 amino acids, the C terminal region (between 2,369 and 2,663 amino acids) containing an important domain for *ANKRD11* protein degradation, which presents D-box sequences as signals for proteasome degradation (23, 24). Thus, the C terminal region play a role in regulating protein abundance, the deletions or nucleotide variants of this regions, being the main mechanism for KBG syndrome, by a dominant negative effect (24). The N-terminal part could be associated with changes protein–protein interaction and homodimer synthesis (24). This gene also has four repeated domains, ANK1, ANK2, ANK3, ANK4 (between the amino acids 167–292) which permit the interactions with other proteins, as histone deacetylases and p160 coactivators, thus controlling the genes expression (4, 23). These effects are important for neuron adaptability and plasticity, also for bone development (25, 26). It is known that *ANKRD11* expression levels are regulated during the cell cycle, with a maximal level at M phase and a rapidly descent after this phase (24).

The duplication seen in our patient, involving the promoter region, may influence gene expression regulation, which could influence the protein abundance and the exact expression during the cell cycle, thus being possible pathogenetic mechanisms for the clinical picture seen in this patient.

In **Table 1** we mentioned the main cases described in the literature with CNVs at 16q24.3 region, involving the *ANKRD11* gene. The phenotypic features of these patients usually have met the diagnostic criteria (6, 7). The great majority of these CNVs are *ANKRD11* deletions, inducing haploinsufficiency for this gene.

At our patient, two major criteria (macrodontia, height below 10th centile) and three minor criteria (hand anomaly, seizures, formal diagnosis of autism) were fulfilled (associated to developmental and speech delay), taking into account the

TABLE 1 | Patients described with CNV of 16q24.3 region involving *ANKRD11* gene.

References	16q24.3 CNVs	Size	Position (hg19)	Dysmorphic features	Internal malformations	DD/ID	Other neuropsychiatric features	Short stature	Skeletal abnormalities
Willemsen et al. (11)	del	378kb	89.12–89.53	Yes	CNS Skeletal Ocular	Moderate DD/ID	Seizures	p3-10	Kyphoscoliosis
Willemsen et al. (11)	del	265kb	89.27–89.53	Yes	CNS	Borderline DD/ID	Speech delay ASD	p10-25	-
Lim et al. (12)	del	240kb	89.35-89.59	Yes	Skeletal	Borderline DD/ID	Seizures, ADHD, Speech delay	<p3	Delayed bone age, narrowing and elongation of iliac wings and body, small femoral heads, small distal tibial epiphyses, and tibiotalar slanting
Isrie et al. (13)	del	221kb	89.34-89.56	Yes	Skeletal	Borderline DD/ID	ADHD, delayed speech and language development	<p3	Clinodactyly, syndactyly
Isrie et al. (13)	del	138kb	89.33-89.47	Yes	Skeletal	Borderline DD/ID	Seizures ADHD	<p3	Short 4th and 5th metacarpals
Scarano et al. (14)	del	140kb	89.28–89.42	Yes	Skeletal, renal, genital	DD/ID	Behavioral features	N	Delayed bone age, ribs abnormalities, joint stiffness, clinodactyly, short metacarpals
Scarano et al. (14)	del	220kb	89.33-89.55	Yes	Skeletal, cardiac	DD/ID	Behavioral features	<p3	Delayed bone age, ribs abnormalities, scoliosis/kyphosis, joint stiffness, clinodactyly
Scarano et al. (14)	del	130kb	89.42-89.55	Yes	Skeletal	DD/ID	Behavioral features	N	Delayed bone age, ribs abnormalities, joint stiffness, clinodactyly
Khalifa et al. (15)	del	197kb	89.38-89.58	Yes	Cardiac, genital, digestive skeletal	DD/ID	Febrile seizures	p10	Delayed bone age, small hands with brachydactyly, partial syndactyly 2nd-3rd toes
Youngs et al. (16)	Del	180kb	89.39-89.57	Yes	-	mild ID	ASD, ADHD, obsessive–compulsive disorder, anxiety, self-injury	<p3	-
Spengler et al. (17)	del	348kb	89.37-89.60	Yes	Skeletal	No	No	<p3	Clinodactyly 5th finger
Novara et al. (18)	del	340kb	89.16-89.50	Yes	Skeletal	ID	Stereotypies bruxism, hand-biting, sleep disorders	<p3	-
Sacharow et al. (19)	del	320 kb	89.28-89.60	Yes	Skeletal, cardiac, genital	ID	Speech delay	p50	Clinodactyly 5th finger
Crippa et al. (20)	dup	89 kb	89.35-89.43	Yes	Skeletal, cardiac, renal	moderate ID		<p3	Short metacarpals
Our patient	dup	113kb	89.54-89.6	Yes	Skeletal, cardiac	DD/ID	ASD, seizures, speech delay	<p3	Low bone mineral density, fracture

Del, deletion; dup-duplication; CNV, copy number variants; DD/ID, developmental delay/intellectual disability; ADS, autism spectrum disorder; ADHD, Attention-Deficit/Hyperactivity Disorder; CNS, central nervous system; N, normal.

criteria of Low et al. (8), which permitted us to affirm the clinical diagnosis of KBG syndrome, even if the genetic testing firstly suggested this diagnosis.

RPL13, another gene involved in this duplication, code for a protein incorporated in the 60S subunit of ribosomes, and was very recently described by Le Caignec et al. to be associated with a

human ribosomopathy, Isidor-Toutain spondyloepimetaphyseal dysplasia, an autosomal dominant disorder (27). These authors showed that *RPL13* gene is highly expressed in osteoblasts and chondrocytes from hypertrophic and remodeling zones in mouse growth plate (27). *RPL13* gene duplication is not known to be associated with human disorder, but the high *RPL13* expression in bones and the association with a skeletal dysplasia in case of heterozygous splice variant could have a role for the very low bone density seen in the present case (27). Also, it is known that *RPL13* gene is a very evolutionary conserved gene and a duplication at this level might have a certain impact, that for the moment we cannot prove. However, the neuropsychiatric picture seen in our patient is not so obvious correlated with *RPL13* gene, the patients with *RPL13* splice mutation described by Le Caignec et al. being without neuropsychiatric pathology (27).

CPNE7 gene is not known to be associated with human pathology and *PGN* is associated with an autosomal recessive form of spastic paraplegia when two alleles are affected (28). In our patient, the CNV involved only one copy of the PGN gene. Therefore, it is less likely to be involved in the etiology of the syndrome in the given case, unless a small variant is not presented on the second allele, this hypothesis being excluded after exome sequencing. Additionally, in the absence of special neurological findings of spastic paraplegia, we considered its role less probable in the aetiologic background of the presented clinical characteristics in our index case.

A particularity of our case is the skeletal involvement as the very low bone density, which has not been clearly described in other cases with KBG syndrome where skeletal involvement is present, but mainly noticed under other phenotypes (kyphoscoliosis, delayed bone age, clinodactyly, short metacarpals, short fingers, costovertebral abnormalities, and hip dysplasia). However, experimental studies on Yoda mutant mice showed that heterozygous missense mutation in *ANKRD11* gene is associated with reduced body size, craniofacial abnormalities and reduced bone mineral density (26). This missense mutation was produced in the C terminal region, affecting a very well conserved repressive transcriptional domain, thus eliminating an inhibitory effect on gene expression, a mechanism that might destabilize the whole protein. This might be another mechanism different than the well-known ANKRD11 haploinsufficiency, responsive for the skeletal phenotype in KBG syndrome. Low mineral density, as observed in our patient has not been described until now in patients with KBG syndrome. This observation raises the question of whether the alteration of the first exon of the *ANKRD11* gene could be more important in determining bone mineral density. Functional studies were not possible to argue this hypothesis. It was also assessed another possible independent event, possibly associated with this duplication, by doing exome analysis, but no pathogenic variants or VUS in genes involved in osteogenesis imperfecta or osteoporosis and also in genes associated with developmental delay/intellectual disability, were observed. A very recent observation about *RPL13* gene and skeletal dysplasia lead to a hypothesis, which must be further evaluated, about the importance of *RPL13* duplication for the extremely low bone density seen in this patient.

Another particularity of this case report is the "genotype first" approach. The clinical phenotype being not specific enough for KBG syndrome, it also indicates other possible pathologies, such as Cornelia de Lange syndrome, fetal alcohol spectrum disorder or Cohen syndrome. Parenti et al. published some cases that demonstrated overlap between Cornelia de Lange syndrome and KBG syndrome, with common features such as intellectual disability, behavioral issues, autism spectrum disorders, and craniofacial abnormalities (microcephaly, synophrys, long eyelashes, and arched eyebrows) (29). In our patient, we observed some similarities to mild form of Cornelia de Lange syndrome, such as microcephaly, cognitive impairment, behavioral problems, autism spectrum disorder, the appearance of the eyebrows, and eyelashes and postnatal growth retardation. Fetal alcohol spectrum disorder was also suggested by some patient's features, such as: microcephaly, dysmorphic signs as long philtrum and thin upper lip, developmental delay/intellectual deficiency and growth restriction (30).

The intellectual delay observed in our case, associated with some dysmorphic signs such as prominent central incisors, microcephaly, and hypotonia, also indicated Cohen syndrome, as differential diagnosis (31). However, the somatic delay observed in our case is in contradiction to the tendency to truncal obesity, often age-dependent, seen in Cohen syndrome.

The exome testing did not show pathogenic or VUS variant in the genes involved in Cornelia de Lange syndrome or Cohen syndrome.

In this patient, the birth was described as complicated, relevant to perinatal ischaemia, which could be a cause for neurologic issues, but the presence of associated dysmorphism and bicuspid aortic valve make this hypothesis less probable.

CONCLUSIONS

This 16q24.3 duplication and the clinical picture of the patient, which could be concordant with KBG syndrome phenotype, led to CNV interpretation as variant of uncertain significance (VOUS) but with arguments for potential pathogenicity which have to be proven, also other potential contributing causes cannot be excluded and functional studies are needed.

To our knowledge, this case is a first genetically confirmed Romanian patient with microduplication involving *ANKRD11*. The patient's presented a subtle clinical picture of KBG syndrome, diagnosis firstly suggested by SNP array analysis. The particularity of the case was the presence of a very low bone mineral density in a patient with 16q24.3 microduplication. *ANKRD11* gene haploinsufficiency is known to be associated with skeletal involvement, usually involving various malformations, short stature or delayed bone age, with an effect on bone mass observed only in experimental studies on mice with induced mutations on the *ANKRD11* gene.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because their exhaustiveness, the original contributions presented in the study are included in the article. Requests to access the datasets should be directed to Miclea Diana, diana.miclea@umfcluj.ro.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Iuliu Hatieganu University of Medicine and Pharmacy. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SB, DM, and PG-S contributed to conceptualisation, methodology, validation, investigation, manuscript writing, and manuscript supervising. CL, CA, and AK contributed to manuscript writing and methodology of the study. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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A Heterozygous Novel Mutation in *TFAP2A* Gene Causes Atypical Branchio-Oculo-Facial Syndrome With Isolated Coloboma of Choroid: A Case Report

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Background: Branchio-oculo-facial syndrome (BOFS) is a rare congenital developmental disorder with highly variable clinical phenotypes in autosomal dominant inheritance. The aim of this study is to identify disease-causing mutations in a Chinese family with predominant coloboma of choroid.

Case report: We described a family (a mother and her daughter) with unclear clinical diagnosis. The mother (proband) presented with bilateral coloboma of choroid, whereas her daughter had a relatively severe phenotype and presented with larger bilateral choroid coloboma and high-vaulted arch. We applied the next generation sequencing (NGS) panel and analyzed 776 genes related to inherited ocular disorders on the proband. Four candidate heterozygous variants in four genes, respectively, were detected in the proband. Validation of these variants were subsequently performed in the family using Sanger sequencing. Among these variants, a novel nonsense mutation c.912C>A, p.(Cys304*) (NM_001042425.2) which in exon 6 of the conserved helix-span-helix domain in *TFAP2A* results in a premature termination codon. It may trigger nonsense-mediated mRNA decay (NMD). Both the affected mother and daughter had this variant, whereas it was absent in the asymptomatic father. Together with the silicon tools and clinical features, we concluded that the variant c.912C>A, p.(Cys304*), was the second reported nonsense mutation in *TFAP2A* gene, which was the disease-causing mutation of the family.

Conclusion: There are many hereditary diseases accompanied by ocular anomalies. For instance, BOFS, patients with atypical features are always at risk of being under-diagnosed. NGS is a powerful method to identify the genetic cause and improve genetic counseling for less clarified hereditary ocular diseases.

Keywords: branchio-oculo-facial syndrome, coloboma of choroid, *TFAP2A* gene, next-generation sequencing, genotype-phenotype

INTRODUCTION

Branchio-oculo-facial syndrome (BOFS; OMIM 113620) is a rare congenital developmental disorder with highly variable clinical features in autosomal dominant inheritance. It is characterized by three main features: branchial skin defects, ophthalmic malformations, and craniofacial anomalies (1). Branchial characteristics include atrophic, hemangiomatous cervical, or supra-auricular. Ocular features are highly variable expressed including microphthalmia, anophthalmia, coloboma of choroid and/or iris, cataract, ptosis, and strabismus. Craniofacial defects include cleft lip (CL) with or without cleft palate (CP), broad nasal bridge, inner ear malformation, hearing loss (conductive and/or sensorineural) and a high forehead (2, 3). Additional symptoms observed include ectodermal anomalies of the hair, teeth, and nails, prenatal and postnatal growth restriction, congenital heart defects, renal malformations, temporal bone anomalies, whereas developmental delay and/or psychiatric are not common (4, 5). The cases of BOFS are distinctive rare, the prevalence of the BOFS is unknown.

TFAP2A (6p34.3) is the gene reported to date responsible for the development of BOFS. It encodes transcription factor AP-2 alpha (AP-2α). AP-2α belongs to a member of the AP-2 family of transcription factors (3). AP-2α is a 52-kD retinoic acid-inducible transcription factor that binds to the consensus sequence 5′-GCCNNNGGC-3′ as a dimer and forms homodimers or heterodimers with other similar family members. AP-2α plays the role of gatekeepers balancing the proliferation and differentiation of embryogenesis (6). It plays a role with other signals to regulate the morphogenesis of the eye, face, body wall, limb, neural tube, and kidney (3, 7). BOFS shows broad phenotypic features ranging from mild to severe forms. No more than 150 cases have been reported with a well-illustrated clinical or molecular diagnosis until now (8). Most individuals with BOFS can be diagnosed correspondence with all three or two features as well as the first-degree affected relative or ectopic thymus (3). Nevertheless, in some cases, the patients showed atypical phenotypic features (9–11).

For the individuals who not meet clinical diagnostic criteria, the molecular genetic testing approaches such as the multigene panel or even exome sequencing could assist clinical diagnosis. The combination of genetic analysis and clinical features is a method for accurate diagnosis, especially helpful for the genetic counseling and prenatal diagnosis.

In the present study, we described a mother and her daughter with an atypical BOFS phenotype that had a heterozygous mutation of the *TFAP2A* gene which was identified by NGS.

CASE PRESENTATION

Clinical Examination

The proband (I-1) (**Figure 1A**) was a 38-year-old Chinese woman, she presented with bilateral choroid colobomas. She accepted the careful ophthalmologic examination at Wuhan Aier Eye Hospital. Her binocular visual acuity was 1.0, bilateral intraocular pressure was 17 mmHg. Fundus examination revealed choroidal coloboma in both eyes. One choroidal coloboma which covered an area of 1 papillary diameter (PD), was located in 1 PD underneath the optic disc of the left eye. The other one which covered an area of 2 PD, was located in 0.5 PD underneath the optic disc of the right eye (**Figure 1B**). She did not have any other defects after a detailed physical

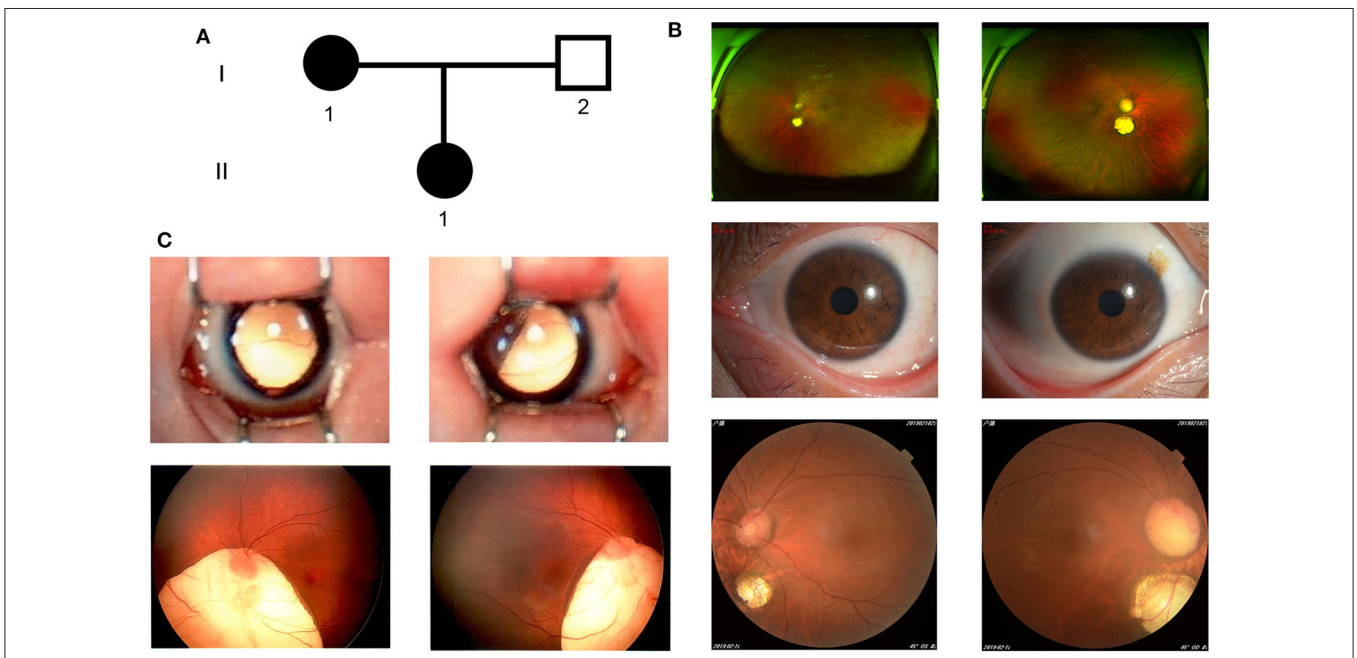


FIGURE 1 | The clinical features of the family. **(A)** The family pedigree. **(B)** Color fundus photographs (Panoramic, anterior, and posterior) of the I-1. **(C)** Color fundus photographs (Panoramic and posterior) of the II-1. left column: left eye; right column: right eye.

inspection. Her daughter (II-1) (**Figure 1A**) was 6 years old and had a relatively severe phenotype presented with larger bilateral choroidal coloboma, plus a high-vaulted arch. One choroidal coloboma existed around the 1/3 inferior quadrant underneath the left eye's optic disc, the other one existed around the 1/4 inferior quadrant underneath the right eye's optic disc (**Figure 1C**). The other physical examinations were unremarkable. The proband's husband (I-2) (**Figure 1A**) had no ocular and other developmental anomalies. I-1 and I-2 were non-consanguineous Chinese couple.

Targeted Next-Generation Sequencing (Targeted NGS)

In order to identify the pathogenic mutation underlying the eye defects in this family, we applied the NGS on the proband with the 4 K captured chip which was described in previously published literature (12). The panel consisted of 4,689 nuclear genes of Mendelian diseases. We focused on the 776 genes related to inherited ocular disorders for the variant analysis (**Table S1**). The genomic DNA was isolated from the 5 ml blood sample with QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). The genomic DNA was fragmented into 200–250 bp by Covaris LE220(Massachusetts, USA). BGISEQ-500 library was enriched by array hybridization, elution, and amplification. The prepared library was sequenced using BGISEQ-500 platform (BGI, Shenzhen, China) which was based on the DNA NanoBalls (DNBs) technology according to the previous paper (13). SOAPnuke was used to generate the "clean reads" (90 bp in length) from the raw sequencing reads. The Burroughs Wheeler Aligner (BWA) was applied to align the clean reads to the GRCh37p10(hg19). SOAPsnp and Samtools software were used to call SNVs and Indels. All the variants were filtered against dbSNP (https://www.ncbi.nlm.nih.gov/SNP), HapMap (https://www.internationalgenome.org/category/hapmap), 1 k Genomes (www.internationalgenome.org), ExAC (https://gnomad.broadinstitute.org/) and 100 Chinese healthy adults database with allele frequency <0.05. The pathogenic impact of the variants was predicted using three algorithms: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and MutationTaster (http://www.mutationtaster.org).

The quality control (QC) of sequence reads generated targeted NGS has been performed following the previous literature (14). The data of QC were summarized in **Table S2**. The size of the target region (776 genes of inherited ocular disorders) was 4.05 Mb, and the average sequencing depth was 154.05-fold. 99.99% of targeted bases were covered and 99.74% reads had an average depth which was at least 10-fold. Four novel heterozygous variants were predicted as candidate variants in the proband for further analysis (**Table S3**). They did not exist in any above databases. The first variant was a nonsense mutation c.912C>A, p.(Cys304*) (NM_001042425.2) in exon 6 of *TFAP2A* gene, and it was supported by 112 out of 240 reads. It caused a premature termination codon (PTC) and might trigger nonsense-mediated mRNA decay (NMD). It is an ocular coloboma-associated gene. The second was a small insertion (c.1130+2dup(NM_003816.2) in intron 11 of *ADAM9* gene, and it was supported by 55 out of 110 reads. This mutation was predicted to disturb 5′- splice sites. Mutations in *ADAM9* gene underline autosomal recessive Cone-rod dystrophy 9 (OMIM 612775), characterized by retinal pigment deposits visible on fundus examination. The third one was a missense mutation c.43G>A, p.(Gly15Ser) (NM_015645.5) in exon 18 of *C1QTNF5* gene, it was supported by 24 out of 47 reads. This variant was "tolerable" which was predicted by SIFT and "polymorphism" by Mutation Taster. Defects in *C1QTNF5* gene are a cause of autosomal dominant late-onset retinal degeneration (OMIM 605670), characterized by night blindness in the fifth or sixth decade of life. The last was a missense mutation c.2791G>A p.(Glu931Lys) (NM_001142800) in *EYS* gene, it was supported by 46 out of 97 reads. This variant was annotated "damaging" by SIFT, "benign" by PolyPhen-2, and "polymorphism" by Mutation Taster (**Table S1**). Mutations in *EYS* gene are responsible for patients with autosomal recessive retinitis pigmentosa 25 (OMIM 602772), characterized by progressive retinal degeneration. The nonsense variant c.912C>A, p.(Cys304*) in *TFAP2A* was assessed as likely pathogenic mutation, the other three variants were assessed as uncertain significance (VUS) based on the ACMG 2015 guidelines.

Sanger Sequencing

Sanger sequencing for the identified variants was performed on the I-1, I-2, and II-1. The primer pairs were listed in **Table S4**. The proband's asymptomatic parents were not available for co-segregation analysis. The results revealed that I-1and II-1 had the heterozygous variants, c.912C>A, p.(Cys304*) in *TFAP2A*, c.43G>A, p.(Gly15Ser) in *C1QTNF5,* c.2791G>A, p.(Glu931Lys) in *EYS* gene, I-2 did not have mutations at these sites. I-2 and II-1 had the heterozygous variant c.1130+2dup in *ADAM9*. I-1 is normal at this site (**Figure 2**). By combining the clinical characteristics, genetic mode, and silicon analysis results, we propose that mutation c.912C>A in *TFAP2A* was likely causative mutation for the features of I-1 and II-1.

DISCUSSION AND CONCLUSION

I-1 and II-1 in our report both had bilateral choroidal coloboma, we analyzed the 776 genes related to inherited ocular disorders with targeted NGS approach. We especially centered on the genes which disturb the function of the pathogenesis of coloboma. Through systematic analysis, we finally took the variant in *TFAP2A* into consideration.

The *TFAP2A* gene maps to chromosome 6p24 and consists of seven exons encoded transcription factor AP-2α with 437 amino acids (NM_001042425). AP-2α contains three conserved domains: a proline and glutamine rich (PG) domain in the N-terminal region, which is responsible for transcriptional activation; a central basic DNA binding (B) domain; and a highly conserved helix-span-helix (HSH) domain in the C-terminal region, which interferes with dimerization and site-specific DNA binding (**Figure 3**). *TFAP2A* mutations or deletions are known to the genetic cause of reported BOFS cases.

To date, 44 mutations in *TFAP2A* have been reported with BOFS according to the HGMD Professional 2019.4 and literature

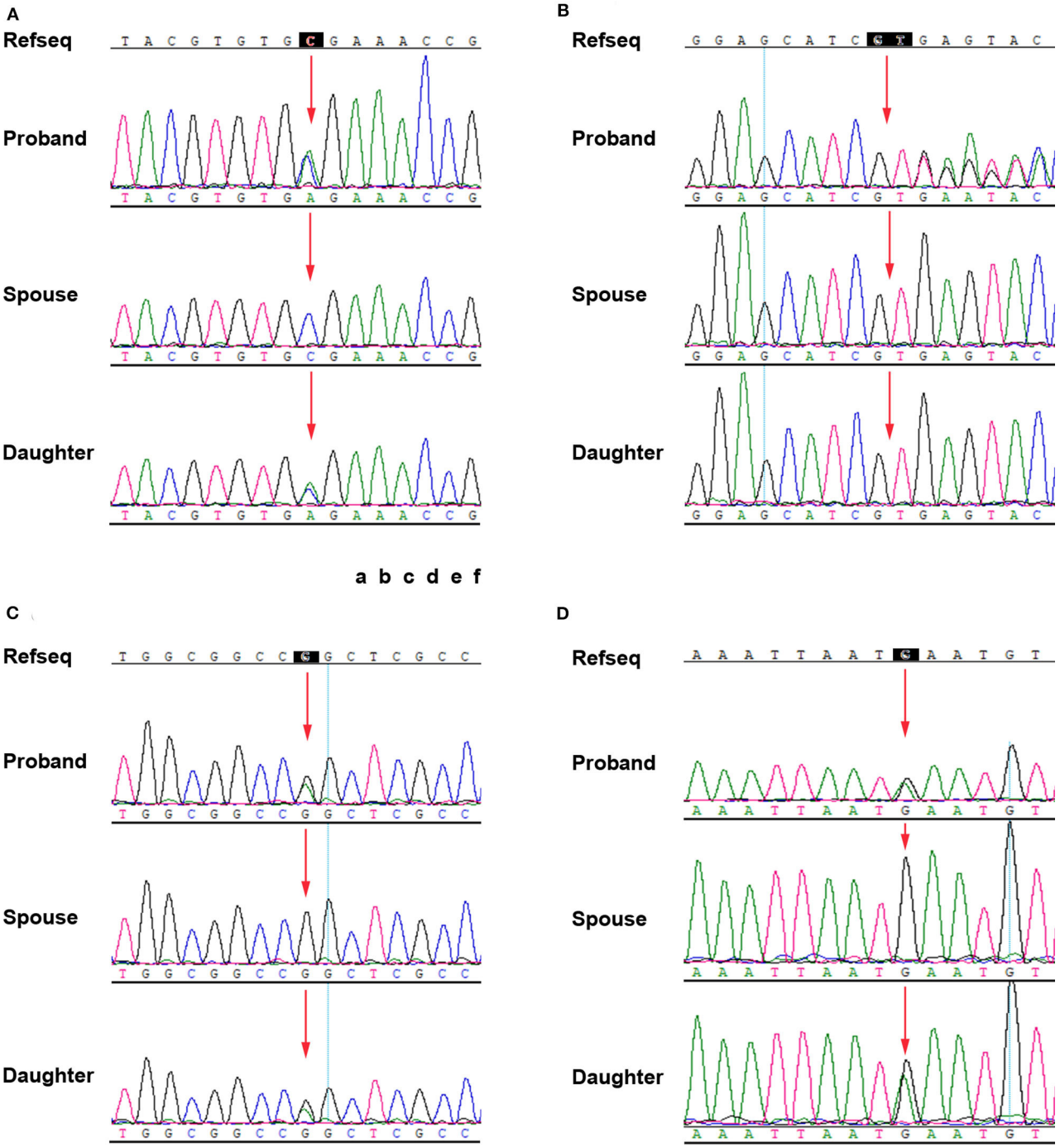


FIGURE 2 | Confirmation of the four mutations by Sanger sequencing. **(A)** *TFAP2A* NM_001042425.2: c.912C>A p.(Cys304*). **(B)** *ADAM9* NM_003816.2: c.1130+2dup. **(C)** *C1QTNF5* NM_015645.5: c.43G>A p.(Gly15Ser). **(D)** *EYS* NM_001142800: c.2791G>A p.(Glu931Lys).

in Pubmed (**Table S5**). It includes 29 missense mutations, one nonsense mutation, one splicing mutation, two regulatory mutations, five small deletions/insertions, one small indel, five gross deletions. The disease-causing mutational hotspot region is in highly conserved DNA-binding domain of AP-2a, encoded by the exons 4 and 5 of *TFAP2A* gene with missense mutations in 90% cases (10).

TFAP2A mutations are associated with BOFS. Like most dominant diseases, BOFS expresses a considerable phenotypic variabilities. The patients displayed variable severity of clinical symptoms, even with the same mutation in the inter-intrafamily due to incomplete penetrance (5, 15–17) and somatic mosaicism (10). Titheradge et al. (18) reported a three generational BOFS family with c.703G >A, p.(Glu235Lys), demonstrating wide

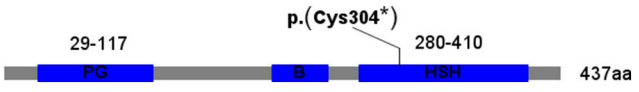


FIGURE 3 | The protein structure of the TFAP2A. PG, proline and glutamine rich domain; B, basic DNA binding domain; HSH, helis span helix domain.

phenotypic spectrum, including lethality. In addition, modifier genes and/or enhancer genes were described to influence the clinical variability (3, 9).

No clear genotype-phenotype correlation has been clarified, however, there is a phenomenon that missense mutations appear with more severe features than the patients with the complete deletion of *TFAP2A* allele. It ascribes that the mutant proteins cause a dominant-negative activity on the wild-type AP-2a protein (19, 20).

The mutation c.912C>A, p.(Cys304*) in the present report is on exon 6, corresponding to HSH domain. A mutation in this domain may influence the ability of DNA binding and hinders the role of transcription.

I-1 and II-1 had mild symptoms, presented with isolated choroid coloboma, without systemic abnormalities. We speculated that this PTC mutation decreased mRNA levels through the mechanism of NMD. Therefore, the clinical features of the patients were milder than that of the patients with missense mutations. Further investigations need to explain why the manifestations are so mild in the patients.

TFAP2A is a retinoic acid (RA)-responsive gene to direct ocular morphogenesis. *Tfap2a* mutants in mice and zebrafish revealed a variable spectrum of eye phenotypes. *TFAP2A* gene regulates lens development and optic fissure closure (9, 21). Almost 83% of published BOFS cases involved the ocular anomaly (15). Individuals with *TFAP2A* mutations can present predominantly ocular phenotypes in the absence of branchial and craniofacial defects. The BOFS cases with predominantly ocular phenotypes were also listed in **Table S5**. Dumitrescu et al. (22) described a family harbored a heterozygous c.1150C>T, p.(His384Tyr) mutation in *TFAP2A* with primarily ocular involvement. Ng et al. (8) reported a family (affected child and mother) with a heterozygous c.253dupC, p.(Leu85Profs*84) mutation in *TFAP2A* gene presented with predominantly ocular anomalies. *TFAP2A* gene plays a role in the morphogenesis of the eye in animal models (23, 24). Ocular abnormalities of *TFAP2A* mutations are also variably expressed. Our two patients had isolated choroidal coloboma and did not have distinct non-ocular phenotypes, which was extremely rare in reported BOFS patients.

There are many hereditary diseases accompanied by ocular anomalies. Especially for the incomplete clinical appearance of the syndrome, they are at risk of being under-diagnosed and inadequately managed. Genetic analysis with NGS panel targeting more comprehensive ocular anomalies related genes helps to facilitate proper clinical diagnosis and improve the efficacy of genetic counseling for these disease groups.

To our knowledge, only one nonsense mutation c.740C>A, p.(Ser247*) in exon 4 (B domain) of *TFAP2A* gene in an affected daughter and mother pair was reported in available literature (16). In our report, the heterozygous mutation c.912C>A, p.(Cys304*) was the second nonsense mutation described to date, which caused the BOFS.

In summary, we applied NGS panel to a patient with bilateral choroidal coloboma who would not be led to a diagnosis of BOFS previously. We identified a novel nonsense mutation in *TFAP2A* in a highly conserved region of the HSH domain, which may be the pathogenic factor of the family. The findings contributed to the understanding of the genotype-phenotype correlation of BOFS. It can offer more knowledge for clinicians to make a clinical diagnosis with incomplete clinical features of the BOFS.

NGS is a powerful method to identify the genetic cause and improve genetic counseling for less clarified hereditary ocular diseases.

DATA AVAILABILITY STATEMENT

The raw datasets generated of this study are deposited on the CNGB Nucleotide Sequence Archive (CNSA: https://db.cngb.org/cnsa; accession number CNP0000402). They are available from the corresponding author on a reasonable application.

ETHICS STATEMENT

This study was approved by the BGI-Shenzhen Ethics Committee (BGI-IRB19097). The proband and her spouse provided written informed consent for participation in the present study. In addition, the couple signed their daughter's informed consent as guardians. This study was performed based on the Principles of the Declaration of Helsinki. The couple provided written informed consent of themselves and their daughter as guardians for information and images to be published.

AUTHOR CONTRIBUTIONS

JM and YW provided clinical data and samples from the patient and her family. BM and HW designed the research and wrote the first draft of the article. XH conducted molecular genetic experimental studies. JM, BM, and SG analyzed data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00380/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Late-Onset Ornithine Transcarbamylase Deficiency and Variable Phenotypes in Vietnamese Females With *OTC* Mutations

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Background: Ornithine transcarbamylase deficiency (OTCD) is an X- linked recessive disorder and the most common error of the urea cycle, caused by the mutations in the *OTC* gene. Due to X-inactivation, 15–20% of female carriers present symptoms of OTCD at late onset. Early diagnosis of OTCD by molecular analysis in females is highly desirable. The aim of the study was to identify the mutations in two unrelated Vietnamese girls suspected with OTCD and the carriers in their families for definitive diagnosis and proper counseling.

Case Presentation: Two patients presented with an acute encephalopathy at the first admission. Biochemical tests revealed hyperammonemia, hyperlactatemia, elevated glutamine level, elevated transaminase, elevated urinary orotic and uracil acid levels, and disorder of prothrombin time. Brain magnetic resonance imaging indicated cerebral edema. Based on the clinical and laboratory results, the two patients were diagnosed with urea cycle disorders. Therefore, the two patients were managed by stopping feeding, with infused glucose, l-carnitine, l-arginine, and sodium benzoate, and with hemofiltration. The two patients were alert and recovered with normal blood ammonia levels after 72 h of treatment. The family history of patient 1 showed that her brother died at 4 days of age due to a coma and dyspnea, while her parents were asymptomatic. Variable phenotypes were observed in three generations of the patient 2's family, including asymptomatic (mother), affected female adults dying at the first symptom (grandmother and aunt), and affected males dying in the first week of life (uncle, cousin, and siblings). Whole-exome sequencing showed two mutations in the *OTC* gene, including one novel missense mutation, c.365A>T, in the patient 1 and one previously reported splicing mutation, c.717+1G>A, in the patient 2. The two mutations are evaluated as likely pathogenic and pathogenic, respectively, according to the recommendations of the American College of Medical Genetics and Genomics (ACMG). Genetic analyses in the families indicated the mothers were heterozygous.

Conclusion: Clinical, biochemical, and molecular findings accurately diagnosed the two patients with late-onset OTCD. Our results explained the genetic causes and proposed the risk in the patients' families, which could be useful for genetic counseling and monitoring in prenatal diagnosis.

Keywords: ornithine transcarbamylase deficiency (OTCD), Vietnamese females, heterozygous females, c.365A>T, IVS7+1G>A

INTRODUCTION

Ornithine transcarbamylase is a pivotal enzyme involved in the urea cycle. It is a mitochondrial enzyme and is responsible for the synthesis of citrulline from carbamyl phosphate and ornithine (1). Ornithine transcarbamylase deficiency (OTCD, OMIM # 311250) is the most defect of the urea cycle, with an estimated prevalence range from 1 in 14,000 (2) to 1 in 80,000 people (3). OTCD is an X-linked recessive genetic disorder and causes heterogeneous clinical symptoms. Hemizygous males have a spectrum of severity ranging from complete OTCD, which presents at the neonatal onset with acute hyperammonemia, to partial OTCD, which presents mild symptoms at late onset (4). Due to a skewed X-chromosome inactivation, leading to the wild-type allele of the two X-chromosome allele in females being randomly inactivated, ∼15% of heterozygous females with OTCD show the mild late-onset phenotypes (5). OTCD is biochemically characterized by profound plasma hyperammonemia, reduced level of citrulline, and elevated glutamine and urinary orotic acid. The clinical manifestations include irregular episodes of vomiting, progressive lethargy, and irritability. The disorder may rapidly progress to include coma, confusion, seizures, hypotonia, hepatomegaly, and edema. Patients may also relapse into clinical signs like protein aversion, headaches, and behavioral changes.

OTCD is caused by mutations of the *OTC* gene, which is located on the short arm of the X chromosome (Xp21.1) and encodes mature OTC proteins composed of 322 amino acids (6). To date, over 500 mutations have been recorded in the *OTC* gene (7). Single base substitutions account for about 70–84% of *OTC* mutations, small-fragment deletions or insertions represent about 12%, and 4% is represented by large-fragment deletions (8). The disease severity depends on the activity of OTC, which is affected by the type and the site of mutations (9, 10). The large deletions, frameshift, and nonsense mutations evidently cause a complete loss of OTC activity, resulting in a neonatal form of the OTCD, while missense and splicing mutations lead to a partial loss of OTC activity, resulting in both of neonatal and late onset of the disease (11). However, missense mutations appearing in the active sites resulted in a nearly complete loss of OTC activity, leading to a severe OTCD (12).

In this study, we report the clinical features and molecular analyses of two Vietnamese girls suspected with OTCD and their families.

CASE PRESENTATION

Clinical Presentation

Patient 1: A 12-month-old girl of non-consanguineous Vietnamese parents presented with fever, cough, anorexia, and lethargy for 2 days, then was referred and managed with a diagnosis of encephalopathy at the local hospital for 1 day. When her consciousness worsened, she was transferred to our emergency. On admission, she presented with mechanical ventilation, shock condition, deep coma, and dilation of both pupils to 4 mm with weak light reaction (**Table 1**). The biochemical investigation revealed hyperammonemia, elevated transaminase, hyperlactatemia, elevated blood glutamine, elevated blood lysine, elevated phenylalanine, elevated urinary orotic and uracil acid levels, and disorder of prothrombin time. The results of brain magnetic resonance imaging (MRI) revealed cerebral edema and abnormal T1W. She was managed by stopping feeding, with infused glucose (10 mg kg^{-1} min^{-1}), L-carnitine (100 mg kg^{-1} day^{-1}), and L-arginine (500 mg kg^{-1} day^{-1}), and with hemofiltration. After 72 h, she was alert and recovered, with normal blood ammonia level. For long-term outcome, at the age of 3, she had a development delay, with a development quotient (DQ) around 50%, and had five recurrent episodes of hyperammonemia. She was the first child and was born at 38 weeks of gestation, with a birth weight of 2.8 kg and normal development. The family history showed that her brother died at 4 days of age due to a coma and dyspnea. Her parents were reported asymptomatic.

Patient 2: A 24-month-old Vietnamese girl presented with vomiting, anorexia, and lethargy for 2 days and then was admitted and managed with a diagnosis of encephalopathy at the province hospital for 1 day. When her consciousness worsened, she was transferred to our emergency. On admission, she presented with a coma, no paralysis, and no convulsion (**Table 1**). The biochemical analyses revealed mild hyperammonemia, elevated alanine aminotransaminase and aspartate aminotransaminase, high glutamine level, elevated lactatemia, elevated urinary orotic and uracil acid levels, normal lysine and phenylalanine levels, and disorder of prothrombin time. The results of the MRI revealed cerebral edema. She was managed by stopping feeding and infused glucose (10 mg kg^{-1} min^{-1}), L-carnitine (100 mg kg^{-1} day^{-1}), L-arginine (500 mg kg^{-1} day^{-1}), and sodium benzoate (500 mg kg^{-1} day^{-1}). She was alert and recovered with normal blood ammonia level after 72 h of treatment. At the age of 4, she had a development delay (DQ = 50%) and 10 recurrent episodes

TABLE 1 | Clinical and molecular analyses of two Vietnamese female patients with OTCD.

	Patient 1	**Patient 2**
CLINICAL SYMPTOMS		
Age of diagnosis	12 months	24 months
Initial symptoms	Fever, cough, anorexia, and lethargy.	Vomiting, anorexia, and lethargy.
Presenting symptoms	Mechanical ventilation, shock condition, deep coma, both of dilated pupils at 4 mm with weak light reaction.	Coma, no paralysis, no convulsion
Family history	Brother died at 4 days of age due to a coma and dyspnea. Parents were asymptomatic.	Uncle, two brothers, and cousin died after birth due to coma and dyspnea. Grandmother died at the age of 35 by a stroke. Aunt died at the age of 21 after delivery due to a coma. Parents and elder sister were asymptomatic.
Initial ammonia levels (μg/dL) (Normal: <50)	1,100	259
AST levels (UI L^{-1}) (Normal: 10–40)	148	53
ALT levels (UI L^{-1}) (Normal: 7–59)	119	69
INR	3.45	3.03
Glutamine levels (μmol L^{-1}) (Normal: 530 ± 81)	1,490	579
Lactatemia levels (mmol L^{-1}) (Normal: 1.1–2.3)	8.3	4.75
Urinary orotic levels	Elevated	Elevated
Uracil acid levels	Elevated	Elevated
Lysine levels (μmol L^{-1}) (Normal: 48–284)	430	135
Phenylalanine levels (μmol L^{-1}) (Normal: 26–91)	116.8	31.7
Brain MRI/CT scanner Findings	Cerebral edema and abnormal T1W	Cerebral edema
Management	Stop feeding, glucose infusion (10 mg kg^{-1} min^{-1}), l-carnitine (100 mg kg^{-1} day^{-1}), and l-arginine (500 mg kg^{-1} day^{-1}), and hemofiltration	Stop feeding, glucose infusion (10 mg kg^{-1} min^{-1}), l-carnitine (100 mg kg^{-1} day^{-1}), l-arginine (500 mg kg^{-1} day^{-1}), and sodium benzoate (500 mg kg^{-1} day^{-1})
LONG-TERM OUTCOME		
Age	3	4
Development	Delay (DQ = 50%)	Delay (DQ = 50%)
Recurrent episodes of hyperammonemia	5	10
MOLECULAR ANALYSES		
Nucleotide change	c.365A>T	c.717+1G>A
Position in the *OTC* gene	Exon 4	Intron 7
Zygosity	Heterozygous	Heterozygous
Maternal status	Carrier	Carrier
Effect	Missense	Donor splice site error
Amino acid change	p.Glu122Val	–
SIFT (score/prediction)	0 (Deleterious)	–
PolyPhen-2 (score/prediction)	1 (Damaging)	–
PANTHER (score/prediction)	4200 (Probably damaging)	–
FATHMM (score/prediction)	−6.61 (Damaging)	–
SNPs&GO (score/prediction)	RI 9 (Disease)	–
Pmut (score/prediction)	0.93(94%) (Disease)	–
Align-GVGD (score/prediction)	C65 (Pathogenic)	–
SNAP2 (score/prediction)	88 (Effect)	–
Mutation assessor (score/prediction)	4.24 (High impact)	–
PROVEAN (score/prediction)	−6.625 (Deleterious)	–
CADD (score/prediction)	34 (Deleterious)	33 (Deleterious)

(Continued)

TABLE 1 | Continued

	Patient 1	Patient 2
Mutation Taster (score/prediction)	0.9999 (Disease causing)	1 (Disease causing)
MaxEntScan	–	0.94 (Mutant) vs. 9.12 (Wild type) (−89.69% variation)
Splice finder	–	60.43 (Mutant) vs. 87.26 (Wild type) (−30.75% variation)
Frequency (1,000 genomes, gnomAD)	0	0
ClinVar	–	Pathogenic (RCV000083542.1)
ACMG classification	Likely pathogenic	Pathogenic
Human gene mutation database	Novel	HGMD ID CS003075

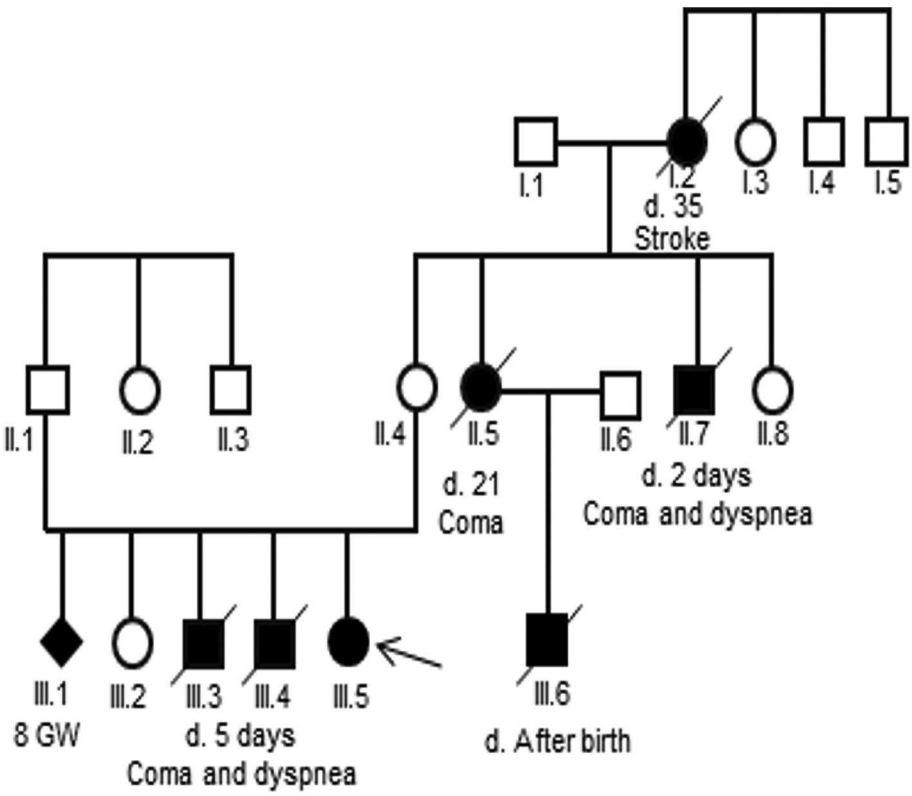


FIGURE 1 | Pedigree of the three-generation family of the patient 2. Generations are shown as I to III. Males are indicated by squares, females by circles, affected members by a shaded black square or circles and the proband by an arrow. Shaded black square with slash indicates death.

of hyperammonemia. Hemofiltration was performed to reduce the blood ammonia level. She was the fourth child of non-consanguineous Vietnamese parents. Her birth weight was 2.9 kg at 38 weeks of gestation. She had normal development at the time of birth.

Analysis of the pedigree disclosed a marked family history of an X-linked maternal inheritance in the family of patient 2 (**Figure 1**). Her uncle and two her elder brothers died at 2 and 5 days, respectively, after birth due to a coma and dyspnea. Her cousin, son of her aunt, also died after birth. However, a skewed X-inactivation may lead to variable clinical features in the females in this family. In detail, her grandmother died at the age of 35 of a stroke, her aunt died at the age of 21 after delivery due to a coma, while her mother and her sister were asymptomatic.

Molecular Investigation

Genomic DNA samples were extracted from peripheral blood samples of the two patients and from avaiable members of their families using QIAamp DNA blood mini kit (Qiagen, Germany), following the manufacturer's protocol. Whole-exome sequencing (WES) was applied to detect variants in the two patients. The DNA libraries were prepared with an Agilent SureSelect Target

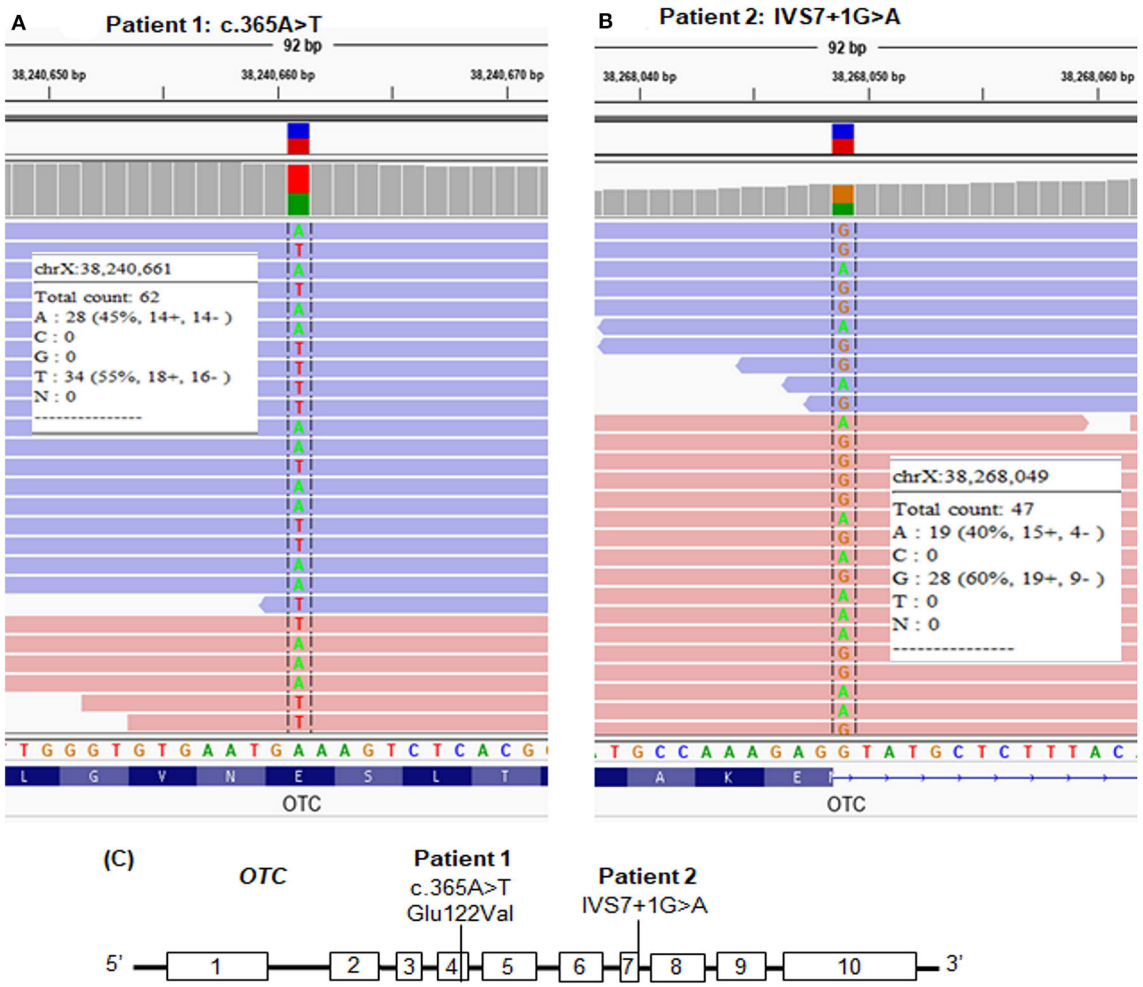


FIGURE 2 | Whole-exome sequencing (WES) paired-end reads are loaded in the IGV genome browser and are shown for each patient. **(A)** The heterozygous mutation, c.365A>T, in patient 1. **(B)** The heterozygous mutation, IVS7+1G>A, in patient 2. **(C)** Location of the point mutations in the *OTC* gene.

Enrichment Kit (Agilent Technologies, CA, USA) according to the manufacture's protocol. The exome libraries were captured and amplified using an Agilent SureSelect Human All exon v6 Kit (Agilent Technologies, CA, USA), then sequenced with the Illumina platform sequencer (Illumina, CA, USA).

The sequencing reads were mapped to the GRCh37/hg19 reference human genome using the Burrows–Wheeler Aligner tool v0.7.12 (13). The duplicate reads were marked and removed using Picard tool v1.130 (http://broadinstitute.github.io/picard/). Variant calling and annotation were performed using the Genome Analysis Toolkit v3.4.0 (14) and SnpEff v4.1g (http://snpeff.sourceforge.net/SnpEff.html), respectively. The population frequency of the variants was estimated by comparing with the Exome Sequencing Project (https://evs.gs.washington.edu/EVS/) and 1,000 Genome (http://browser.1000genomes.org). Variants with a minor allele frequency > 0.01 were excluded. The pathogenicity of the variants was evaluated based on the information in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar) and *in silico* analyses. Sorting Intolerant from Tolerant (SIFT) (15) and Polymorphism Phenotyping v2 (PolyPhen-2) (16) were applied to predict the effects of missense variants. The mutation Taster program (17) and Combined Annotation Dependent Depletion (CADD) (18) were used for the pathogenic prediction of the candidate variants. Human Splicing Finder (HSF) software (19) and MaxEntScan program (http://hollywood.mit.edu/cgi-bin/Xmaxentscan_scoreseq.pl) were applied to estimate the effect of the splice site candidate variant on the splice site strengths and the presence of putative splicing regulatory elements. The pathogenicity of the missense candidate variant was further evaluated using the SNAP2 (https://www.rostlab.org/services/snap/), Align-GVGD (http://agvgd.hci.utah.edu/agvgd_input.php), PANTHER (http://www.pantherdb.org/tools/hmmScoreForm.jsp), SNPs & GO (https://snps.biofold.org/snps-and-go/snps-and-go.html), protein variation effect analyzer (PROVEAN) (20), FATHMM (http://fathmm.biocompute.org.uk/inherited.html), mutation assessor (http://mutationassessor.org/r3/), and Prediction of Pathological Mutations on proteins (Pmut) (http://mmb.irbbarcelona.org/PMut/analyses/new). The read depth of the candidate variants

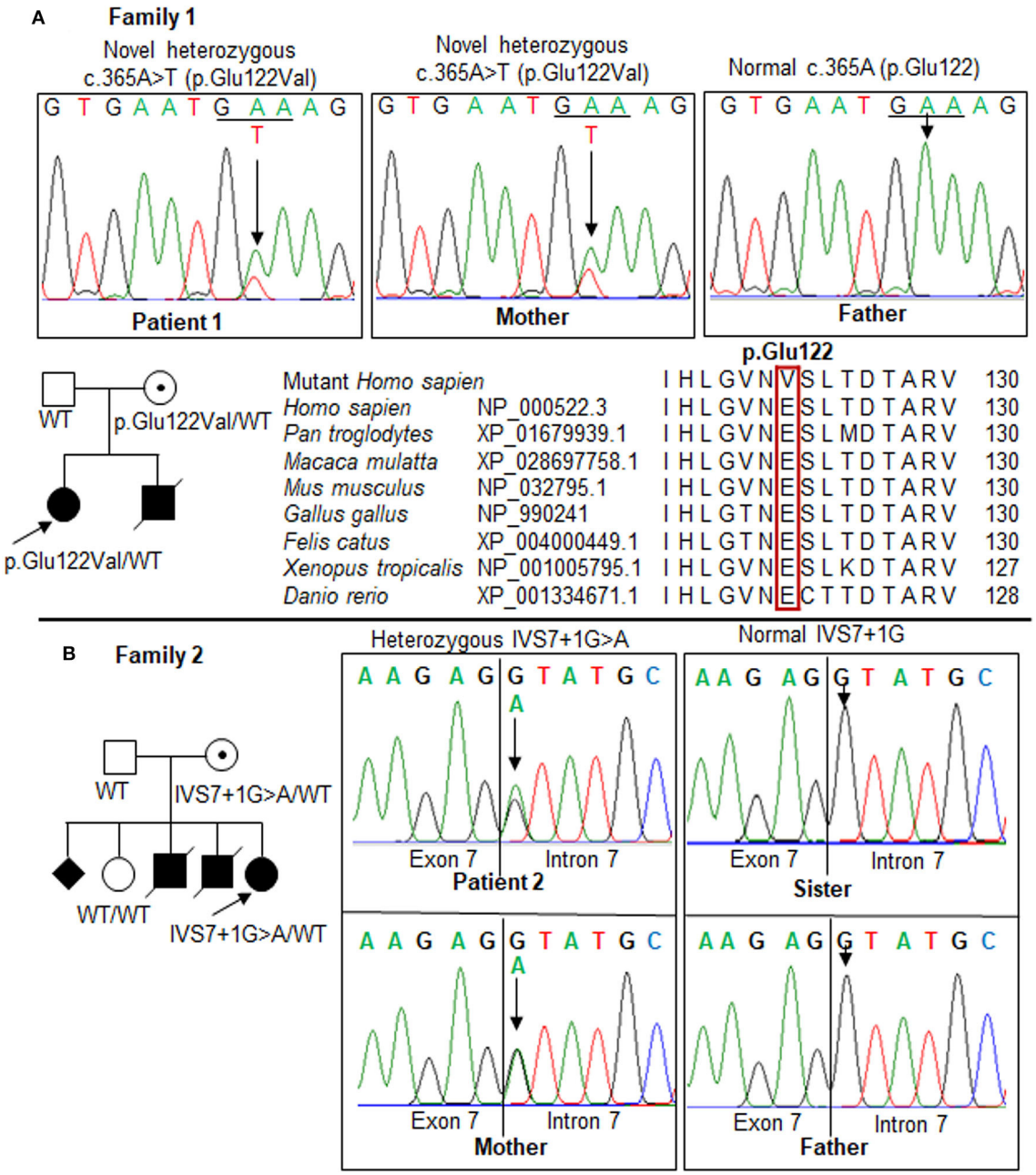


FIGURE 3 | Mutations in the *OTC* gene in the two families. Pedigrees of the two families show the affected (*solid*), unaffected (*open*), and asymptomatic carrier (*circle with dot*) members. *Squares*, males; *circles*, females; *slashed symbol*, deceased. The genotype is shown *underneath each symbol*; WT, wild-type. **(A)** Patient 1 and her mother carried a novel heterozygous missense mutation, c.365A>T (p.Glu122Val), in exon 4. Multiple-sequence alignment of the amino acid sequences of *OTC* from different species revealed a complete conservation of the p.Glu122 residue across species (*red box*). **(B)** Identification of a mutation, IVS7+1G>A, in the *OTC* gene in the family of patient 2. The results showed a maternal carrier, while the father and sister were normal. The GenBank reference sequences of the human *OTC* gene are NM_000531.6 for the mRNA sequence and NC_000023.11 for the exon-intron boundary.

was checked using the Intergrative Genomics Viewer (IGV 2.6.3) (21).

We screened the candidate genes associated with hyperammonemia and elevated glutamine and selected the variants which had a frequency $\leq$ 1% and were evaluated as disease causing by *in silico* analyses. We found a novel missense mutation, c.365A>T, in exon 4 of the *OTC* gene in patient 1 (**Table 1** and **Figures 2A,C**) and a reported splice site mutation, IVS7+1G>A, in intron 7 of the *OTC* gene in patient 2 (**Table 1** and **Figures 2B,C**). The two mutations were heterozygous. The read depth for variation c.365A>T was 62, with the mutant allele T accounting for 55%. The read depth for variation IVS7+1G>A

was 47, with the mutant allele A accounting for 40%. These mutations were confirmed by Sanger sequencing (**Figure 3**). Such molecular investigation supports a definitive diagnosis for the two patients with OTCD.

In patient 1, the replacement of A to T at position 365 leads to switch glutamate (Glu) at residue 122 to valine (Val). Sanger sequencing revealed the mother carries the mutation, but the father does not. The proband and mother were heterozygous for this mutation. The p.Glu122Val mutation was predicted to be "disease causing" by the Mutation Taster (**Supplementary Table 1**), SNP&GO, and PMut analyses; "probably damaging" by the PolyPhen-2, PANTHER, and FATHMM analyses, "deleterious" by the SIFT, PROVEAN, and CADD analyses; "high effect" by the SNAP2 and Mutation assessor analyses; and "pathogenic" by the Align-GVGD analysis (**Table 1**). The amino acid sequences of OTC from human and other species were compared to identify the conservation at the mutant position. A glutamate residue at the position 122 was completely conservative among distinct species (**Figure 3A**). The alternation of Glu to Val at the conservative position may lead to a change in the protein structure, which may affect the enzyme stability or activity. The c.365A>T has not been previously reported in the 1,000 Genomes Project, Exome Variant Project, Human Genome Mutation Database, ClinVar database, Genome Aggregation Database, and in-house WES database ($n = 54$); therefore, c.365A>T is a novel mutation in the *OTC* gene. This variant is classified as likely pathogenic according to the ACMG criteria (**Supplementary Table 2**).

In patient 2, a heterozygous splice site mutation, c.717+1G>A (IVS7+1G>A), was identified at the first nucleotide of intron 7 of the *OTC* gene. This mutation was previously reported as a pathogenic variant in ClinVar (RCV000083542.1, rs66500027) and predicted as a disease-causing variant in the Mutation Taster analysis (**Supplementary Table 1**) and a deleterious variant in the CADD analysis (**Table 1**). This alternation disrupted a highly conserved GT dinucleotide sequence at the splice donor site. Sanger sequencing showed that the IVS7+1G>A mutation was heterozygous in patient 2 (**Figure 3B**). IVS7+1G>A may affect normal splicing of exon 7. The G>A substitution at the first nucleotide in intron 7 resulted in a lower maximum entropy (MaxENT) score than wild-type (0.94 vs. 9.12, respectively). Human Splicing Finder analysis indicated that the IVS7+1G>A mutation could cause an alteration of the wild-type donor site, most probably affecting splicing (−30.75% variation). Inherited genetic analysis revealed the carrier as the mother, while the father and sister were not. The c.717+1G>A variant is considered as pathogenic according to the ACMG recommendations (**Supplementary Table 2**).

DISCUSSION

Our patients presented the typical signs and symptoms of urea cycle disorders such as anorexia, lethargy, vomiting, coma, bilaterally dilated pupils, cerebral edema, development delay, hyperammonemia, elevated glutamine, elevated urinary orotic acid, and disorder of prothrombin time (7). In addition, neonatal death of the males and the variable phenotypes in the females in the patients' families support an X-linked maternal inheritance. In the urea cycle disorders, only OTCD is inherited in an X-linked manner; therefore, the two patients were suspected with OTCD. Molecular analysis is the first choice to obtaining an accurate diagnosis of these patients (22). In this study, by using WES, we identified one missense mutation, c.365A>T, in patient 1 and one splicing mutation, IVS7+1G>A, in patient 2. Such results established a definitive diagnosis of the two patients with OTCD. The onset of symptoms of our patients was from 12 to 24 months old, respectively, therefore, we classified them to late onset, as suggested by Hediger et al. (23).

Hyperammonemia and high brain glutamine can affect the central and peripheral nervous system (24). The early neurological effects like anorexia and vomiting and progressive central nervous system dysfunction like lethargy and coma occurred in our patients. Neurological damage is further confirmed in patients 1 and 2 by cerebral edema, which was also reported in OTCD patients in the studies of Chongsrisawat et al. (25) and Hershman et al. (26). Acute hyperammonemia may induce liver function impairment. The most frequently liver-related complication in OTCD is acute liver failure with coagulopathy (27). Classification of the acute liver injury in patients 1 and 2 is liver dysfunction due to INR>2, as described by Gallagher et al. (28). A total of 75, 50, and 40% of neonatal males and patients with moderate and mild OTCD, respectively, showed liver injury, but only 9% of asymptomatic patients showed liver dysfunction in the study of Gallagher et al. (28). In the study of Laemmle et al. (29), acute liver failure was also reported in nine of nine male patients, but 6 in 15 symptomatic females and none in five asymptomatic females with OTCD; therefore, the percentage of symptomatic females showing liver injury ranged from 40 to 50%. In contrast, two of the two (100%) symptomatic female showed liver dysfunction in our study. The number patients in this study is small. This is a weakness of our study.

A novel heterozygous missense mutation, c.365A>T (p.Glu122Val), was predicted as causing disease in patient 1 based on the 12 computational algorithms (Mutation Taster, CADD, PolyPhen-2, PANTHER, FATHMM, SNPs&GO, PMut, Aling-GVGD, SNAP2, Mutation Assessor, PROVEAN, and SIFT). The switching of a negative charge hydrophilic glutamate to a hydrophobic valine in the polypeptide chain might have caused the clumping together due to staying away from the water, resulting in impaired folding of the protein. Residue Glu122 is involved in the subunit interactions with residue Arg92, which maintains the stabilizing of the tetrahedral carbon in the loop that connects β strand 3 with α-helix 3 (30, 31). Moreover, the mutation occurred in the highly evolutionary conserved position; hence, the replacement of Glu 122 to Val potentially impacted the normal configurations of the protein structure.

To the best our knowledge, a total of 12 pathogenic missense mutations have been reported in exon 4 of the *OTC* gene (**Supplementary Table 3**). These mutations are amorphic, which causes the loss of function of OTC. The majority of which (9/12, 75%) presents at late onset in either males or females. Only three of which (25%) appeared at neonatal onset in male. In

the patient 1's family, the brother of the proband died due to a coma and dyspnea; therefore, we suspect that the brother also carried the mutation p.Glu122Val. Although the mother of the proband is a carrier of the heterozygous Glu122Val mutation, she had no apparent clinical symptoms until now. This could be explained by the random X- chromosome inactivation or the severity of the mutation (25, 32). At residue 122, the replacement of Glu with Gly was previously identified in late-onset OTCD in a Chinese family of Han nationality (33) and in a Spanish male (12). The alternation p.Glu122Gly was predicted to cause partial deficiency by eliminating the intersubunit connections between the side-chain carboxylate of Glu122 and the guanidinium group of Arg92 (12). Glycine is also a hydrophobic like valine. In this manner, the mutation p.Glu122Val may reduce enzyme activity, resulting OTCD in patient 1. However, p.Glu122Gly resulted in a late-onset of OTCD in 13-month-old Spanish male (12), while p.Glu122Val may cause a neonatal-onset of OTCD in patient 1's brother.

A heterozygous splice site mutation, IVS7+1G>A, appeared on the first nucleotide of intron 7 in patient 2. The alternation of within GT dinucleotide at the splice donor sequence may induce exon skipping, partial exon loss, intron retention, and cryptic donor splice site activation, in which exon skipping is common (34). The size of exon 7 and intron 7 is short (54 and 80 nucleotides, respectively); hence, exon 7 skipping may occur, leading to a truncated mRNA chain. Splice site mutations accounted for about 8% of all disease-causing mutations in late-onset OTCD (35). The mutation IVS7+1G>A was reported in a neonatal-onset male with OTCD (36). Our case was the second to undergo the mutation IVS7+1G>A. Except IVS2+1G>A which cause the late-onset in males (7, 37), most of +1G>A mutations cause the neonatal-onset in males and the late-onset in females (10), in which the neonatal-onset disease was more prevalent than the late phenotypes. This trend also observed in the family of patient 2 with variable phenotypes in three generations, including asymptomatic (mother), affected female adults dying at the first symptom (grandmother and aunt), and affected males dying in the first week of life (uncle, cousin, and siblings). We suggested they might also have OTCD, but misdiagnosed.

Approximately 80% of heterozygous females are mostly healthy; however, 20% of those are symptomatic hyperammonemia, in which adult heterozygous females can have hyperammonemia episodes after changing to high protein intake, acute illness, trauma, pregnancy, delivery, or corticosteroid use (38). Therefore, the mothers of patients 1 and 2 are at high risk of OTCD and need to have routine medical monitoring for proper management. Torkzaban et al. (39) summarized that, when the diagnosis of maternal OTCD is known prior to pregnancy, this reduces the maternal and neonatal morbidity and mortality compared to when the diagnosis is obtained during pregnancy. In detail, 31% (5/16) of undiagnosed prior to pregnancy maternal OTCD died, while no one (0/20) was observed in the diagnosed prior to pregnancy group. In consequence, the detection of the carrier status in the mothers of patient 1 and 2 may significantly improve the maternal outcomes of their subsequent pregnancies, as suggested by Torkzaban et al. (39). Moreover, any new offspring from the carriers should also undertake genetic testing to determine the status of their OTC alleles for appropriate genetic counseling and early medical intervention.

Genetic testing identified the mutations in the *OTC* gene in the patients, leading to an accurate diagnosis of the patients with OTCD. Based on these findings, the patients obtained an appropriate management, such as treatment with the drugs for lowering of blood ammoniac level like sodium benzoate, arginine, or citrulline, intake of a protein-restricted diet, and a plan for liver transplantation in severe conditions. The positive genetic results are also useful for genetic counseling. Genetic testing helped to identify the asymptomatic female carriers who might be at risk of hyperammonemia in the future. In addition, genetic testing provided an answer about the family history of the patient 2 and useful information when parents are planning for a new child. Therefore, genetic testing, especially next generation sequencing, is an established test method in clinical managements (40, 41). Integrating genetic and clinical data enhances the care quality for the patients as well as their families (40–43).

CONCLUSIONS

In conclusion, two heterozygous mutations, c.365A>T and IVS7+1G>A, in the *OTC* gene were detected for the first time in two Vietnamese girls with OTCD. Females carrying these mutations should be considered at risk for hyperammonemia episodes and need appropriate counseling and surveillance. Males in these families need emergency treatment plan after delivery. Further studies using animal models are required for the analysis of the detail effect of the mutations with OTCD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Institute of Genome Research (No. 18/QD-NCHG, Institute of Genome Research Institutional Review Board, Hanoi, Vietnam). For minors, written informed consent for sample collection and genetic analyses were signed by their parents. Written informed consent was obtained from the minor(s)' parents, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

H-HN and NK conceptualized, designed the study, and wrote and reviewed the manuscript. CD provided patients'

clinical information and reviewed the manuscript. N-LN performed whole-exome sequencing, analyzed data, and wrote and reviewed the manuscript. TT carried out Sanger sequencing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00321/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Novel Compound Heterozygous Mutations in *CRTAP* Cause Rare Autosomal Recessive Osteogenesis Imperfecta

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Whole-exome sequencing (WES) has advantages over the traditional molecular test by screening 20,000 genes simultaneously and has become an invaluable tool for genetic diagnosis in clinical practice. Here, we reported a family with a child and a fetus presenting undiagnosed skeletal dysplasia phenotypes, while the parents were asymptomatic. WES was applied to the parents and affected fetus to identify the genetic cause of the phenotypes. We identified novel compound heterozygous mutations consisting of a single-nucleotide variant (SNV) and a large deletion in the *CRTAP* gene (NM_006371.4:c.1153-3C > G/hg19 chr3:g.32398837_34210906del). Genetic alterations of *CRTAP* are known to cause osteogenesis imperfecta (OI) in an autosomal recessive manner. Further examination of the proband's elder sibling who was diagnosed as OI after birth found that she shares the inherited compound heterozygous mutations of *CRTAP*; thus, the findings support the disease-causing role of *CRTAP* mutations. Through the *in vitro* molecular test and *in silico* analysis, the deleterious effects of the splicing-altering SNV in *CRTAP* (c.1153-3C > G) on gene product were confirmed. Collectively, our WES-based pathogenic variant discovery pipeline identifies the SNVs and copy number variation to delineate the genetic cause on the proband affected with OI. The data not only extend the knowledge of mutation spectrum in patients with skeletal dysplasia but also demonstrate that WES holds great promise for genetic screening of rare diseases in clinical settings.

Keywords: whole-exome sequencing, *CRTAP*, skeletal dysplasia, osteogenesis imperfecta, splicing-altering variant

INTRODUCTION

Skeletal dysplasias are a series of heterogeneous genetic disorders that affect primarily cartilage and bone but also have a significant effect on joints and muscles (Krakow, 2015). According to the 2019 Nosology and Classification of Genetic Skeletal Disorder, skeletal disorders comprise 461 different conditions that are sorted into 42 groups based on their clinical and/or molecular

phenotypes (Mortier et al., 2019). Among them, 425 conditions are shown to be associated with pathogenic variants in one or more of 437 different genes. Mutations in different loci within one gene can cause distinct types of skeletal disorders, while mutations in different genes can lead to the same disease (Foldynova-Trantirkova et al., 2012; Maioli et al., 2019). Therefore, genetic testing is indispensable in conjunction with clinical examination and radiographs for diagnostic confirmation. The encoding proteins of those disease-causing genes have diverse functions involving different signaling pathways, such as NOTCH, BMP, or TGFβ signaling, which are essential for skeletal development, growth, or homeostasis (Krakow, 2015). Interestingly, some pathogenic variants in mitochondrial-associated proteins have been shown to cause skeletal dysplasias, which is not common for mitochondria-related disorders (Mehawej et al., 2014; Dikoglu et al., 2015; Girisha et al., 2019; Peter et al., 2019). In addition to the protein-coding gene, the epigenetic alteration, first included in 2019 Nosology, indicates a novel gain-of-function variant in microRNA miR-140, which causes autosomal dominant skeletal dysplasia (Grigelioniene et al., 2019; Mortier et al., 2019). Although there has been substantial progress in understanding the genetic landscape of skeletal dysplasias, many more genes or mutations may be involved given the complicated phenotypes and the heterogeneous nature of the disorders. Given this complexity, genetic diagnosis of skeletal dysplasia has been a great challenge due to the involvement of a variety of genetic alterations (Zabel and Spranger, 2017; Mortier et al., 2019).

Among all skeletal disorders, osteogenesis imperfecta (OI) is the most common one that presents with decreased bone density, and the severe forms may present in the newborn period with fractures sustained *in utero* (Krakow, 2015). OI is a collective term for a group of rare connective tissue disorders that share similar skeletal deformities and are characterized by low bone mass and extremely fragile bones. Due to its phenotypical and genetic heterogeneity, recent nosology has classified OI into types ranging up to XIX (Mortier et al., 2019; Rossi et al., 2019). The incidence of OI varies across different populations, ranging from 0.3 to 1.5 per 10,000 births (Orioli et al., 1986; Kuurila et al., 2002; Stevenson et al., 2012; Folkestad et al., 2016), with the majority of defects in type I collagen structure and synthesis. Autosomal dominant mutations in *COL1A1* and *COL1A2*, coding for the α1(I) and α2(I) chains of type I collagen, account for 85–90% of all cases (Van Dijk and Sillence, 2014). In addition to the autosomal dominant inheritance, mutations in the genes involved in collagen-related metabolic pathways, such as posttranslational modification, folding and cross-linking, bone mineralization, and osteoblast differentiation with collagen insufficiency, are known to cause an autosomal recessive form of OI (Barnes et al., 2006; Forlino and Marini, 2016). Cartilage-associated protein (CRTAP), which forms a trimeric complex with P3H1 and cyclophilin B for 3-hydroxylation of proline 986 residue of collagen α1(I) and proline 707 residue of collagen α2(I) (Morello et al., 2006; Mehawej et al., 2014), respectively, was found to cause an autosomal recessive form of OI (Morello et al., 2006; Girisha et al., 2019). *CRTAP* deficiency leads to disruption of chain alignment and helical folding and results in defects of collagen synthesis (Pyott et al., 2011). The affected OI patients who were attributed to *CRTAP* mutations have been classified as type VII in the Online Mendelian Inheritance in Man (OMIM) database (OMIM #610682). Notably, the symptom and severity of OI are highly heterogeneous from case to case, even among individual patients carrying the same variant within the family (Forlino and Marini, 2016). Therefore, among the clinically indistinguishable cases, especially in the perinatal period, the genetic testing of OI is a critical and necessary step for diagnostic confirmation (Marini et al., 2017).

The traditional genetic carrier screening is based on an individual's ethnic background or family history and is offered with a limited number of causative mutations leading to the specific diseases (Lazarin et al., 2013). This strategy has some restrictions, especially in the situation where the proband's phenotype is not well defined and/or the causative genetic defects to the disease are highly heterogeneous. Therefore, expanded carrier screening has been recommended and requires a more comprehensive technique to identify carriers for a broader array of diseases (Edwards et al., 2015; Antonarakis, 2019). With the advances of sequencing technology over the past decade, whole-exome sequencing (WES) shows the potential to be widely used in the procedure of expanded carrier screening in a clinical setting, especially for rare diseases that have undergone exhaustive evaluations and for which an etiologic diagnosis is still unable to be provided (Posey et al., 2016; Sawyer et al., 2016). WES screens thousands of genes simultaneously by sequencing DNA in the protein-coding regions of an individual's genome (also known as exomes). Although WES sequence accounts for less than 2% of the entire human genome, it contains more than 85% of known disease-causing variants (Sims et al., 2014; Salk et al., 2018). Importantly, in addition to single-gene Mendelian disorders, several studies recently showed that WES enables identification of multilocus pathogenic variations in families with complex clinical indications and/or variable severities (Karaca et al., 2018; Alves et al., 2019). Results from these studies further highlight the promising land of WES in genomic medicine.

Here, we report an affected fetus with complex and undiagnosed skeletal phenotypes, short long bones and multiple severe fractures, while the parents are phenotypically asymptomatic. We applied the WES pipeline and identified that the proband harbored a novel compound heterozygous single-nucleotide variant (SNV) and deletion in the *CRTAP* gene. The mutations were validated by Sanger sequencing (Sanger-seq) and array comparative genomic hybridization (aCGH). The proband's elder sibling, who was diagnosed as OI, was also confirmed to have the compound heterozygous mutations in the *CRTAP* gene. In particular, we uncovered the novel splicing variant in the *CRTAP* gene as a deleterious mutation by showing the reduced mRNA level as a consequence of mutation-mediated mRNA decay. We further assessed and confirmed the pathogenicity of the novel compound mutations in *CRTAP* that cause OI. Our findings uncover novel pathogenic variants that explain the underlying genetic basis for phenotypic expansion and provide strong support to apply the WES pipeline in genetic diagnosis, especially for highly heterogeneous rare diseases like skeletal dysplasia.

MATERIALS AND METHODS

Subjects and DNA Isolation

This study included a family with two individuals affected with skeletal dysplasia. The parents (**Figure 1A**, Individuals I and II) and proband (**Figure 1A**, Individual IV) requested a molecular genetic testing procedure for future prenatal diagnosis. The family underwent clinical examinations at the Fetal Medicine Center (FMC, Taiwan) and the Department of Pediatrics, Chi Mei Medical Center (Taiwan). Informed consent was obtained from all participants. The proband's sibling (**Figure 1A**, Individual III) had a clinical diagnosis of OI after birth based on radiographic findings. Due to frequent long bone fractures, she has been on pamidronate therapy since the age of 1 year and 4 months. The parents were asymptomatic and had no family history of skeletal dysplasia. Written and signed informed consent was obtained from all subjects of this study or their legal guardians and approved by the Institutional Review Board (IRB) of the National Cheng Kung University Hospital. Written informed consent for the publication of any potentially identifiable images or data included in this article was obtained from the individuals or their legal guardians.

Genomic DNA was isolated from peripheral blood (Individuals I, II, and III) and umbilical tissue (proband, Individual IV) using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany; cat. no.: #51104) supplemented with Buffer ATL (QIAGEN; cat. no.: #19076) according to the manufacturer's instructions.

Library Preparation, Exome Enrichment, and Sequencing

Approximately 100 ng DNA was sheared using the Covaris S2 system (Covaris Inc., Woburn, MA, United States) to generate fragmented DNA mainly between 150 and 300 bp. The fragmented DNA was used for library construction using the KAPA HyperPrep Kit (KAPA Biosystems, Wilmington, MA, United States; cat. no.: #07962312001). The quantity of library was determined using the Qubit dsDNA HS assay kit with the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, United States). A total of 1,000 ng mixed DNA libraries was captured using the SeqCap EZ MedExome Enrichment Kit (Roche NimbleGen Inc., Madison, WI, United States; cat. no.: #07676581001), which targets ∼19,390 genes covering 47 Mb genomic sequences. The captured exomes were subsequently quantified by the KAPA Library Quantification Kit (KAPA Biosystems; cat. no.: #07960336001), followed by sequencing using NextSeq 550 (Illumina, San Diego, CA, United States) with NextSeq 500/550 High Output v2 kit (300 cycles: 2 × 150 bp paired-end reads) according to the protocol provided by the vendor.

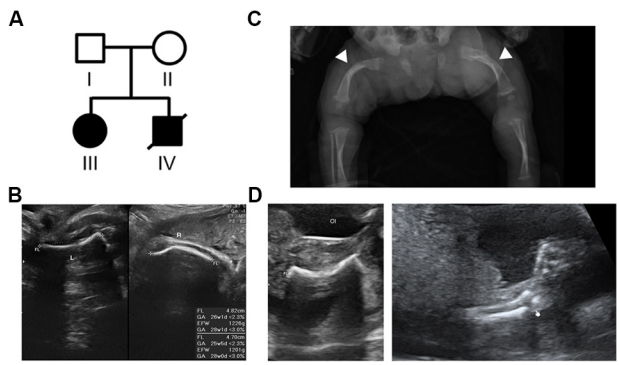


FIGURE 1 | Pedigree of the family with skeletal dysplasia and their phenotypes. **(A)** Pedigree of the family with skeletal dysplasia. **(B)** Prenatal ultrasound images of patient III show both short left (L) and right (R) femurs. **(C)** Radiography of patient III after birth shows osteopenia and curvature and fractures of the femurs (arrow heads) with widening of the distal diaphysis. **(D)** Ultrasound images of the proband (patient IV) show the short and fractured femur (left) and fractured radius (right).

Bioinformatics Pipeline for Mapping and Variant Calling

An in-house bioinformatic pipeline was built to process sequencing reads. Briefly, the sequencing reads were first trimmed using the Trimmomatic tool (base quality ≥ Q20), followed by a quality check using the FastQC tool. Next, the trimmed reads were mapped against the human reference genome (hg19,[1]) using the BWA-MEM algorithm (Li and Durbin, 2009). The output SAM file was then converted to a BAM file, and only the unique mapped reads were used for variant calling using the Genome Analysis Toolkit (GATK) platform[2] according to the "Best Practices" guidelines (version 4) from the Broad Institute. GATK-Mutect2 and FilterMutectCalls were conducted to identify SNVs and insertions and deletions (indels) using default parameters. The output Variant Call Format (VCF) file was annotated with various public databases, including dbSNP[3], SIFT[4], PolyPhen-2[5], MutationTaster[6], PROVEAN[7], FATHMM[8], gnomAD database[9], 1000 Genomes projects[10], and ClinVar[11], using the ANNOVAR program (Wang et al., 2010). In addition, the minor allele frequency (MAF) was calculated from 996 Taiwanese whole-genome sequencing (WGS) VCF data (Lin et al., 2018) and used as a plugin in our pipeline to delimitate pathogenic variant(s).

Detection of Copy Number Variation (CNV)

To analyze the regional DNA copy changes from each WES sample, the CNVkit (Talevich et al., 2016) that uses

[1]http://genome.ucsc.edu
[2]https://software.broadinstitute.org/gatk/
[3]https://www.ncbi.nlm.nih.gov/projects/SNP/
[4]https://sift.bii.a-star.edu.sg/
[5]http://genetics.bwh.harvard.edu/pph2/
[6]http://www.mutationtaster.org/
[7]http://provean.jcvi.org/index.php
[8]http://fathmm.biocompute.org.uk/
[9]https://gnomad.broadinstitute.org/
[10]https://www.internationalgenome.org/
[11]https://www.ncbi.nlm.nih.gov/clinvar/

both the targeted reads and the non-specifically captured off-target reads to infer copy number evenly across the genome was applied, and this was generated by the BWA-MEM algorithm. Briefly, the target and off-target coverages of each sample were calculated based on the read counts per bin using the BAM file as input. The averages of target and off-target coverages from normal populations served as a reference. Normalization of samples to reference was performed and converted to log2 values. The segmentation was then conducted using the Circular Binary Segmentation algorithm by the default setting. Finally, a scatter plot was drawn using the normalized log2 value of each bin together with the segments.

In addition, the raw WES read counts of each exon within chr3p22.3 (31573992. . .36986548) were extracted and normalized to the total mapped read counts to obtain the CNV ratio within the target region. This was followed by summing up all normalized read counts in a given gene and the maternal-to-paternal gene ratio or proband-to-paternal gene ratio across the chr3p22.3, which was then plotted into a graph according to the genomic coordinates. The deleted region identified from the WES data was confirmed by aCGH using the CytoOneArray® (Phalanx Biotech, Taiwan), which is designed to identify chromosomal microduplications and microdeletions, especially in the disease-related genomic regions, using the genomic DNAs from both parents and proband.

Sanger Sequencing

The primers used for amplifying genomic regions of *CRTAP* and *MESP2* are as follows: *CRTAP*, forward sequence 5′-CAA ATG GAG TGG AAG CCG AGG T-3′, reverse sequence 5′-GGG CTG TTG GGA AAA GGA CAA-3′; and *MESP2*, forward sequence 5′-GAG CCC AAG CCG CAC-3′, reverse sequence 5′-GGG TTC CTT CCA TTC TCC C-3′. The PCR products were sequenced on the ABI PRISM 3130XL Genetic Analyzer.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from the paternal, maternal, and normal control's buffy coat using TRIzol (Invitrogen, Carlsbad, CA, United States) and purified by ethanol precipitation. Reverse-transcription and quantitative PCR assays were performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, United States; cat. no.: #4368813).

For quantification of *CRTAP* mRNA in pre-mRNA and mature mRNA levels, KAPA SYBR Fast qPCR kit (KAPA Biosystems, Wilmington, MA, United States; cat. no.: #KK4973) was used, and the beta actin (*ACTB*) level was used as an internal control. All reactions were run on the SYBR program at the default setting using an Applied Biosystems StepOnePlus Real-Time PCR system in a 96-well plate format. Primer sequences for specific genes are as follows: for pre-mRNA of *CRTAP*, forward sequence 5′-GAC CAC ACT CCA GAA GGA GC-3′, reverse sequence 5′-ACT TCA GGG AGG ACT CAG CC-3′; for mature mRNA of *CRTAP*, forward sequence: 5′-GAC AAG GTC ATG CAG CAG AA-3′, reverse sequence 5′-CTG AAC GCC AAG AGG AAG TC-3′; and for *ACTB*, forward sequence 5′-GCC CTG AGG CAC TCT TCC A-3′, reverse sequence 5′-CGG ATG TCC ACG TCA CAC TT-3′.

RESULTS

Case Presentation

This study included a family with two affected offspring with skeletal disorders, while both the parents are aged in their 20s, healthy (**Figure 1A**, Individuals I and II), and without a family history of skeletal disorders. The proband's elder sister (**Figure 1A**, Individual III) presented short limbs on prenatal ultrasound images (**Figure 1B**) and was diagnosed with OI after birth because of curvature and fractures of bilateral femurs on X-ray (**Figure 1C**). Due to frequent long bone fractures, she received regular intravenous pamidronate therapy from the age of 1 year and 4 months, and the follow-up bone mineral density and fracture rates significantly improved. The aborted proband (**Figure 1A**, Individual IV) exhibited short long bones and multiple fractures on ultrasound images at 21 weeks of gestational age (**Figure 1D**), indicating symptoms which are related to OI and more severe than that observed in his sibling. The pedigree of normal parents with two affected offspring implies an autosomal recessive inheritance underlying the skeletal dysplasia within this family.

Identification of Candidate Mutations in Proband Using WES

To identify the genetic cause of skeletal disorder in this family, we first screened *COL1A1* and *COL1A2* genes using Sanger sequencing, the two most common genes that are known to cause OI, but this revealed no mutations. Next, we applied WES for the trio study using the proband's umbilical DNA and parents' blood DNA to examine the genetic basis of the skeletal disorder. The sequencing was conducted on the illumine NextSeq 550 platform at a sequencing depth of ∼100×. The sequencing reads covered 99.8% of human exonic regions, while the uniformity and on-target rate were 97.0 and 82.8%, respectively. The mapping reads were subjected to the GATK (McKenna et al., 2010) for variant calling with the default parameters of the Mutect2 tool.

With the attempt to identify causative genetic mutation(s), we developed a "pathogenic variant discovery pipeline" (**Figure 2A**) to annotate the sequence variants obtained from exome sequencing. The number of variants generated after each filtering step in our pipeline (**Figure 2A**) is depicted and shown in the Sankey diagram (**Figure 2B**). In general, the WES procedure discovers 130–134 k variants per individual. Among all the variants, we focused on those belonging to the clinically relevant genes derived from the OMIM database and obtained around 27–28 k variants per individual (**Figure 2B**). Next, we selected variants that lead to amino acid changes or splicing errors and reduced the number to ∼2 k variants per individual. This was followed by filtering the remaining variants using a MAF less than 5% in the Taiwanese population (data derived from Taiwan Biobank, Academia Sinica, Taiwan

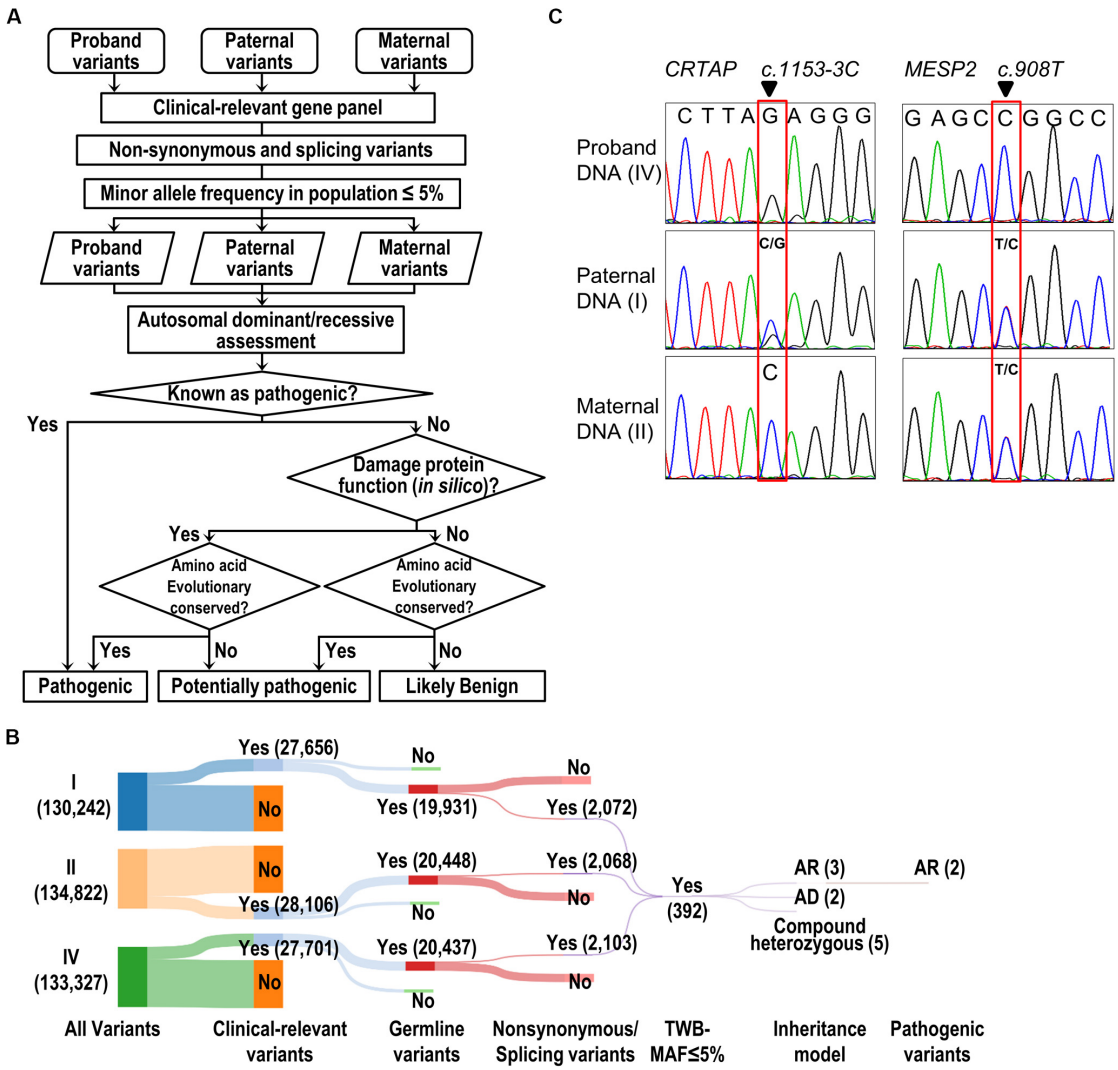


FIGURE 2 | Identification and validation of pathogenic variants. **(A)** The flow of the pathogenic variant discovery pipeline. The genetic variants were obtained by GATK variant calling algorithm. The SIFT, PolyPhen-2, and MutationTaster were used for prediction of the effect of a variant on the damage of protein function. Human Splicing Finder and dbscSNV were used to predict the consequence of splicing variant. The evolutionary conservation was scored by the phastCons tool. **(B)** The Sankey diagram shows the reduced volume of variants from the trio study (Individuals I, II, and IV). The number of variants is shown in parentheses. **(C)** Validation of WES results by Sanger-seq at *CRTAP* and *MESP2* loci.

(Lin et al., 2018), from which we obtained 392 variants merged from all three family members. We further filtered variants to meet with the recessive inheritance according to the family transmission pattern. Finally, we evaluated the predicted impacts of variants on the gene product or protein function, together with the conservation of genomic positions of each variant, and therefore pinpointed two candidate variants: a splicing variant (c.1153-3C > G) in the *CRTAP* gene and a missense variant (c.908T > C, p.Leu303Pro) in the *MESP2* gene (**Figure 2B** and **Table 1**).

The Sanger sequencing was performed to confirm that the proband was homozygous at the position c.1153-3C > G in *CRTAP* gene and c.908T > C in *MESP2* gene (**Figure 2C**). The father carries heterozygous variants in *CRTAP* (c.1153-3C > G) and *MESP2* (c.908T > C), while the mother harbors wild-type *CRTAP* and a heterozygous variant in *MESP2* (c.908T > C) (**Figure 2C**).

Confirmation of Causing Compound Heterozygous Mutations of CRTAP (c.1153-3C > G/deletion) in the Proband

Notably, we found that the mother showed the homozygous wild type at position c.1153-3C of the *CRTAP* gene. As the proband showed homozygous paternal mutant alleles, it is hard to explain the missing allele transmitted from mother to the offspring (**Figure 2C**). We suspect that the mother may have a deletion within this region. To analyze CNV, we applied the

TABLE 1 | Detailed information of variants in *CRTAP* and *MESP2* genes.

Gene	Coordinate[a]	Mutation	Type	Amino acid change	Population MAF (Taiwanese)[b]	Population MAF (EAS)[c]
CRTAP	chr3:33183884	NM_006371.4 c.1153-3C > G	Splicing altering	-	0.001	<0.0001
MESP2	chr15:90320496	NM_001039958.2 c.908T > C	Missense	p.Leu303Pro	0.034	0.020

[a] *Genomic coordinate from GRCh37 (also known as hg19).* [b] *The population MAF was derived from Taiwan Biobank (Academia Sinica, Taiwan). MAF, minor allele frequency.* [c] *The population MAF was derived from the 1000 Genomes Project. EAS, East Asian.*

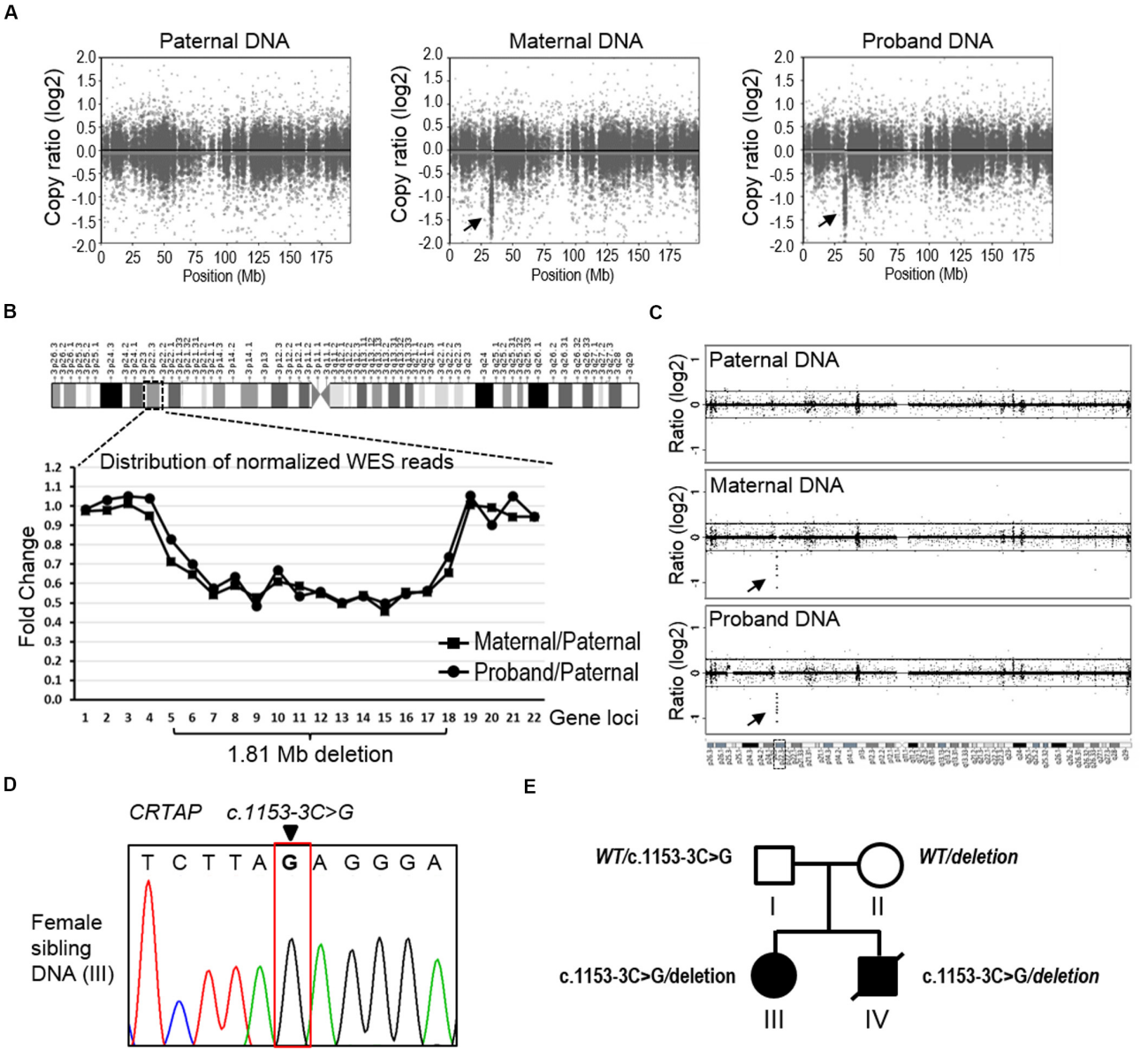


FIGURE 3 | The detection of copy number variation of the *CRTAP* gene. **(A)** The CNVkit was conducted to call CNV using WES data from the father (left), mother (middle), and proband (right). The scatter plot of chromosome three shows a ∼1.81 Mb deletion at the p22.3 region in both maternal and proband samples. The arrows indicate discrete copy number segments across chromosome 3. **(B)** The distribution of normalized WES reads shows the 1.81 Mb deletion that covers 14 gene loci across the chr3p22.3 in the maternal and proband DNA. The ratio of normalized read counts, maternal/paternal or proband/paternal, of each gene was plotted in the graph according to the genomic loci of the successive 22 genes in chr3p22.3. *CRTAP* is the no. 13 gene (*X*-axis). **(C)** The data of aCGH show a deletion in the maternal and proband DNA in the chr3p22.3 region. The arrows indicate the region of detected deletion. **(D)** Sanger-seq at the *CRTAP* locus from the elder sibling of the proband (Individual III). **(E)** The summary of mutation and inherited genotype of *CRTAP* (c.1153-3C) in the family with osteogenesis imperfecta.

CNVkit, a tool developed specifically to call CNV using data from WES or targeted-seq (Talevich et al., 2016), to search for the explanation for the missing maternal allele. Results from the CNVkit clearly showed that the mother carried a ∼1.81 Mb deletion at chr3p22.3 (hg19, chr3:g.32398837_34210906del), and she passed this deletion to the proband, while the father did not have any CNV at this region (**Figure 3A**). In parallel, we plotted the copy number ratios using normalized read counts from the mother or proband compared to that of the father's (**Figure 3B**). Consistent with the results from the CNVkit, we found both mother and proband exhibited this 1.81 Mb deletion covering 14 gene loci on chr3p22.3 (**Figure 3B**). The regional deletion was confirmed by aCGH (**Figure 3C**). Collectively, these data showed that the father is a carrier of the *CRTAP* splicing mutation (c.1153-3C > G), while the mother carries a 1.81 Mb deletion on the chr3p22.3 region that contains the *CRTAP* gene. The proband thus has inherited compound heterozygote mutations that are one deletion and one SNV of the *CRTAP* gene from the parents. Notably, the identified SNV and large deletion of *CRTAP* have not hitherto been reported in the literature, nor have they been collected in the Human Gene Mutation Database[12] (**Supplementary Table 1**), thus indicating that the compound heterozygous variants (c.1153-3C > G/deletion) represent novel *CRTAP* mutations.

In addition, we examined the elder sibling of the proband who was diagnosed with OI and found that she harbored the same *CRTAP* compound heterozygous variants (c.1153-3C > G/deletion, **Figure 3D**), indicating the disease-causing role of the *CRTAP* mutations (**Figure 3E**).

TABLE 2 | Prediction of functional consequence caused by *CRTAP* and *MESP2* mutations and the pathogenicity classification[a].

Gene Mutation		*CRTAP NM_006371.4 c.1153-3C > G*	*MESP2 NM_001039958.2 c.908T > C*
Category	**Tool**	**Score (consequence)**	**Score (consequence)**
Splicing altering effect	dbscSNV_ADA	0.999 (splicing altering)	–
	dbscSNV_RF	0.932 (splicing altering)	–
	Human splicing finder	Broken WT acceptor site	–
Protein function	SIFT	–	0.02 (deleterious)
	PolyPhen-2	–	0.33 (benign)
	PROVEAN	–	−1.83 (neutral)
	FATHMM	–	−2.17 (deleterious)
Gene/protein function	MutationTaster	Disease causing	Disease causing
Nucleotide conservation	PhastCons	0.911 (conserved)	0.841 (conserved)
Variant classification	ACMG	Pathogenic PS3, PM2, PM3, PM4	Benign PM1, BS1, BS2, BP6

[a]*Variant classification is based on the criteria addressed in ACMG standards and guidelines. dbscSNV, database of splicing consensus single-nucleotide variants; SIFT, the sorting intolerant from tolerant; PolyPhen-2, polymorphism phenotyping v2; PROVEAN, protein variation effect analyzer; FATHMM, functional analysis through hidden markov models; –, not applicable.*

Exclude the Involvement of MESP2 Mutation (c.908T > C) in the Clinical Phenotype of the Proband

The missense mutation of *MESP2* (c.908T > C, p.Leu303Pro) is located in a critical functional domain, and the affected amino acid of MESP2 (p.Leu303) is highly conserved across species (data not shown), and this was also indicated by the phastCons analysis (0.841) (Siepel et al., 2005) on the conservation of genomic position (**Table 2**). However, the mutation was predicted to be tolerated by PolyPhen-2_HDIV (0.331) and PROVEAN (−1.83), has been observed in 23 healthy adults in homozygous state, and reported a MAF of 0.0047 in the gnomAD exomes database. Furthermore, *MESP2* (c.908T > C, p.Leu303Pro) was classified as a benign variant based on the ClinVar database or according to the American College of Medical Genetics and Genomics (ACMG) guidelines (**Table 2**). Taken together, we thus excluded the involvement of the *MESP2* (c.908T > C) mutation in the clinical phenotype of the proband and dropped it from further analysis.

Biological Effect and Clinical Significance of CRTAP Mutation

The genomic position of the *CRTAP* mutation (c.1153-3C) was highly conserved as shown by phastCons (0.911) analysis (Siepel et al., 2005), and the mutation was predicted to be "disease causing" using the MutationTaster tool (**Table 2**). To determine the effect of the splicing variant of the *CRTAP* gene, the Human Splicing Finder and Database of Splicing Consensus Single-Nucleotide Variants (dbscSNV) were used to predict the consequence of *CRTAP* (c.1153-3C > G) mutation. The *CRTAP* c.1153-3C is located at the -3 position upstream from exon seven in intron six (**Figure 4A**). The C > G at the -3 position was predicted to damage the splicing process and break the original splicing acceptor site by dbscSNV and Human Splicing Finder, respectively, which resulted in intron six retention (**Table 2** and **Figure 4B**). Sequence analysis indicates that the intron retention leads to a premature termination codon after five additional amino acids downstream from the junction of exon–intron six, and thus, it may induce nonsense-mediated mRNA decay (NMD) (Isken and Maquat, 2007; Hug et al., 2016). On the other hand, the deletion of one allele of the *CRTAP* gene has been shown to decrease the expression level of mature mRNA (Morello et al., 2006).

It is expected that deletion of *CRTAP* results in reduction of both pre-mRNA and mature mRNA, whereas the intron retention-mediated NMD is supposed to decrease mature mRNA without changing the pre-mRNA level. To this end, we examined the expression levels of pre-mRNA (**Figures 4A,B**, F1 and R1) and mature mRNA (**Figures 4A,B**, F2 and R2) of *CRTAP* using quantitative RT-PCR in the paternal sample harboring the heterozygous *CRTAP* c.1153-3C > G mutation and the maternal

[12]http://www.hgmd.cf.ac.uk/ac/index.php

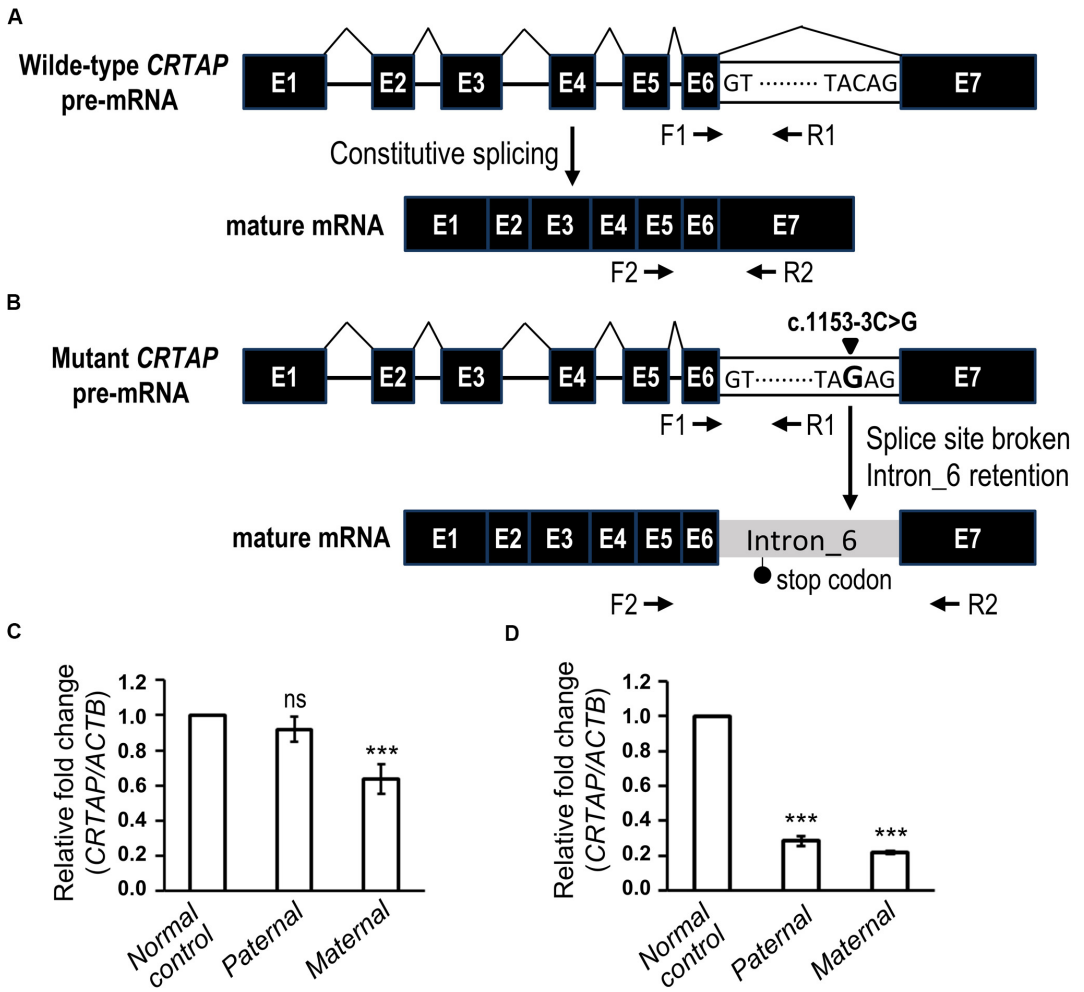


FIGURE 4 | The biological effect of the splice variant of *CRTAP* (c.1153-3C > G). **(A,B)** Scheme depicting the consequence of RNA splicing of wild-type *CRTAP* pre-mRNA **(A)** or mutant *CRTAP* (c.1153-3C > G) pre-mRNA **(B)**. The mutation of the consensus splice acceptor site of *CRTAP* results in a broken splice site and intron six retention and generates a premature termination codon (stop codon). The arrows indicate the primers designed for amplification of pre-mRNA (F1/R1) or mature mRNA of *CRTAP* (F2/R2). **(C,D)** Quantitative PCR analysis shows the relative expression of the *CRTAP* pre-mRNA level **(C)** and mature mRNA level **(D)** of the paternal, maternal, or normal control sample. Error bars represent SEM, $N = 3$. *P*-values are calculated with a two-tailed *t*-test. $^{***}P < 0.001$; ns, not significant.

sample harboring the heterozygous *CRTAP* deletion. In line with our prediction, the paternal sample expressed the same level of *CRTAP* pre-mRNA as the normal control (**Figure 4C**), while the paternal mature mRNA level was dramatically reduced to the same level as the maternal sample (**Figure 4D**). In contrast, both *CRTAP* pre-mRNA and mRNA levels of the maternal sample were lower compared to those of the normal control (**Figures 4C,D**). These data demonstrated that the splicing mutation in the *CRTAP* gene (c.1153-3C > G) results in a splicing error and a dramatic decrease of *CRTAP* mRNA.

According to the guidelines of sequence variant interpretation made by the ACMG (Richards et al., 2015), we confirmed that the splicing mutation in the *CRTAP* gene (c.1153-3C > G) significantly decreased the gene product [as a strong evidence of pathogenicity 3 (PS3)], resulted in length change of the gene product [as a moderate evidence of pathogenicity 4 (PM4)], and were detected in trans with a pathogenic variant at very low frequency (as evidence of PM2 and PM3). Therefore, the *CRTAP* c.1153-3C > G mutation was classified as a pathogenic variant (**Table 2**).

DISCUSSION

Due to the strong clinical and genetic heterogeneities, searching for the causative mutations of skeletal dysplasia remains a task with big challenges. With the advance of next-generation sequencing (NGS) technology, attempts have been made to implement and apply it to offer a comprehensive strategy in clinical practice (Liu et al., 2019; Yang et al., 2019). In the current study, through integration of WES and molecular analyses, we identified novel pathogenic compound mutations in patients affected with OI: a splicing error-causing mutation (NM_006371.4:c.1153-3C > G) and a large deletion in the *CRTAP*

gene (hg19, chr3:g.32398837_34210906del). The pathogenicity of the novel mutation was also evaluated according to the latest guidelines of sequence variant interpretation made by ACMG (**Table 2**).

OI is a collective term for a group of rare connective tissue syndromes characterized by extremely fragile bones. It has been reported that *COL1A1* and *COL1A2* mutations account for around 90% of all cases of OI in an autosomal dominant manner (Van Dijk and Sillence, 2014), while the remaining mutations of the *CRTAP* (Type VII, OMIM #610682) and *P3H1* (Type VIII, OMIM #610915) genes are responsible for rare and severe to intermediate OI in an autosomal recessive manner (Byers and Pyott, 2012). Previous studies demonstrated that CRTAP protein is required for 3-hydroxylation of proline 986 residue of collagen α1(I) (Morello et al., 2006), and its deficiency leads to defects of collage type I biosynthesis through disrupting chain alignment initiation and helical folding (Pyott et al., 2011). Among the reported 23 *CRTAP* pathogenic mutations, the majority of them are SNVs (14) and small indels (8) and result in premature termination codons, while the remaining one is a gross deletion of the promoter to the intron 1 region in the *CRTAP* gene (**Supplementary Table 1**). The data suggest that the amount of gene products (protein) is the key factor influencing the collage type I biosynthesis. Here, we report the findings of novel compound heterozygous *CRTAP* mutations (c.1153-3C > G/deletion) identified in the proband and his sibling. By using quantitative RT-PCR, our results revealed a dramatic loss of *CRTAP* mRNA expression through both chromosomal deletion of one allele and probably the NMD of mRNA from the splicing variant. It has been shown that null mutations with a dramatic reduction of *CRTAP* gene products as well as 3-hydroxylation of the Pro986 residue of collagen α1(I) cause severe OI (Marini et al., 2010). Given that the CRTAP protein mutually stabilizes P3H1 in the collagen prolyl 3-hydroxylation complex (Chang et al., 2010), the reduction of CRTAP expression thus has great impact on the collage type I biosynthesis and leads to the observed OI phenotype. Consistent with clinical indications, the sibling was diagnosed as OI with mitral regurgitation and developmental delay, who is now under pamidronate infusion therapy. Notably, the mother harbored a heterozygous 1.81 Mb deletion in chr3p22.3 that covers 14 gene loci, but she is clinically asymptomatic. Among genes within this region, only two genes, *CRTAP* and *GLB1*, have been reported to be associated with genetic diseases, both of which are manifested in an autosomal recessive manner. As the mother is a heterozygote for the deletion in chr3p22.3, our WES data confirmed that she does not carry any pathogenic variant in the other allele of the *GLB1* gene, explaining the observation of a clinically normal phenotype.

The majority of variants in the human population are rare due to *de novo* mutations occurring in each generation (1000 Genomes Project Consortium et al., 2012, 2015). Most of these rare variants are non-functional or neutral, and as such, they could accumulate in human populations (Harpak et al., 2016; Lappalainen et al., 2019). In line with the previous findings, we analyzed the variants in the Taiwanese population (Lin et al., 2018) and found similar features. Among 38.9 million variants discovered from WGS in 996 Taiwanese individuals, 31.9 million variants (81.8%) are of low to rare frequency (MAF < 5%). Nevertheless, there are 3,008 (0.0094%) known disease-causing variants that correspond to 2,354 genes in these low- to rare-frequency variants. Although purifying selection may remove a number of deleterious variants from populations (Karczewski et al., 2019), some deleterious variants will be retained and predispose to a disease in an autosomal recessive manner. Taking skeletal dysplasia as an example, we found that 64 out of 3,008 known disease-causing variants are involved in skeletal dysplasia, while 54 variants (84.4%) cause skeletal dysplasia by autosomal recessive inheritance. Most importantly, the MAF of the *CRTAP* mutation identified in this study is 0.1% in Taiwanese people; therefore, we shall take extra precautions to evaluate low-MAF allele (0.1-5%) for the possible pathogenicity leading to autosome recessive diseases in the population. The expanded carrier screening is of importance to identify such variants in couples before pregnancy. Once the couples are found to be at risk for transmitting certain types of diseases, *in vitro* fertilization and preimplantation genetic testing (PGT) for monogenic disorders may be considered to stop transmitting deleterious variants into the next generation (Daar et al., 2018; Fu et al., 2019; van der Schoot et al., 2019).

In summary, our data uncover novel compound pathogenic variants that explain the underlying genetic basis for OI in a Taiwanese family. We not only expand the mutation scale of skeletal dysplasia but also provide a molecular insight into the effects of these mutations. In addition, we hope to draw more attention to the abundant low-frequency variants leading to autosomal recessive diseases. The proper application of WES technology in the expanded carrier test shall provide indispensable value to reduce the detrimental effect of these variants on the human population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB of National Cheng Kung University Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Y-AT and HS conceived and designed this study. Y-AT, C-MC, and HS processed, analyzed, and interpreted the data. L-YW, I-WL, and W-HT contributed in the assessment and interpretation of the clinical symptoms and data. I-WL and W-HT contributed in

the recruitment of patients in the hospital. Y-AT and HS wrote the manuscript. All authors contributed to the improvement of the manuscript and read the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00897/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Genetics

CASE REPORT
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Next-Generation Sequencing Revealed Disease-Causing Variants in Two Genes in a Patient With Combined Features of Spherocytosis and Antley-Bixler Syndrome With Genital Anomalies and Disordered Steroidogenesis

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Conventionally, patients with combined rare diseases are often difficult to diagnose. This is because some clinicians tend to consider the multiple disease symptoms as the presentation of a complicated "syndrome." This pattern of thinking also confines their way of filtering pathogenic mutations. Some real pathogenic mutations might be ignored due to not covering all disease presentations. Here we report the case of a girl who was suffering from spherocytosis and Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis. She remained undiagnosed even after targeted gene detection before. However, after performing next-generation sequencing and analyzing the sequencing data, we identified two mutations: c.2978T > A in *ANK1* and c.1370G > A in *POR*. Our findings and experiences in diagnosing these mutations could contribute to the existing knowledge on the clinical and genetic diagnosis of patients with disease presentations in multiple systems.

Keywords: combined disease symptoms, multi-system involved, congenital adrenal hyperplasia, hereditary spherocytosis, POR, ANK1

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INTRODUCTION

Hereditary spherocytosis (OMIM 182900), with a prevalence of 1/2,000, is a hemolytic disorder characterized by the presence of spherical erythrocytes on a peripheral blood smear (Gallagher, 2005; Perrotta et al., 2008; Yocum et al., 2012). Clinical presentations include anemia, jaundice, and splenomegaly with heterogeneous severity (Perrotta et al., 2008). Genes related to hereditary spherocytosis include *ANK1*, *SLC4A1*, *SPTB*, *EPB42*, and *SPTA*. Among them, *ANK1* is the most common disease-causing gene (Yocum et al., 2012). The *ANK1* gene (OMIM*612641) encodes

human erythroid ankyrin containing 1,880 amino acids. It combines tetramers of spectrin with the cytoplasmic domain of band three to form a spectrin-actin based membrane skeleton. This skeleton further interacts with the plasma membrane to stabilize the erythrocyte membrane (Gallagher, 2005). Defects in ankyrin synthesis or structure might result in instability of the erythrocyte membrane, thus causing hereditary spherocytosis.

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders characterized by impaired cortisol biosynthesis due to deficiencies in the various enzymes in the adrenal steroidogenesis pathway (Hannah-Shmouni et al., 2017; El-Maouche et al., 2017; Witchel, 2017). The candidate genes include *CYP21A2*, *CYP11A1*, *CYP11B1*, *CYP17A1*, *HSD3B2*, *StAR*, and *POR* (El-Maouche et al., 2017; Hannah-Shmouni et al., 2017). CAH due to *POR* deficiency is a rare variant of CAH. *POR* (OMIM*124015) encodes cytochrome P450 oxidoreductase. It is a flavoprotein that donates electrons to all microsomal P450 enzymes, including 17OH and 21OH. Therefore, combined 17OH and 21OH deficiency could be observed in the patients (El-Maouche et al., 2017). The female patients with *POR* deficiency always present prenatal virilization, as the alternative pathway of producing androgens is not blocked (Arlt et al., 2004). Besides, some of these patients present skeleton malformations, such as craniosynostosis, radioulnar, and midface hypoplasia. This skeleton malformation presentation involved a form of CAH called Antley-Bixler syndrome or Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis (OMIM 201750) in OMIM.

Herein, we report a Chinese patient presenting combined features of CAH and Antley-Bixler syndrome. Deficiencies in two related genes – *ANK1* and *POR* – were identified by whole-exome sequencing. To our knowledge, patients carrying multiple causative gene mutations are uncommon since most patients are diagnosed as having a "syndrome" when they have symptoms involving multiple systems. Here, we share our experience in the diagnosis of this rare case.

CASE PRESENTATION

Subject and Clinical Features

The study was approved by the ethics committee of the Capital Institute of Pediatrics. Written informed consent was obtained from the patient's parents for the publication of this report and any accompanying images.

The patient was admitted to our hospital due to CAH. The patient was a female aged 10 years and six months. She was the first child born to non-consanguineous parents. She was born by normal cesarean delivery with a birth weight of 3,200 g (P25–P50) and a birth height of 51 cm (P50–P75). Her mother was diagnosed with polycystic ovary syndrome during pregnancy. Our patient presented clitoromegaly at birth with chromatosis of the vulva but not the skin. An ovarian cyst with a size of 5.7 × 3.7 × 4.3 mm was observed on the patient's right ovary when performing a pelvic ultrasound 23 days after birth. A karyotype test showed a normal karyotype of 46 autosomal chromosomes and two sex chromosomes. A biomedical test indicated slight abnormality with increased K+ (5.7 mmol/l) and decreased Na+ (133.9 mmol/l). The concentration of the adrenocorticotropic hormone (ACTH) was 3.09 pg/ml and cortisol was 4.4 μg/dl at 8 am. In a sex hormone test, the luteinizing hormone (LH) was 38.9 μl U/ml, the follicle-stimulating hormone (FSH) was 7.7 l U/l, testosterone (T) was 123.9 ng/dl, and estradiol (E2) was 36.5 pg/dl. When the patient was three years old, she had a clitoridectomy. At the age of five, the patient received gene detection by a gene panel in another hospital. However, the gene result revealed no possible pathogenic mutations (the original samples and results are missing). An ACTH stimulation test identified cortisol (10.5 μg/dl), Testosterone (16.4 ng/dl), and 17-OHP (19.050 ng/ml). Hydrocortisone (5 mg/bid) has been used to treat the patient until present, although no significant progress has been observed. When the patient was 5 years and 6 months old, she was admitted to our hospital due to "fever, fatigue, and pale skin lasting more than 1 week." She was found to have hepatosplenomegaly during a physical test, her liver was palpable at 2 cm and her spleen was palpable at 4 cm below the costal margin. In a routine blood test, her white blood cell count (WBC) was 13.67 × 10 E9/l, hemoglobin (Hb) was 49 g/l, and reticulocyte (Ret) was 0.065. A blood smear revealed that the patient's proportion of spherocytes was 7% and seven nucleated red blood cells per 100 white blood cells could be counted. In an erythrocyte osmotic fragility test, hemolysis began at 0.56% salt solution (normal control at 0.42%) and complete hemolysis began at 0.32% salt solution (normal control at 0.22%). In liver function tests, total bilirubin (TB) was 30.9 μmol/l, direct bilirubin (DB) 12.1 μmol/l, and lactate dehydrogenase (LDH) 382 U/l. A routine bone marrow test showed significantly active bone marrow proliferation and active erythroid proliferation, as well as normal folic acid, vitamin B12, ferritin, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, and pyruvate kinase tests. Moreover, Coomb's test showed negative results. The patient was then clinically diagnosed with hereditary spherocytosis. The patient was then not treated and her jaundice relapsed. She received an ultrasound-guided percutaneous cholecystolithotomy and partial splenic artery ligation when she was 10 years old. The patient's condition soon improved and her jaundice did not return.

Physical Examination During Admission

The patient's weight was 29 kg (P25–P50) and her height was 144.5 cm (P25–P50). Her blood pressure was 100/60 mmHg and her bone age was normal, as was her mental status. A café-au-lait spot with a diameter of 2 cm was observed at the patient's waist. She was in Tanner stage I of puberty. Arachnodactyly with long and slender fingers (**Figure 1a**) and toes (**Figure 1b**) was observed. The fourth and fifth metacarpal bones of the patient's hands were short and thick. Stiffness was observed in the elbow and knee joints. The patient could not make a fist due to joint contracture. Bony swelling was observed on the patient's sacrum.

Imaging Tests

X-ray examination: foot AP axial view: the bilateral fourth and fifth metatarsal bones of the patient were short, the fourth

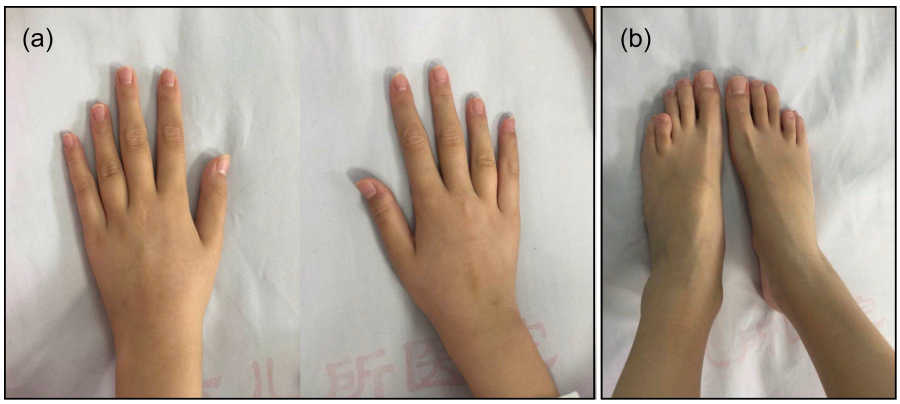


FIGURE 1 | Arachnodactyly with long and slender fingers **(a)** and toes **(b)** could be observed. The bilateral fourth and fifth metacarpal bones and metatarsal bones were all short.

phalanxes were short, and the bone mineral density of both feet was relatively low. Knee AP view: spiking of the bilateral tibial intercondylar eminence was observed. Lumbar spine AP view: slight scoliosis. Elbow AP view: mean trabecular plate density was relatively low. Contrast-enhanced CT scanning: no obvious abnormality was observed in the adrenal glands. However, the intrahepatic bile duct expansion, common bile duct expansion, and splenomegaly were observed.

Laboratory Analyses

Auxiliary examinations: the patient had normal serum electrolytes, while a sex hormone test showed that her FSH was 3.23 IU/l, her LH was less than 0.1 IU/l, and her E2 was less than 18.35 pmol/l. She had testosterone (T) of 559 ng/ml (14–76), prolactin (PRL) of 28.61 ng/ml, progesterone (PROG) of 0.25 nmol/l, and parathyroid hormone (PTH) of 14.5 pg/ml. Her 17-hydroxyprogesterone (17-OHP) was 2.14 ng/ml, her dehydroisoandrosterone (DHEA) was 0.8 ng/ml, her androstenedione was less than 0.3 ng/ml, and her dihydrotestosterone was 13.86 pg/ml. Her cortisol at 8 am, 4 pm, and 0 am were 2.25, 4.9, and 15.71 μg/dl, respectively. Her renin, angiotensin, 1-angiotensin, and 2-aldosterone were 3.08 ng/ml, 37 pg/ml, 40 pg/ml, and 132 pg/ml, respectively at recumbent position, and 3.69 ng/ml, 23 pg/ml, 37 pg/ml, and 282 pg/ml, respectively at erect position. Uterus and ovaries ultrasound images demonstrated that the length of the patient's uterus was 1.65 cm with a diameter of 0.5 cm, and her cervix length was 1.6 cm. The patient's endometrium could not be delineated clearly, not excluding the possibility that a primordial uterus existed.

Treatment

An ultrasound-guided percutaneous cholecystolithotomy and partial splenic artery ligation were performed. After the operation, the intrahepatic bile duct and common bile duct were expanded, the distal common bile duct narrowed, diffuse enlargement was observed, and the middle and lower parts of the spleen were infracted. Hydrocortisone (5 mg/bid) was taken orally by the patient for 6 days until she was discharged.

METHODS

Whole-Exome Sequencing

Proband's DNA was sequenced to discover the causal gene. Next-generation sequencing was performed by Running Gene Inc. following the manufacturer's protocol. DNA was isolated from peripheral blood using a DNA Isolation Kit (Bioteke, AU1802) and 1 μg genomic DNA was fragmented into 200–300 bp lengths using a Covaris Acoustic System. The DNA fragments were then processed by end-repairing, A-tailing and adaptor ligation, a four-cycle pre-capture polymerase chain reaction (PCR) amplification, and targeted sequences capture. Captured DNA fragments were eluted and amplified by 15-cycle post-capture PCR. The final products were sequenced with 150 bp paired-end reads on an Illumina NovaSeq platform according to the standard manual.

The raw data converted by NovaSeq were filtered and aligned against the human reference genome (hg19) using the BWA Aligner[1]. The single-nucleotide polymorphisms (SNPs) were called by using the Genome Analysis ToolKit (GATK) software[2]. Variants were annotated using ANNOVAR[3]. The effects of single-nucleotide variants (SNVs) were predicted by the SIFT, Polyphen-2, and MutationTaster programs. All variants were interpreted according to the standards for the interpretation of sequence variations recommended by ACMG (American College of Medical Genetics and Genomics) and categorized as pathogenic, likely pathogenic, variants of unknown clinical significance (VUS), likely benign, or benign. The associated phenotypic features of candidate genes were analyzed against the patient's phenotype. Core phenotypes were extracted and used to acquire a gene list of the virtual panel by the OMIM database[4] and Mingjian (211.149.234.157/login). Re-annotation was conducted according to the virtual panel.

[1]http://bio-bwa.sourceforge.net/
[2]https://software.broadinstitute.org/gatk/
[3]http://annovar.openbioinformatics.org/en/latest/
[4]http://omim.org/

Sanger Sequencing

The candidate causal genes discovered via whole-exome sequencing (WES) were confirmed by Sanger sequencing, and co-segregation analyses among the family were also conducted. The primers were designed using Primer Premier 5.0 (Premier Biosoft). PCR was conducted to amplify the fragments covering the mutated sites. The PCR products were further purified with a Zymoclean PCR Purification Kit and then sequenced with an ABI 3730 DNA Sequencer. The Sanger sequencing results were analyzed by Chromas Lite v2.01 (Technelysium Pty Ltd., Tewantin, QLD, Australia).

RESULTS

A heterozygous mutation (c.2978T > A) in *ANK1* (NM_000037) and a homozygous mutation (c.1370G > A) in *POR* (NM_000941) were identified by next-generation sequencing (**Supplementary Figure S1**), which resulted in amino acid alterations p.I993N and p.R457H, respectively. According to validation by Sanger sequencing in the proband's family, the proband's parents were both carriers of heterozygous mutation c.1370G > A in the *POR* gene. Neither parent had mutation c.2978T > A in *ANK1* (**Figure 2**).

According to the results, variant c.2978T > A in *ANK1* was proven to be a *de novo* mutation by Sanger sequencing (PS2). This variant was also absent in the control (PM2) and is predicted to be deleterious by multiple *in silico* algorithms (PP2): it was predicted to be disease-causing by MutationTaster (>0.99), deleterious by PROVEAN (−6.85), damaging by SIFT (0.00), and probably damaging by Polyphen-2 (1.000). The patient's phenotype was also specific for the disease, as she presented spherocytosis (PP4). Therefore, according to ACMG standards, this variant could be interpreted as likely pathogenic. Mutation c.1370G > A is a previously established pathogenic variant (PS1), and there is well-established functional studies supported the damaging effect of this variant (PS3). For instance, this mutation is classified as pathogenic according to ACMG guidelines.

DISCUSSION AND CONCLUSION

Hereditary spherocytosis is a common congenital hematologic disorder and also a common reason for inherited chronic hemolysis in Western countries. Hereditary spherocytosis has heterogeneous clinical presentations. In most cases, patients are asymptomatic or only present anemia. However, for patients have mild disease courses, 20–30% of these patients have mild splenomegaly and reticulocytosis, while for patients have moderate disease courses, 60–70% of them typically presented asymptomatic anemia, splenomegaly, and jaundice during infections in their childhood (Perrotta et al., 2008).

The structure of the erythrocyte membrane contains band-3 protein; protein 4.1; protein 4.2; ankyrin; spectrin; glycophorin A, B, C, and D; Rhesus (Rh) complex; CD47; Landsteiner-Wiener glycoprotein; dematin; tropomyosin; adducin; and tropomodulin. Deficiency in any of these proteins may result in the disassociation of the erythrocyte membrane structure and subsequently reduce the surface-to-volume ratio, which finally causes hereditary spherocytosis (Eber and Lux, 2004). Since ankyrin-1 encoded by ANK1 mediates the linkage of band-3 protein with spectrin, it plays an essential role in the stabilization of the membrane. Deficiency in ankyrin-1 protein may also result in the decreased assembly of spectrin, regardless of the spectrin synthesis (Hanspal et al., 1991).

In our case, the patient presented hereditary spherocytosis due to mutation in *ANK1*. The proband was identified with heterozygous mutation c.2978T > A, which results in amino acid change p.I993N. Residue I993 is a highly conservative residue in ankyrin-1 protein among several species (**Figure 3**). The conservation of this residue indicates its importance in evolution. Besides, isoleucine is a non-polar neutral amino acid, while asparagine is a polar neutral amino acid. The alteration in polarity might change the interaction between amino acids within the structure or between ankyrin and other proteins, thus affecting the function of ankyrin. Moreover, residue resides in a domain called ZU5 (from residue 911 to residue 1,015). Experiment of Mohler et al. (2004) shows that this domain could help in the binding of spectrin. The occurrence of variation in this domain

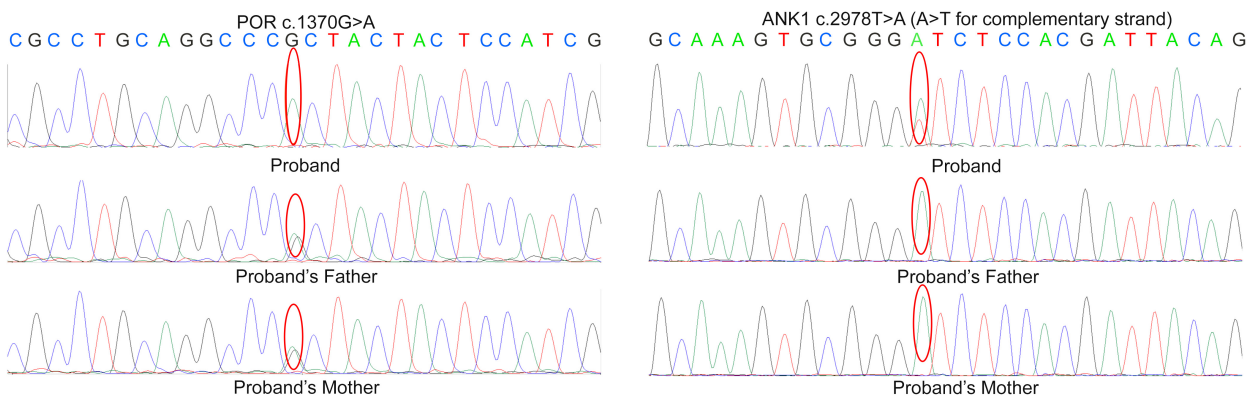


FIGURE 2 | Sanger sequencing result in the proband's family. According to the result, the parents of proband are both the carrier of c.1370G > A variant in *POR* but doesn't carry the mutation c.2978T > A in *ANK*.

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Human                          LRVVIPPRTCAAPTRITCRLVKPQKLSTPPPLAEEEGLASRIIALGPTGAQFLSPVIVEI   993
Mouse                          LRVVIPPRTCAAPTRITCRLVKPQKLNTPPPLAEEEGLASRIIALGPTGAQFLSPVIVEI   1034
Chimpanzee                     LRVVIPPRTCAAPTRITCRLVKPQKLSTPPPLAEEEGLASRIIALGPTGAQFLSPVIVEI   1034
Zebrafish                      LRVIIPPRTCAAPTRITCRLVKPQKLPTPPPLVEGEGLASRIISLGPASMQFLGPVIVEI   1077
Sheep                          LRVVVPPRTCAAPTRITCRLVKPQKLPTPPPLAEEEGLASRVIALGPTGVQFLSPVIVEI   1033
Southern_pig_tailed_macaque    LRVVIPPRTCAAPTRITCRLVKPQKLSTPPPLAEEEGLASRIIALGPTGAQFLSPVIVEI   1034
                               ***::********************** *****.* ******:*:***:. ***.******
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FIGURE 3 | Sequencing alignment of residue I993 in ANK1 protein. Result shows that this residue is extremely conserved among species.

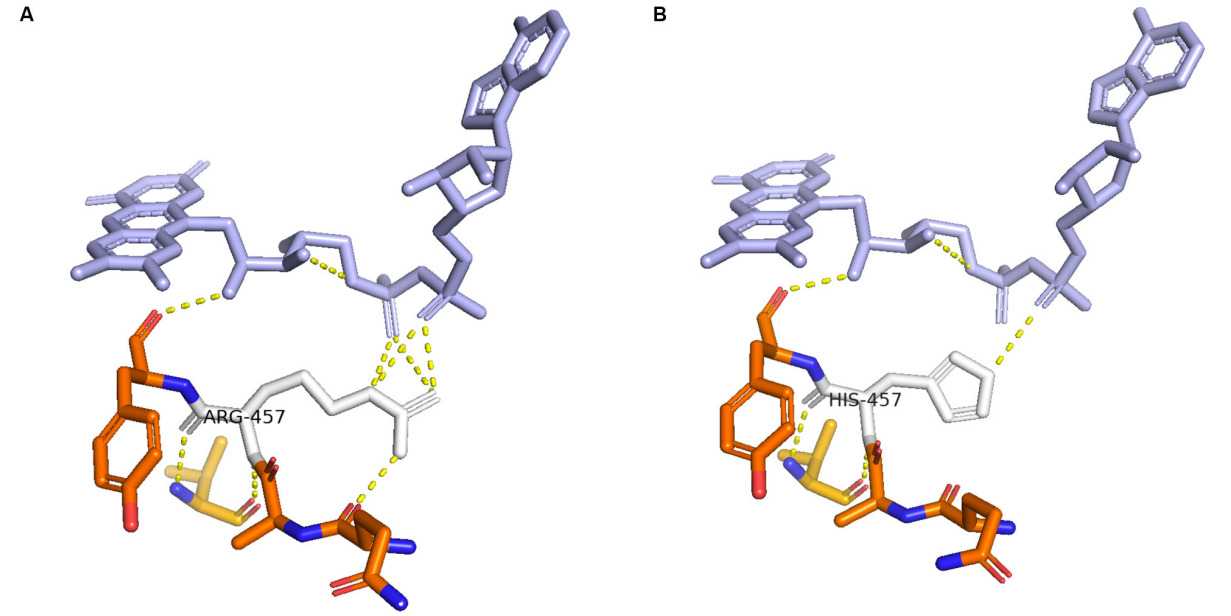


FIGURE 4 | Structure of cytochrome P450 oxidoreductase before and after mutation. **(A)** Crystal structure of wild-type P450 oxidoreductase (pbd id:5FA6). **(B)** Crystal structure of mutant P450 oxidoreductase (pdb id:3QFR). The attraction between P450 oxidoreductase and FAD was found significantly decreased.

TABLE 1 | Summarized clinical information of patients carrying mutation c.1370G > A (p.R457H) in the POR gene.

Report	Sex		Clinical Presentation
Adachi et al. (2004)	M	Com Het	Cardiac arrest due to upper airway obstruction twice during infancy. Craniosynostosis, radiohumeral synostosis, carpal and tarsal bone fusion, malformed and simple ear, arachnodactyly.
Adachi et al. (2004)	F	Com Het	Bilateral radiohumeral synostosis, midfacial hypoplasia, malformed ears, and genital ambiguity including clitoromegaly and labial fusion. No secondary sex characteristics appeared at 11 years of age.
Fukami et al. (2005)	M	Com Het	Craniosynostosis, midfacial hypoplasia, multiple digital joint contractures, arachnodactyly, scoliosis at 13 years of age, micropenis, cryptorchidism, hypospadias.
Fukami et al. (2005)	M	Com Het	Craniosynostosis, midfacial hypoplasia, radiohumeral synostosis at 10 years of age, multiple digital joint contractures, arachnodactyly, micropenis, cryptorchidism.
Fukami et al. (2005)	M	Com Het	Craniosynostosis, midfacial hypoplasia, multiple digital joint contractures, arachnodactyly, cryptorchidism.
Fukami et al. (2005)	F	Hom	Craniosynostosis, midfacial hypoplasia, multiple digital joint contractures, clitoromegaly, labial fusion.
Fukami et al. (2005)	F	Het	Craniosynostosis, midfacial hypoplasia, multiple digital joint contractures, arachnodactyly, labial fusion.
Fukami et al. (2005)	F	Hom	Craniosynostosis, midfacial hypoplasia, multiple digital joint contractures, clitoromegaly, labial fusion.
Fukami et al. (2005)	F	Com Het	Craniosynostosis, midfacial hypoplasia, conduction deafness at 13 years of age, multiple digital joint contractures, arachnodactyly, clitoromegaly, labial fusion.
Fukami et al. (2005)	F	Hom	Proximal interphalangeal joint, clitoromegaly.
Arlt et al. (2004)	F	Com Het	Clubfeet, ambiguous genitalia, clitoromegaly, labial fusion, marfanoid habitus, scoliosis, arachnodactyly, dysplastic ears, slim limbs, unilateral oophorectomy.
Flück et al. (2004)	M	Com Het	Craniosynostosis, hypertelorism, mild choanal narrowing, radiohumeral synostosis, midface hypoplasia, arachnodactyly, rocker-bottom feet, clitoromegaly, labial fusion.
Bai et al. (2017)	F	Hom	Labial fusion, ovarian cyst without special skeletal deformities.

Com Het, compound heterozygous; Hom, homozygous; Het, heterozygous.

may disrupt the association between β-spectrin and ankyrin-1, thus dissociating the erythrocyte membrane structure.

Congenital adrenal hyperplasia refers to a series of disorders in adrenal steroidogenesis that can affect the biogenesis of glucocorticoid, mineralocorticoid, or sex steroid production. CAH consists of two forms: a severe form and a mild form. The severe form affects nearly one in 15,000 births worldwide, while the prevalence of the mild form is one in 1,000 or even higher in certain ethnic groups (Merke and Kabbani, 2001).

Enzymes involved in cortisol biosynthesis include 21-hydroxylase, 11β-hydroxylase, 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase (type II), steroidogenic acute regulatory protein, P450 cholesterol side-chain cleavage enzyme, and P450 oxidoreductase (*POR*). The initial two enzymes in cortisol biosynthesis, 17OH and 21OH, are functionally active only when the electron transport cytochrome P450 oxidoreductase exists. Therefore, patients with *POR* deficiency present combined 17OH and 21OH deficiency. The patient in our study was identified as carrying homozygous mutation c.1370G > A, which caused amino acid variation p.R457H. This mutation is a reported variant that is located in the FAD binding domain. The guanidinium group of residue R457 was proven to form a salt bridge with the pyrophosphate group of FAD (Wang et al., 1997; Xia et al., 2011). Alteration in H457 weakens the interaction between FAD and POR according to the molecule structure model (**Figure 4**). Since this variant is a known disease-causing mutation, the clinical presentation is also summarized as **Table 1** (Adachi et al., 2004; Arlt et al., 2004; Flück et al., 2004; Fukami et al., 2005; Bai et al., 2017). According to the summarized information, nearly all patients carrying mutation R457H are diagnosed as having Antley-Bixler syndrome, except for one patient, who did not present any skeletal malformation. Our patient presented arachnodactyly, multiple digital contractures, scoliosis, and clitoromegaly, as in most of the reported patients; therefore, she could be diagnosed as having Antley-Bixler syndrome.

In this study, we reported a girl presenting both spherocytosis and Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis. Patients presenting more than one diseases, especially hereditary diseases, are not a common affair for the clinicians. Therefore, when handling cases presenting multi-system symptoms, some of the clinicians would tend to treat it as a rare syndrome not several separated diseases. This thought might impede the analysis of genetic tests. We hope our experience in diagnosing this case would delight other clinicians in handling patients with disease presentations in multiple systems. Moreover, the cooperation between doctors in different department is also important. In our case, the clitoromegaly of the patient was treated when she was 3 years old in other hospital without a clear diagnosis. She was admitted to the hematological department of our hospital at the age of 5 only due to the complaint of spherocytosis. However, since the patient was found to have arachnodactyly during admission she was then sent to the department of endocrinology. After the next-generation sequencing and the examination of clinicians in both departments, she was finally diagnosed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Capital Institute of Pediatrics. Written informed consent was obtained from the patient's parents for the publication of this report and any accompanying images.

AUTHOR CONTRIBUTIONS

SF and RL were in charge of the diagnosis of spherocytosis and also the writing works concerning the presentations of spherocytosis. They also gave advices when doing the gene analysis. FS, MD, HY, and XC were responsible for the diagnosis of CAH and all the description parts of CAH in this article. They also gave advices when doing the gene analysis. XS took the charge of performing gene sequencing and the analysis and also the genetic parts of this article. All authors agreed to be accountable for the content of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00976/full#supplementary-material

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Conflict of Interest: XS was employed by the company Running Gene Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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An Initial Survey of the Performances of Exome Variant Analysis and Clinical Reporting Among Diagnostic Laboratories in China

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Exome sequencing has become an effective diagnostic method for *Mendelian* disorders. But the quality of services differs widely across laboratories in China, particularly in variant classification, even with the adoption of the ACMG guidelines. As an effort of quality control and improvement for better clinical utilization of exome sequencing, we assessed the exome data analysis and clinical reporting among Chinese laboratories. Five raw datasets of real clinical samples with associated phenotypes were sent to 53 laboratories. The participants independently performed secondary analysis, variant classification, and reporting. The first round of results was used for identifying problems associated with these aspects. Subsequently, we implemented several corrective actions and a training program was designed based on the identified issues. A second round of five datasets were sent to the same participants. We compared the performances in variant interpretation and reporting. A total of 85.7% (42/49) of participants correctly identified all the variants related with phenotype. Many lines of evidence using the ACMG guidelines were incorrectly utilized, which resulted in a large inter-laboratory discrepancy. After training, the evidence usage problems significantly improved, leading to a more consistent outcome. Participants improved their exome data analysis and clinical reporting capability. Targeted training and a deeper understanding of the ACMG guidelines helped to improve the clinical exome sequencing service in terms of consistency and accuracy in variant classification in China.

Keywords: exome sequencing, variant analysis, variant interpretation, clinical reporting, laboratory performance

INTRODUCTION

Exome sequencing has proven to be an effective strategy for gene discovery and clinical diagnosis for patients with Mendelian disorders, which herald a new era of genomic medicine (Yang et al., 2013, 2014; Lee et al., 2014; Retterer et al., 2016; Wright et al., 2018). However, genome-wide variant analysis is highly complex and challenging for every clinical laboratory. From

processing massive raw data to reporting variants associated with phenotype, the bioinformatics pipeline consists of a series of steps and data transformations using multiple algorithms, software, databases, and operation environment (Roy et al., 2018). There is no standard for how to use these components to analyze the outputs of different sequencing platforms, and for how these tools are combined and used in context for patient care (Brownstein et al., 2014). Many issues have been reported regarding the inconsistency of variant calling, variant annotation, variant filtration, variant classification, and variant reporting in clinical diagnostic settings. For example, a study analyzed the same set of raw sequence data with different alignment and variant-calling pipelines, showing that there was a significant discrepancy in SNV and indel calling across exomes and demonstrating fundamental methodological variation between commonly used tools (O'Rawe et al., 2013). Annotation discrepancy has also been reported when using different transcript sets or software in annotation (McCarthy et al., 2014), splicing variants were the category with the greatest discrepancy (McCarthy et al., 2014; Zhao and Zhang, 2015). To facilitate the clinical implementation of genomic medicine, it is important to obtain a robust, accurate, and consistent variant analysis pipeline.

In terms of variant classification, previous reports revealed extensive discordance between laboratories (Brownstein et al., 2014; Landrum et al., 2016; Pepin et al., 2016). A total of 17% of variants in ClinVar had non-uniform classifications from multiple submitters, demonstrating the need for standardized guidelines for variant interpretation. In 2015, the American College of Medical Genetics and Genomics (ACMGG) and the Association for Molecular Pathology (AMP) published the variant classification guidelines (Rehm et al., 2015), which significantly helped the variant classification process. However, the guidelines did not solve all of the problems, discordant results remain across laboratories (Amendola et al., 2016; Harrison et al., 2017). In China, exome sequencing technology has been adopted very rapidly. In addition to common issues, China is confronted with unique challenges: (1) a lack of professional, clinical, and medical geneticists, the equivalent of ABMGG-certified medical geneticists in the United States; (2) a lack of genetic counselors who play important roles in collecting/delineating clinical information, variant classification, report drafting, and genetic counseling for both physicians and patients; and (3) a lack of regulations like the Clinical Laboratory Improvement Amendments of 1988 (CLIA 88) in the United States. With these challenges in mind, the National Center of Clinical Laboratories (NCCLs) organized an initial assessment focusing on exome variant analysis and clinical reporting in China, as part of the effort to assess the performances of Chinese laboratories offering clinical diagnostic tests using exome. The assessment helped to identify issues related with the ability of laboratories to adapt to the ACMG/AMP guidelines. NCCLs also conducted a training program after the first survey and educated the laboratories on how to apply ACMG-AMP criteria correctly and move toward more consistent variant interpretations.

MATERIALS AND METHODS

Raw Sequence Dataset

The initial survey involved three probands and two trios. The clinical manifestation and/or family history of these test cases suggested a possible genetic disease.

Genomic DNA was isolated from peripheral blood samples using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The target regions for proband-only samples were captured by the ClearSeq Inherited Disease panel kit (cat No.5190–7519, Agilent Technologies, Santa Clara, CA). The whole exome for trio samples were captured by the SureSelect Human All Exon V6 kit (cat No.5190–8864, Agilent). Sequencing was performed on Hiseq X Ten (Illumina, San Diego, CA) using paired-end 150-bp reads according to the manufacturer's protocol. Raw data (FASTQ files) were generated via the on-board Hiseq control software and reporter software (Illumina).

Surveys and Analysis

Chinese laboratories that offer exome sequencing for routine clinical molecular testing were invited to participate in the survey. Participation was voluntary and free of charge. Participating laboratories downloaded the FASTQ datasets from a central portal. Laboratories were asked to process the data using their validated pipelines for alignment, variant calling, annotation and filtration, and variant interpretation and reporting as they would normally do for a clinical case. Participants were asked to provide a detailed description of the bioinformatic pipelines and quality metrics. In addition to the results of the variant classification, the laboratories were also asked to provide individual lines of evidence following the ACMG/AMP guidelines. The NCCLs collected all the results. The participants were asked to provide an explanation for missing identifying the causative variants.

After identifying the problems based on the initial assessment, a training program was designed and conducted, focusing on training the laboratories on how to apply the ACMG-AMP criteria correctly. The training included a one time in-person training course with workshops plus months of online educational lectures. After training, another five cases with putative disease-causing variants were sent to the same laboratories. The criteria applied by each laboratory were tracked to identify to what extent training improved the consistency and accuracy of variant interpretation.

RESULTS

Clinical Findings and Genetic Variants in Survey Samples

The initial set (labeled as -1) and the second set (labeled as -2) of clinical cases are shown in **Supplementary Table S1**, which was provided to the participating laboratories along

with the FASTQ files. **Table 1** lists the phenotype-related variants in each case. All variants were confirmed by Sanger sequencing.

Participants and Performance

A total of 53 clinical laboratories from hospitals or commercial entities providing genetic testing participated in this survey. The necessary experience with WES data analysis was at least one year for these participates. At the deadline of the survey, 49 laboratories returned the results meeting the requirements. Each laboratory provided the variants associated with a phenotype, clinical diagnostic reports for each sample, and a brief description of their bioinformatics pipeline. Most participants (69.4%, 34/49) were from a commercial entity. As a result, 85.7% (42/49) of participating laboratories correctly identified all the variants related with a phenotype. A total of 42 laboratories reported the intended variant nomenclature as shown in **Table 1**. However, seven labs failed to identify eight disease-causing variants. One lab was a hospital laboratory, the other six were commercial laboratories. They subsequently provided the reasons for missing the variants which are summarized in **Table 2**. Two institutions (lab1 and lab2) identified the correct variants but reported the wrong ones due to clerical errors, exposing the quality control gap. One laboratory (lab3) missed the variants for sample 1815 due to a bug in the bioinformatics pipeline that resulted in the random termination of the annotation in dealing with trio data. The problem was fixed afterward. One institution (lab5) missed calling the indel variant in *FBP1*(NM_000507.3) c.720_729delTTATGGGGCC in sample 1815. Two institutions (lab4 and lab6) reported wrong variants by using the wrong

TABLE 1 | Genetic variants in surveyed samples.

Survey sample ID[a]	Intended variants and nomenclature	Variant type	Zygosity	Inheritance mode	Variant classification	Phenotype
Survey 1–1811	NM_000441.1(SLC26A4):c.281C > T(p.Thr94Ile)	Missense	Het	AR	P	Deafness, autosomal recessive 4, with enlarged vestibular aqueduct(OMIM:600791)
	NM_000441.1(SLC26A4): c.919-2A > G	Splicing site	Het	AR	P	
Survey 1–1812	NM_000507.3(FBP1):c.720_729 delTTATGGGGCC(p.Tyr241Glyfs)	Frameshift	Het	AR	P	Fructose-1,6-bisphosphatase deficiency (OMIM:229700)
	NM_000507.3(FBP1): c.490G > A(p.Gly164Ser)	Missense	Het	AR	P	
Survey 1–1813	NM_000257.3(MYH7):c.2167C > G(p.Arg723Gly)	Missense	Het	AD	P	Cardiomyopathy, familial hypertrophic 1 (OMIM: 192600)
Survey 1–1814	NM_001854.3(COL11A1): c.3816 + 1G > A	Splicing site	Het	AD/*De novo*	P	Marshall syndrome (OMIM 154780)/ Stickler syndrome II (OMIM 604841)
Survey 1–1815	NM_017780.3(CHD7): c.1666-2A > G	Splicing site	Het	AD/paternal	LP	CHARGE syndrome(OMIM:214800)
Survey 2–1911	NM_006662.2 (SRCAP):c.7303C > T (p.Arg2435Ter)	Non-sense	Het	AD/*De novo*	P	Floating-Harbor syndrome (OMIM:136140)
Survey 2–1912	NM_000277.1(PAH):c.1197A > T (p.Val399 =)	Synonymous	Het	AR	P	Phenylketonuria (OMIM: 261600)
	NM_000277.1(PAH): c.728G > A (p.Arg243Gln)	Missense	Het	AR	P	
Survey 2–1913	NM_012123.4(MTO1):c.1291C > T(p.Arg431Trp)	Missense	Het	AR	LP	Combined oxidative phosphorylation deficiency 10 (OMIM: 614702)
	NM_0121233.4(MTO1):c.1390C > T(p.Arg464Cys)	Missense	Het	AR	LP	
Survey 2–1914	NM_000275 (OCA2):c.1503 + 5G > A	Missense	Het	AR	P	Albinism, oculocutaneous, type II (OMIM:203200)
	NM_000275 (OCA2):c.1441G > A (p.Ala481Thr)	Missense	Het	AR	VUS	
Survey 2–1915	IL2RG:c.664C > T(p.Arg222Cys)	Missense	Het	XLR	P	Severe combined immunodeficiency, X-linked (OMIM: 300400)

Surveys 1–1811, 1812, and 1813 are proband-only raw data. Surveys 1–1814 and 1815 are trio raw data.

phenotype. One participating laboratory (lab7) called a wrong indel variant (*FBP1* c.718_727delCCCCATAAGG).

Bioinformatics Pipeline Used by Participants

Generally, a bioinformatics pipeline consists of the following major steps: alignment, pre-variant call, variant calling, variant annotation, and filtration. The bioinformatics pipeline and its components used by participating laboratories for each step were highly variable as shown in **Table 3**.

The most used population frequency database was the 1000 Genomes project (94%), followed by ExAC (88%), gnomAD (63%), dbSNP (80%), ESP6500 (51%), and an in-house database (51%). A variety of knowledgebases and databases were used, including but not limited to: ClinVar (84%), OMIM (94%), Human Gene Mutation Database (HGMD) (84%), HPO (20%), CHPO (6%), COSMIC (16%), Orphanet (8%), an in-house annotation (22%), dbNSFP (69%), LOVD (14%), and DECIPHER (10%). A diverse range of pathogenicity prediction tools were utilized often in combinations of more than two.

Comparison of Clinical Reports Between Two Surveys

Forty-five laboratories submitted clinical reports for each case in the first survey. The elements of the report are summarized in **Table 4**. It is notable that some laboratories did not clearly state the variants and associated information. Some important elements, such as tests performed, limitations of test methods, recommendations for genetic counseling, and further testing were reported by approximately only half of the laboratories (55.56%). A third of laboratories reported secondary findings following the ACMG 59 genes list (Kalia et al., 2017). Only two institutions indicated that they provide reanalysis of data after one year or after three months.

A training meeting was subsequently conducted in order to standardize the content and format of clinical reports. The

TABLE 2 | Summary of the reasons for wrong variants.

Lab No.	Sample ID	Expected variant	Wrong variant reported	Reason for missing the right variant
1	Survey 1–1814	*COL11A1* (NM_001854.3):c.3816 + 1G > A	*COL11A1* (NM_001854.3):c.4554 + 1G > C	Clerical error
2	Survey 1–1815	*CHD7* (NM_017780.3):c.1666-2A > G	*CHD7* (NM_017780.3:c.1666-2AA > G	Clerical error
3	Survey 1–1815	*CHD7*(NM_017780.3):c.1666-2A > G	False-negative	Bug in annotation script
4	Survey 1–1812	*FBP1*(NM_000507.3): c.490G > A/ c.720_729delTTATGGGGCC	*ABCD1*(NM_000033):c.1489-6delC	Wrong filtration based on wrong phenotype capturing
5	Survey 1–1812	*FBP1*(NM_000507.3): c.490G > A/ c.720_729delTTATGGGGCC	*NDUFAF6*(NM_152416):c.420 + 2_420 + 3insA	Not called by their bioinformatics pipeline
6	Survey 1–1815	*CHD7*(NM_017780.3):c.1666-2A > G	*COL11A2*(NM_080680.2):c.688G > T	Wrong filtration based on wrong phenotype capturing
7	Survey 1–1812	*FBP1*(NM_000507.3): c.720_729delTTATGGGGCC	*FBP1*(NM_000507.3): c.718_727delCCCCATAAGG	Wrong nomenclature for indel variant
8	Survey 1–1815	*CHD7*(NM_017780.3):c.1666-2A > G	*HSPG2*(NM_005529.6):c.4213G > C/c.9019G > A	Wrong inheritance mode

TABLE 3 | Bioinformatic pipeline elements used by participating laboratories.

Bioinformatic pipeline steps	Tools used (%)
Data quality control	FastQC (65%), in-house developed tools (16%), QualiMap (8%), Fastp (16%), Trimmomatic (18%), cutadapt (6%)
Sequence alignment	BWA-MEM (90%), SENTIEON (12%)
Pre-variant calling processing	Picard (57%), SAMtools (33%), GATK (69%)
Variant calling	GATK HaplotypeCaller (78%), SAMtools (10%), Freebayes (8%), SENTIEON (12%), DNAnexus (2%), Vardict (2%), Lofreg (2%)
Annotation	Annovar (82%), snpEff (10%), Ensembl VEP (12%), Annotools (2%), IVA (2%), and in-house developed tools (8%)
Pathogenicity prediction	dbscSNV (24%), SPIDEX (4%), GERP + + (6%), SIFT (39%), PolyPhen-2 (31%), LRT (8%), MutationTaster (16%), FATHMM (10%), MetaSVM (4%), MetaLR (14%), CADD (12%), InterVar (24%), REVEL (12%), M-CAP (8%), RadialSVM (4%)
Filtration	Annovar (49%), Phenolyzer (37%), Exomiser (33%), Phenomizer (4%), Ensembl VEP (2%), gemini (2%), Tgex (4%), Varseq (1%), in-house filtration (10%), IVA (2%)

TABLE 4 | Comparison of content for the clinical reports between the two surveys.

Report elements	Percent of report (*n* = 45) in survey 1		Percent of report (*n* = 43) in survey 2	
Patient name	44	97.78%	43	100%
Patient sex	45	100%	43	100%
Patient date of birth or age	45	100%	43	100%
Clinical symptoms and family history	34	75.6%	43	100%
Test indications	34	75.6%	43	100%
Specimen number	42	93.33%	43	100%
Date specimen collected	14	31.11%	30	69.77%
Date specimen received	36	80%	35	81.40%
Specimen type	42	93.33%	41	90.35%
Title	41	91.11%	43	100%
Laboratory contact information	25	55.56%	43	100%
Ordering physician	31	68.89%	40	93.02%
Signatures	38	84.44%	43	100%
Date of report issued	40	88.89%	43	100%
Current/total page	35	77.78%	40	93.02%
Test performed (e.g., WES/panel/WGS)	28	62.22%	42	62.22%
Description of test method	13	28.89%	27	62.79%
Sequencing instrument and sequencing information	11	24.44%	26	60.47%
Description of bioinformatics pipeline	14	31.11%	14	32.56
Technical parameters (e.g., mean depth of coverage across the target region, coverage on target region)	18	40%	24	55.81%
Limitations and validity of test method	35	77.78%	42	97.67%
Test results	45	**100%**	43	100%
Human reference genome build (e.g., UCSC hg19)	21	46.67%	28	65.12%
Chromosome number and gene coordinates	36	80%	36	90.70%
Gene name	45	100%	43	100%
Exon or intron number	25	55.56%	34	79.07%
Transcript	43	95.56%	43	100%
Nomenclature at both the nucleotide (genomic and cDNA) and protein level using HGVS nomenclature	45	100%	43	100%
Zygosity	44	97.78%	43	100%
Variant classification (pathogenic)	45	100%	43	100%
Mode of inheritance (AD, AR)	43	95.56%	41	95.35%
Parental inheritance	38	84.44%	38	88.37%
A summarized conclusion	27	60%	33	76.74%
Test interpretation	40	88.89%	35	90.70%
Recommendations for genetic counseling and further testing	25	55.56%	38	88.37%
References	35	77.8%	32	74.42%
Statement of laboratory's availability	20	44.4%	24	55.81%

reports submitted for the second round of surveys had a much improved consistency as shown in **Table 4**.

Performance and Improvement of ACMG-AMP Variant Classification

All participating laboratories claimed that they assessed the pathogenicity of variants following the ACMG/AMP guidelines (Richards et al., 2015), but the outcomes were quite different, and the concordance rate differed greatly from variant to variant. Of the seven variants (**Figure 1A**) in the first survey, the classification of four variants were concordant across laboratories, with only one variant in exact agreement and three variants only differing in P versus LP. The classifications of the remaining three variants (*FBP1*:c.490G > A, *SLC26A4:*c.281C > T, and c.720_729del) were quite discordant, the classification crossed clinical categories (i.e., P/LP versus VUS). For 3.3% (11/335) of the variant assessments, the ACMG evidence listed by the laboratories did not support the classification chosen.

A total of 16 different lines of evidence were invoked for variant classifications in the survey. PM2 (absent from controls), PP3 (*in silico* evidence), and PVS1 (variant predicted null where loss of function is a mechanism of disease) were the most frequently used lines of evidence in the first survey. Lines of evidence that should have been used but were not are plotted

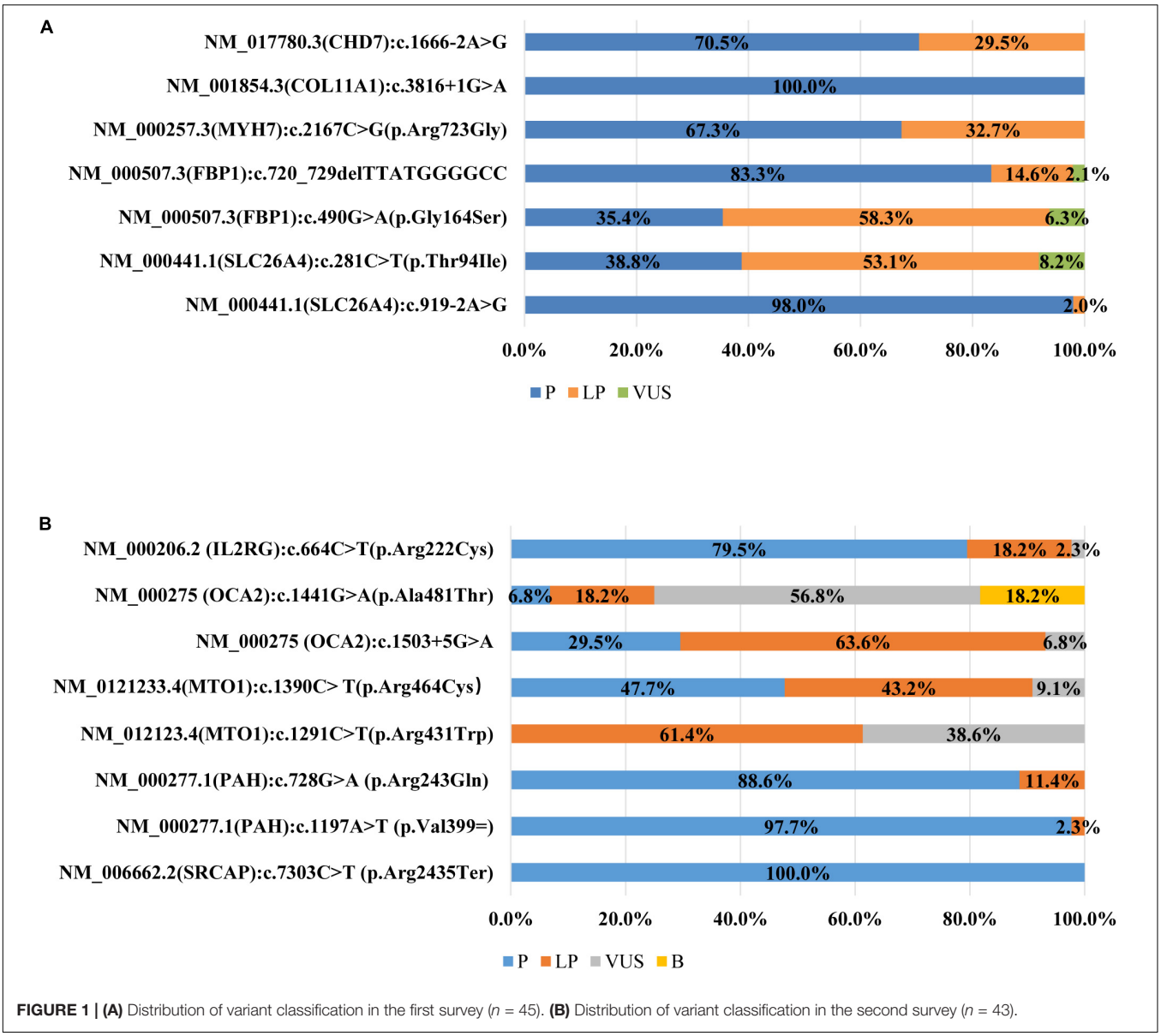


FIGURE 1 | (A) Distribution of variant classification in the first survey (*n* = 45). **(B)** Distribution of variant classification in the second survey (*n* = 43).

in **Figure 2A (category 1 error) and** those that should not have been used but were are plotted in **Figure 2B (category 2 error).** Category 1 error occurred in over 30% of instances for some lines of evidence. PP1 (segregation data) (47.52%), PM3 (allelic variant in-trans) (32.07%), and PS4 (case-control difference) (34.11%) were among the most significantly misused lines of evidence as part of category 1 error. PP4 (using phenotype to support variant claims) (42.86%) was also the most inconsistently used evidence among the laboratories. Category 2 error occurred in over 15% of instances for some lines of evidence. PP5 (reputable source) was still used for variant classifications (32.94%), though ACMG-AMP had proposed that laboratories discontinue the use of this criteria. PS1 (same amino acid change) (19.24%), PP3 (functional prediction) (19.24%), PM1 (mutational hot spot and/or critical and well-established functional domain) (19.24%) were among the most significantly misused lines of evidence as part of category 2 error. The misusage of PP3 was due to 'double counting' when PVS1 had already been used. Some laboratories used PS1 when the same missense variant had been reported before. Some used PS1 instead of the PM5 (novel missense at the same position) criteria for the MYH7 c.2167C > G (p. Arg723Gly) variant. The misusage of PM1 was due to a misunderstanding of the well-established functional domain. Only three laboratories correctly up- and downgraded lines of evidence (up-graded for PM3 and PP1 and down-graded for PM3, PS4, and PS3).

In the second survey, three of eight variants had consistent classifications across laboratories (**Figure 1B**). While the classification for the other five variants were still discordant, the ACMG evidence codes listed by the institutions all supported their chosen classification and the overall error rates for some lines of evidence were greatly reduced compared to the first

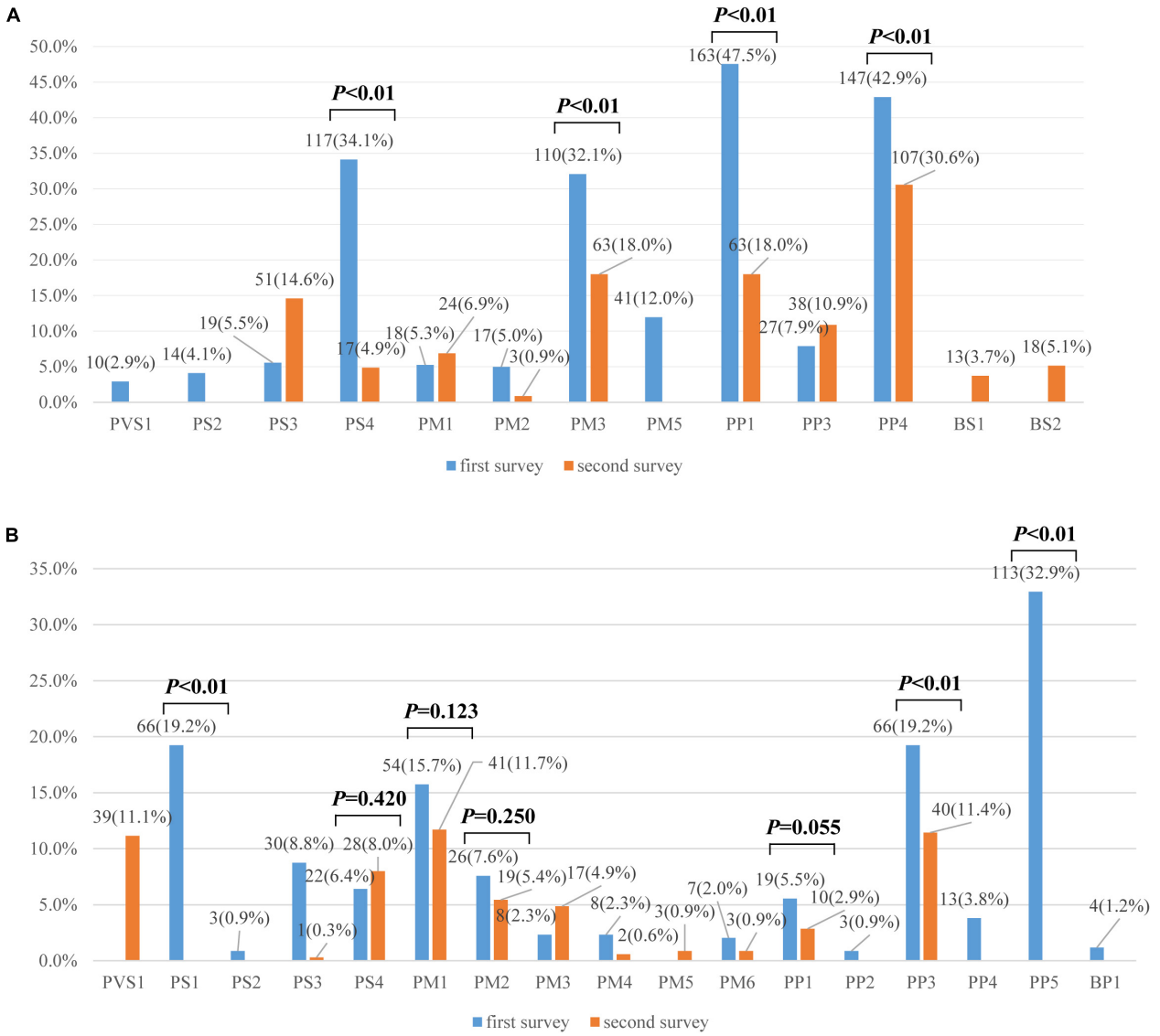


FIGURE 2 | (A) Percentages of category 1 errors across lines of evidence between the first and second surveys (n = 335). **(B)** Percentages of category 2 errors across lines of evidence between the first and second surveys (n = 335). Legend: Lines of evidence that should have been used but were not are category 1 errors, those that should not have been used but were are category 2 errors.

survey (**Figures 2A,B**). For example, the percentage of category 1 error dropped from 47.52%to 18.00% for PP1, from 32.07% to 18.0% for PM3, and from 34.11% to 4.86% for PS4. Yet there was still a high error rate for PP4 (30.57%). The rates for category 2 error dropped to 0 for both PS1 and PP5, and dropped from 19.24% to 11.43% for PP3. The laboratories were still relatively confused on how to use PM1. PVS1 (Null variants) (11.14%) was also a significantly misused line of evidence as part of category 2 error, this is because the disease mechanism can be difficult for *SRCAP* mutations. More laboratories modified the strengths of evidence to make it more appropriate for certain lines of evidence (four lines of evidence - PS2, PM3, PP1, and PP4 were upgraded and three lines of evidence - PM3, PS4, and PS3 were downgraded.

DISCUSSION

In 2015, ACMG and AMP published the guidelines for variant classification (Richards et al., 2015). In 2017, the Chinese translation of the guideline was published and it was recommended that all Chinese clinical laboratories adopt the guidelines for clinical diagnostic services (Wang et al., 2017). However, we knew very little about how well the guidelines were followed and most importantly, whether the guidelines were used correctly in actual practice across different laboratories in China. These concerns are particularly pressing since we do not have a well-established professional training and quality assurance program for molecular diagnostic testing in China, yet the volume of clinical diagnostic tests is the largest in the world

due to a large patient population and the relatively low cost of testing in China (Hu et al., 2018). For this reason, we conducted this survey to identify problems in our current practice including issues with the secondary data analysis, variant classification, and clinical reporting.

The Secondary Analysis of Exome Data

There is no uniform or recommended bioinformatics pipeline for clinical exome testing in China. Most laboratories developed their own pipeline by combining different open-source algorithms or commercial software for secondary analysis of exome data. Thus, it is difficult to perform a detailed and direct comparison between laboratories. Instead, we looked for the final reportable variants. As a result, the survey exposed several errors due to the bioinformatics pipeline used, the errors included wrong variant calling, wrong variant annotation, and incorrect filtering. In addition, even for laboratories using similar software components, there was no consensus on quality control (QC) metrics. The summary of quality metrics provided by participating institutions are provided in **Supplementary Figure S1**. Developing a robust and cohesive diagnostic pipeline to achieve optimal NGS testing quality is still a challenge for some Chinese diagnosis laboratories. The problems were rooted in the fact that there was a lack of vigorous bioinformatics pipeline validation before the required implementation of standards and guidelines for routine use (Rehm et al., 2013; Hegde et al., 2017; Roy et al., 2018). Ultimately, the lack of validation was due to the lack of accreditation for clinical laboratories and certification for personnel, which in turn should be overseen by a regulatory body like CLIA. Our findings indicated that implementing routine proficiency testing for the bioinformatics portion of the NGS assay is necessary in China. Several corrective actions for the improvement of the secondary analysis pipeline could be implemented based on specific issues exposed during proficiency testing. A future proficiency test could consider sending raw data with VUS or negative results to laboratories to see whether the laboratories correctly identify VUS or negative results, and consider sending repeated reference material such as NA12878 and other clinical samples so that participating laboratories submit raw datasets (i.e., BAM and VCF). Detailed inter-laboratories data quality parameters such as coverage consistency and precision of variant calling would be informative for the continued standardization and improvement of the secondary analysis of exome data. There will be insights into what the impact of using different tools is and whether the discrepancies were related to software or whether it was due to inexperience in the application of these tools (i.e., setting thresholds for variant calling and FPs). If it is necessary, training entailing information about bioinformatics pipeline calling could also be performed in the future.

Variant Classification

It has been a major challenge to accurately and consistently classify variant pathogenicity. The ACMG/AMP guidelines provided a great framework for variant clinical interpretation and helped to significantly reduce discrepancies (Amendola et al., 2016; Garber et al., 2016; Niehaus et al., 2019). But the implementation of the guidelines does not eliminate the issues. For example, even with a consensus effort of using the ACMG guidelines, 21% and 29% of variants showed intra- and inter-laboratory discordance, respectively (Amendola et al., 2016). Similar discordance rates (22–25%) were reported by Garbor et al. and 28% of variants remained discordant after a harmonization effort (Garber et al., 2016). The main purpose of our survey was to identify the problems associated with the proper use of the lines of evidence for variant classification following the ACMG/AMP guidelines in China. Since only a limited number of variants were assessed by the laboratories, the findings are qualitative rather than quantitative. Garbor et al. pointed out that the discrepancies were mainly due to the time difference between when the variant was assessed, the availability of internal data, and the use of different AF cutoffs. While these are important factors affecting the accuracy of variant classification, the problems in China, as this survey found, are more rudimentary. Many discrepancies were due to the incomplete understanding or misunderstanding of the meaning of the evidence. For example, the ACMG evidence codes listed by eight institutions did not support their chosen classification for some variants, due to inexperience in using the rules for combining criteria to classify sequence variants. PS1 was often used when the exact variant was previously reported as pathogenic. PP3 was used when PVS1 was already invoked. Many laboratories did not keep up with guideline updates so PP5 continued to be used, up and downregulations were not practiced when the information was available. The findings of our initial survey underscore the importance of training for personnel in Chinese clinical laboratories. Significant reductions in both category 1 and 2 errors in evidence utilization after the training supported the benefit of such activity conducted by the NCCLs. But there are still other issues: 1) though ClinGen's Sequence Variant Interpretation Expert Panels are publishing specifications of the guidelines to further clarify how certain criteria are applicable for a given gene or disease, some Chinese laboratories do not pay enough attention to updates and do not apply them for variant interpretation for a given gene or disease. For example, the ClinGen PAH Expert Panel published specifications to the ACMG/AMP variant interpretation guidelines for the PAH gene[1]. It specified that the *in vitro* enzyme activity $< 50\%$ compared to wild-type control or RT-PCR evidence of mis-splicing could be used as PS3 (functional evidence). However, PS3 was not used by 13.6% (6/44) of laboratories in variant classifications for the PAH gene in the second survey. Also, there was a lot of uncertainty associated with the use of PS3. It was not used in variant classifications for 14.57% of variants in the second survey, illustrating that the evidence is still challenging for many laboratories; 2) up and downgrading evidence became the main cause of discordance for lines such as PP1, PM3, and PS2/PM6. Many laboratories had not implemented the upgraded guidelines or had difficulty in judging when it was appropriate to up or downgrade the evidence, or literature searching was incomplete so some raw evidence was missed. On the other hand, we trained laboratories on how to interpret the loss of function PVS1 in

[1]https://www.clinicalgenome.org/affiliation/50015

the ACMG/AMP variant criterion (Abou Tayoun et al., 2018). In a future survey, we will examine the correct usage of PVS1 in upgrading and downgrading evidence; 3) in China, genetic tests are not only offered by a few hospital laboratories who have clinical and research experience with genetic disorders, but also by laboratories or commercial entities without an extensive knowledge about genetic disorders. Also given the fact that most ordering physicians were not well-trained in medical genetics, the patients did not receive a thorough phenotypic evaluation and differential diagnosis, and thus the orders were often accompanied with very limited clinical information. Consequently, many laboratories were not able to properly use PP4, either due to unavailable insufficient information or the incapability of physicians in performing clinical assessment. For example, in this survey, about half of the laboratories (19/44) did not use PP4 evidence when we provided sufficient clinical information that were well-matched with the phenotype of Floating-Harbor syndrome (FHS). In addition, there was a lack of effective interaction with the ordering physician, so when the phenotypic information was incomplete, the lab would miss the variant. This is the case for the lab that missed the "obvious" variant *CHD7*:NM_017780.3, c.1666-2A > G, because they thought the provided information was the "whole observable phenotype", and assumed that the patient did not have any other abnormalities. The lack of personnel with clinical experience will be a significant problem for genetic testing in China if a certification program is not implemented. It should be noted that the number and type of variants in the surveys were limited and not all relevant issues had been identified by this initial assessment. But improving the understanding of the guidelines can help with certain issues right away. Our near-future efforts will focus on further training for personnel and the development of additional practice guidelines to assist with the application of the ACMG-AMP guidelines in Chinese laboratories. During the training we will illustrate the importance of data and experience sharing, NCCLs will organize a clinical consortium to facilitate fair data sharing and resolve inter-laboratory discrepancies, as was already implemented by ClinGen (Harrison et al., 2017). In the future survey, a wider variety of variants including benign variants will be distributed to more laboratories by NCCLs, this effort will help to clarify common usage errors and identify challenges in Chinese genetic laboratories.

Clinical Reporting

Although specific international guidelines (Aziz et al., 2015; Richards et al., 2015) and Chinese standards (Huang et al., 2018) have been published and recommended for clinical reporting, our survey also identified many issues with the reports. It was noted that most clinical reporting did not include all the essential information on performed NGS-based testing, including description of the test method, limitations, the validity of the test method, description of genetic counseling, recommendations for next steps, and the human reference genome build (as shown in **Table 4**). Our training and the feedback of the survey results to the participating laboratories emphasized the essential elements of the report and assisted the participating laboratories in helping them to continuously improve the format and content of their clinical reports. Our work has been useful in helping laboratories develop a clinical report process with the clinical significance on any relevant findings clearly and concisely stated and comprehensible to clinicians.

The overall purpose of this initial survey in China was to investigate the performance of exome variant analysis and clinical reporting and to identify the shortcomings of the process and its product. We have used training as one of the solutions to amend the identified problems. We also implemented several corrective actions based on the specific issues exposed during the surveys including the adjustment and improvement of the secondary analysis pipeline, the implementation of appropriate validation, and a more vigorous process of variant interpretation and reporting. In conclusion, the practice of NGS-based exome sequencing for genetic disease in China is still in the early stages of its development. The initial survey revealed a significant number of issues associated with but not unique to the clinical exome test in Chinese laboratories. We also illustrated the approaches that can help to achieve optimal exome testing quality in Chinese diagnostic laboratories.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

KZ drafted the manuscript. YS and JL revised the manuscript. JW, DH, YH, and GL contributed to the acquisition and interpretation of data. KZ, YS, and JL designed the work. All authors contributed to the manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.582637/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Two Novel Mutations of *ANKRD11* Gene and Wide Clinical Spectrum in KBG Syndrome: Case Reports and Literature Review

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Background: KBG syndrome (OMIM #148050) is a rare, autosomal dominant inherited genetic disorder caused by heterozygous mutations in the ankyrin repeat domain-containing protein 11 (ANKRD11) gene or by microdeletion of chromosome 16q24.3. It is characterized by macrodontia of the upper central incisors, distinctive facial dysmorphism, short stature, vertebral abnormalities, hand anomaly including clinodactyly, and various degrees of developmental delay. KBG syndrome presents with variable clinical feature and severity among individuals. Here, we report two KBG patients who have different novel heterozygous mutations of ANKRD11 gene with wide range of clinical manifestations.

Case presentation: Two novel heterozygous mutations of ANKRD11 gene were identified in two unrelated Korean patients with variable clinical presentations. The first patient presented with short stature and early puberty and was treated with growth hormone and gonadotropin-releasing hormone agonist without adverse effects. He had mild intellectual disability. In targeted exome sequencing, a novel *de novo* frameshift variant was identified in ANKRD11, c.5889del, and p. (Ile1963MetfsX9). The second patient had severe intellectual disability with epilepsy. He had normal height and prepubertal stage at the age of 11 years. He had behavioral problems such as autism-like features, anxiety, and stereotypical movements. Whole exome sequencing (WES) was performed, and the novel heterozygous mutation, c3310dup, p. (Glu110GlyfsTer5) in ANKRD11 was identified.

Conclusion: KBG syndrome is often underdiagnosed because of its non-specific features and phenotypic variability. Performing a next—generation sequencing panel, including the ANKRD11 gene for cases of developmental delay with/without short stature may be helpful to identify hitherto undiagnosed KBG syndrome patients.

Keywords: ANKRD11 gene, KBG syndrome, phenotype (mesh), growth hormone, intellectual disability—genetics

INTRODUCTION

KBG syndrome (OMIM #148050) is a rare, autosomal dominant inherited genetic disorder that is caused by heterozygous mutations in the ankyrin repeat domain-containing protein 11 (ANKRD11) gene (Sirmaci et al., 2011) or by microdeletion of chromosome 16q24.3 that includes the ANKRD11 gene (Willemsen et al., 2010; Isrie et al., 2012). The syndrome is characterized by macrodontia of the upper central incisors, distinctive facial dysmorphism (including triangular face, brachycephaly, synophrys, and hypertelorism), skeletal abnormalities (costovertebral anomalies, brachydactyly, scoliosis, short stature), developmental delays, and behavioral problems (Brancati et al., 2006; Morel Swols et al., 2017). In 2011, Sirmaci et al. demonstrated that mutations in the ANKRD11 gene caused KBG syndrome. To date, more than 200 patients with KBG syndrome have been reported; this has enabled clinicians to update the clinical features and genetic aspects of the condition (Scarano et al., 2019). Patients with KBG syndrome have diversiform phenotypes and various degrees of intellectual disability (ID), even within families where multiple members are affected (Morel Swols and Tekin, 2018). The Deciphering Developmental Disorders (DDD) study in the United Kingdom reported that about 1% of patients with an undiagnosed neurodevelopmental disorder were found to have mutations in the ANKRD11 gene (Wright et al., 2015). KBG syndrome is still likely to be underdiagnosed because of its various and non-specific symptoms, phenotypic overlap with other syndromes, and often mild clinical manifestations. Here, we report two cases of KBG syndrome; both were identified with different novel heterozygous variants of the ANKRD11 gene and showed a broad range of clinical manifestations.

CASE PRESENTATIONS

Patient 1

The patient was born at term via a cesarean section to healthy and non-consanguineous Korean parents, with a birth weight of 2,520 g (−1.76 standard deviation score, SDS) and without any perinatal problems. He had a medical history of recurrent otitis media and had undergone surgery to insert tympanostomy tubes in both ears. At the age of 5.5 years, he had a short stature and his bone age was more than 3.5 years behind the chronological age (according to the Greulich and Pyle atlas). At that time, he had been diagnosed with growth hormone (GH) deficiency (peak GH levels from two different GH stimulation test results were 7.71 and 4.65 ng/mL) and had been treated with GH. He showed a significant increase in growth velocity and height SDS (−3.04 to −1.91 SDS) after 3 years of GH treatment.

At the age of 8.9 years, the volume of his testes began to increase, and other clinical signs of puberty appeared. He was diagnosed with precocious puberty through a gonadotropin-releasing hormone (GnRH) stimulation test. His bone age was still delayed at that time, but the lag behind chronological age had decreased by 1 year. The combination treatment of GnRH agonist (triptorelin) with GH began at the age of 10 years because of the rapid progression of puberty. He was treated with GH and GnRH agonist for 3 years; at the time of discontinuance of the GnRH agonist, his bone age was similar to the chronological age and his height SDS had increased to −0.88 SDS. He had no adverse events related with GnRH agonist treatment; moreover, his growth velocity did not decrease during treatment (**Figure 1**). As he was growing up, distinctive facial features suggesting KBG syndrome gradually became apparent. He showed macrodontia of the upper central incisors, a dysmorphic face including prominent and high nasal bridge, anteverted nostrils, hypertelorism, long philtrum, and brachydactyly of both hands (**Figure 2A**).

Targeted exome sequencing (TES) was performed and a novel frameshift variant was identified in ANKRD11, c.5889del, p. (Ile1963MetfsX9), which was confirmed with Sanger sequencing (**Figure 3A**). As the parental genetic tests revealed that the parents did not have this frameshift, the variation was identified as a *de novo* variant. This variant was categorized as pathogenic by the American College of Medical Genetics and Genomics (ACMG) guideline (PVS1, PS1, PS2, PM2) (Richards et al., 2015) and, to the best of our knowledge, has not been reported earlier. Regarding the neurologic and behavioral aspects, the patient had mild learning disability and had been taken methylphenidate for attention deficit hyperactivity disorder (ADHD) since childhood. However, he was able to participate in normal school life without any help.

Patient 2

The patient was referred to our rare disease center at the age of 11 years to undergo evaluation for an undiagnosed neurodevelopmental disorder. He grew up in a child protective facility, so there was no information on his parents or birth history. He had seizures and progressive developmental delay from 3 months of age. He visited the genetics clinic of another hospital that performed tests for evaluation of developmental delay including chromosome analysis, skeletal survey, metabolic work up, brain magnetic resonance imaging (MRI), and electroencephalography (EEG). At that time, he was suspected with acrofacial dysostosis, catania type.

Despite taking an antiepileptic drug (oxcarbazepine 6.6 mg/kg/day), he had frequent focal epilepsy with secondary generalized convulsions. Brain MRI scans at the age of 3 months and 10 years showed no significant abnormal findings. EEG findings at the age of 3 months and 6.5 years suggested partial seizure disorder originating from the left occipital area. At the age of 7, his total intelligence quotient (TIQ), which was evaluated using the Korean version of the Wexler Intelligence (K-WISC III) and the Visual Motor Integrated Development Test, was about 26; he was considered to have a severe ID. He had behavioral problems such as autism-like features, anxiety, and stereotypical movements.

At the time of referral, he had normal height (149 cm, 0.77 SDS) and weight (42 kg, −0.09 SDS), and showed macrodontia of the permanent upper central incisors; dysmorphic face features such as triangular face, thick eye lashes and eye brows, prominent nasal bridge, long philtrum, and brachycephaly; and hand anomalies including brachydactyly and clinodactyly of the 5th finger (**Figure 2B**). His bone age was delayed compared to

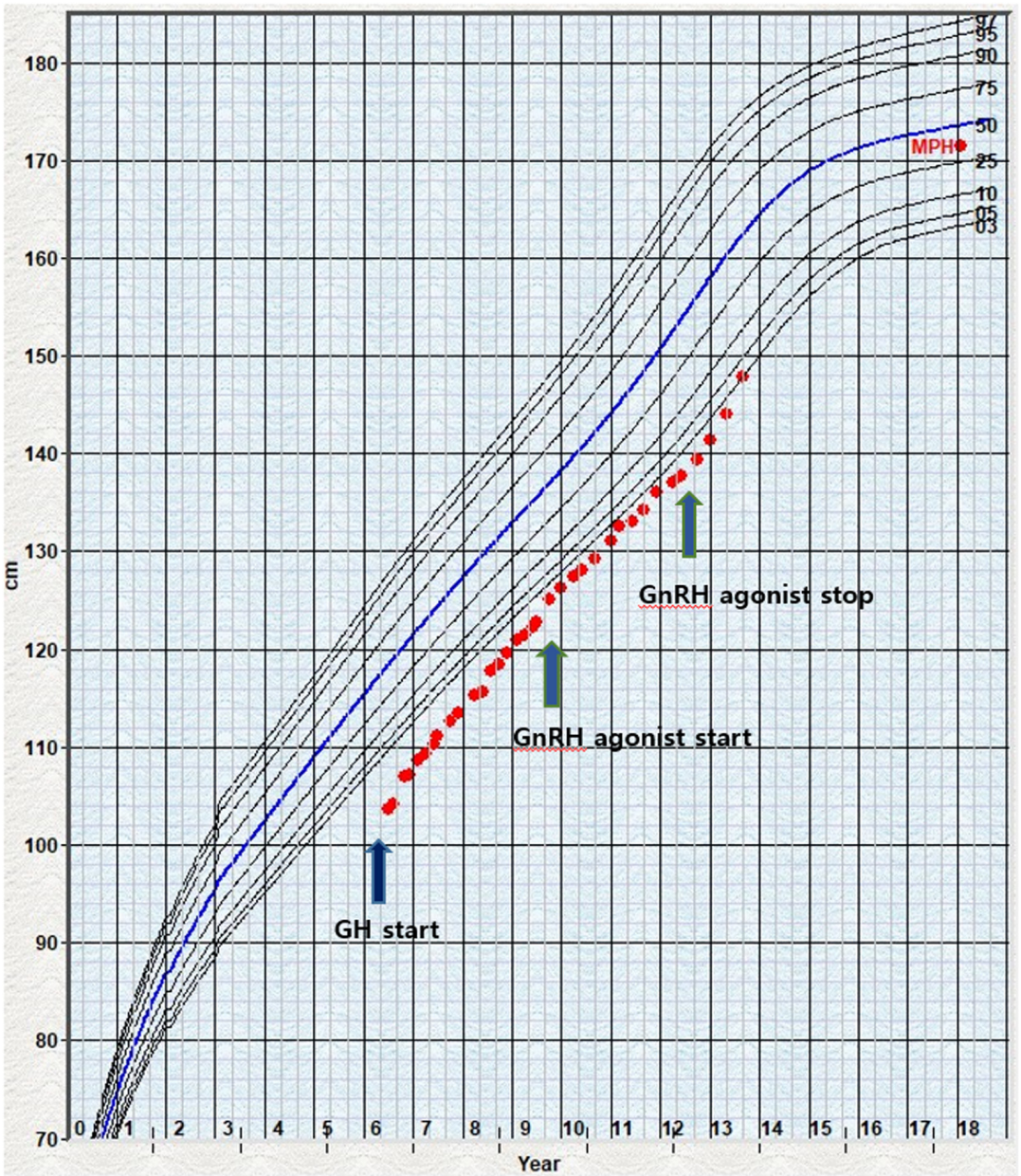


FIGURE 1 | Growth chart of the patient 1 under GH and GnRH agonist treatment. The dot indicates the height measurement. The arrow marks the beginning of GH and GnRH treatment start or stop. GH, growth hormone; GnRH, gonadotropin-releasing hormone; MPH, mid-parental height.

the chronological age according to the Greulich and Pyle atlas and there were no signs of puberty. He attended a special-education school with quite some assistance from his caregiver. He continued to take antiepileptic drugs (oxcarbazepine 33 mg/kg/day and levetiracetam 7 mg/day) and antipsychotic drugs (risperidone 3 mg/day and haloperidol 1.5 mg/day).

Whole exome sequencing (WES) was done, and a novel heterozygous variant, c3310dup, p. (Glu110GlyfsTer5) in ANKRD11 was identified (**Figure 3B**). In this variant, the amino acid glutamic acid is changed to glycine to result in a stop codon leading to nonsense-mediated decay, thereby causing haploinsufficiency of ANKRD11. This variant was categorized as likely pathogenic according to the ACMG guideline (PS1, PM2, PM4, PM5) (Richards et al., 2015).

Mutational Analysis

A DNA sample was obtained from peripheral blood leukocytes by using a Chemagic Magnetic Separation Module I (Chemagic MSM I, BaesWeiler, Germany) extraction robot with DNA Blood 200 μL Kit. TruSight One (Illumina Inc., San Diego, CA) for patient 1 and SureSelect Human All Exon V5 (Agilent Technologies, Santa Clara, CA) for patient 2 were used for



FIGURE 2 | (A) Dysmorphic features of patient 1. Macrodontia of the upper central incisors, a dysmorphic face including prominent and high nasal bridge, anteverted nostrils, hypertelorism, long philtrum, and brachydactyly of both hands. **(B)** Dysmorphic features of patient 2. Macrodontia of the permanent upper central incisors; dysmorphic face features such as triangular face, thick eye lashes and eyebrows, prominent nasal bridge, long philtrum, and brachycephaly; and hand anomalies including brachydactyly and clinodactyly of the 5th finger.

library preparation, and sequencing was done on the Illumina NextSeq500 platform (Illumina Inc., San Diego, CA) generating 2 × 150 bp paired-end reads. Alignment of sequence reads, indexing of the reference genome (hg19), and variant calling were performed with a pipeline based on the Genome Analysis Tool kit (GATK) Best Practices. Alignment was done with the BWA-mem (version 0.7.12), duplicated reads were marked with Picard (version 1.96)[1] and local alignment, base quality recalibration, variant calling was performed with a GATK (version 3.5), and annotation was done with VEP88 (Variant Effect Predictor), dbNSFP v3.3.

DISCUSSION AND CONCLUSION

There is currently no consensus regarding the clinical diagnostic criteria for KGB syndrome; however, several diagnostic criteria have been suggested (Skjei et al., 2007; Ockeloen et al., 2015; Low et al., 2016; Morel Swols et al., 2017). Postnatal short stature (defined as height below 3–10 percentile) is a major criterion, as 41–83% cases were described to show this characteristic in previous reports (Reynaert et al., 2015; Low et al., 2016; Morel Swols et al., 2017).

In 2015, Reynaert et al. reported first responses to GH treatment in KBG syndrome and suggested that GH would be a promising treatment for short stature in KBG syndrome, regardless of the presence of GH deficiency (Reynaert et al., 2015; Ge et al., 2020). Thereafter, some KBG syndrome patients with short stature have been treated with GH; preliminary evidence supports that GH treatment may be effective in enabling patients with KBG syndrome catch up to the normal growth rate (Goldenberg et al., 2016; Kang et al., 2019; Scarano et al., 2019).

Advanced puberty was also reported in a few patients with KBG syndrome (Goldenberg et al., 2016). Scarano et al. (2019) mentioned that early sign of puberty may influence short stature in KBG syndrome. It is not clear why the loss of function mutations of ANKRD11 gene causes short stature and advanced puberty. Our first patient was treated with GH and GnRH agonist due to GH deficiency combined with early and rapid progressive puberty. In prior reports, one male patient had been treated with GH and combined GnRH agonist, but the patient's treatment started at 12.5 years of age and was stopped because of decreased growth velocity at 13.8 years (Reynaert et al., 2015). In another patient, treatment with GnRH agonist was interrupted after 2 months due to a local adverse effect (Scarano et al., 2019). In our case, the first pubertal signs presented when the patient was 8.9 years old, and the results of the GnRH test were consistent with a diagnosis of early puberty. With the accumulation of clinical data and the increase in the number of cases wherein KBG is diagnosed at a young age, the frequency of early puberty is likely to increase. Therefore, the careful monitoring of puberty-related signs in patients with KBG syndrome at pre and peripubertal ages is important. Further experience with GnRH agonist and/or GH treatment in KBG syndrome, including our case, will help establish guidelines about puberty and growth in patients with KBG syndrome.

ID is an important characteristic of KBG syndrome. The role of ANKRD11, which typically regulates transcription by binding

[1]http://picard.sourceforge.net

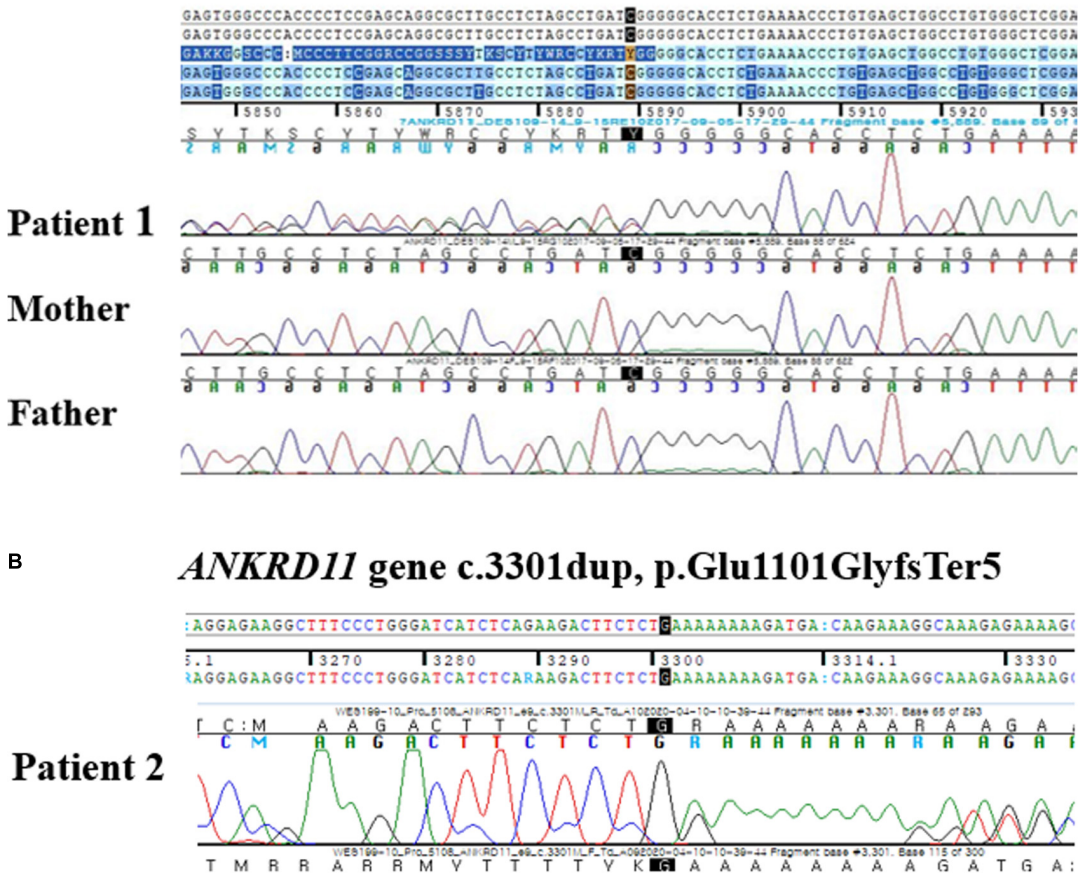


FIGURE 3 | (A) Electropherograms of the patient 1 and his parents. A *de novo* heterozygous frameshift mutation at position 5889 (c.5889del) in the *ANKRD11* gene was found in the patient 1 and was not inherited from his parents. **(B)** Electropherograms of the patient 2. A heterozygous single base pair duplication at position 3301 (c.3301 dup) in the *ANKRD11* gene.

chromatin-modifying enzymes such as histone deacetylases, in the development of the central nervous system, is not yet fully understood (Gallagher et al., 2015; Walz et al., 2015). Recently, Ka and Kim (2018) demonstrated a fundamental role of ANKRD11 in regulating pyramidal neuron migration and dendritic differentiation via the BDNF/TrkB signaling pathway in the developing mouse cerebral cortex. Haploinsufficiency of ANKRD11 due to microdeletion of chromosome 16q24.3 or loss of function mutation in the ANKRD11 gene results in the KBG syndrome phenotype, which includes neurodevelopmental problems. Alfieri et al. reported a low mean TIQ score (66.0 ± 16.2, represented as mean ± SD) in 17 patients with KBG syndrome (Alfieri et al., 2019), and van Dongen et al. (2017) showed similar mean TIQ distribution (66.5 ± 15.6 in 12 children, 62.5 ± 7.1 in 6 adults). As mentioned above, most patients have moderate to mild ID and more than half of the adults with KBG syndrome have jobs and are self-sufficient in ordinary life (Goldenberg et al., 2016; Low et al., 2016). Our second patient had severe ID (TIQ 26 at 7 years of age) with the lowest known TIQ in KBG syndrome up to date. Unfortunately, we did not have information about other environmental or perinatal factors related to ID, such as fetal alcohol exposure or perinatal asphyxia.

In addition to ID, some behavioral and psychopathologic problems such as ADHD, autism-like features, and impulsivity are major issues in patients with KBG syndrome (Novara et al., 2017). In recent study of 17 pediatric patients with KBG syndrome, there was a higher prevalence of obsessive-compulsive traits, tic disorders, depressive mood, and ADHD as compared with the general population; obsessive-compulsive traits, in particular, did not seem to be related to cognitive level (Alfieri et al., 2019). Similarly, another study also mentioned that ADHD-like behavioral problems associated with KBG syndrome could not be directly related or explained with a specific intelligence profile (van Dongen et al., 2017). In our cases, the first patient was treated for ADHD, and the second patient also had behavioral problems such as ASD, anxiety, and stereotypical movements.

As seen in our cases, some of the features of KBG syndrome including macrodontia and facial dysmorphism (triangular face, synophrys, thick eyebrow) have become more apparent over

time. Facial dysmorphism in KBG syndrome are overlapping with that seen in other syndromes such as Cornelia de Lange syndrome or Kabuki syndrome. As it is not easy to suspect KBG syndrome based only on the characteristic gestalt, KBG syndrome is considered vulnerable to underdiagnosis. Recently, the number of cases of KBG syndrome being diagnosed before 6 years of age through a next-generation sequencing (NGS) panel or WES has increased. In these cases, the clinical symptoms of KBG syndrome such as macrodontia and short stature were not clear. Developmental delay, especially speech delay and congenital malformations such as congenital heart defect or urogenital anomalies, hearing loss, and feeding difficulties are the main clinical manifestations in younger patients (Goldenberg et al., 2016; Low et al., 2016; Gnazzo et al., 2020). Low et al. recommended including the ANKRD11 gene in the NGS panel for developmental delay (Low et al., 2016). Early diagnosis through NGS or TES can enable clinicians to monitor possible problems, such as behavioral disorders, short stature, and hearing impairment. Genetic counseling is also of vital importance in the management of this condition. Early interventions may improve the patient's quality of life.

In conclusion, we report two novel mutations of the ANKRD11 gene with different phenotypes of KBG syndrome. GH treatment and combined GnRH agonist treatment are effective in a child with KBG syndrome with short stature and early puberty. Moreover, our case presented with severe ID, seizures, and psychopathologic problems without short stature despite KBG syndrome being generally characterized by mild to moderate ID. Considering the various phenotypes of KBG syndrome, a NGS panel including the ANKRD11 gene for developmental delay with/without short stature may be helpful to identify patients with undiagnosed KBG syndrome.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

Ethics board approval and consent was obtained for this work from the Institutional Review Board at the Inha University Hospital, Incheon, Korea (2020-03-039). The patients' parent or the parental agent provided written informed consent. Written informed consent was obtained from the patient's parent or the parental agent for publication of this case report and any accompanying images.

AUTHOR CONTRIBUTIONS

SJK analyzed and interpreted the data, prepared and corrected the manuscript. YSK and JEL designed the study. AY, J-SL, and DGK involved in the revision of this manuscript. All authors read and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Targeted Next-Generation Sequencing in the Diagnosis of Facial Dysostoses

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Background: Defects in the development of the first and second pharyngeal arches and their derivatives result in abnormal formation of the craniofacial complex, consequently giving rise to facial dysostoses (FDs). FDs represent a group of rare and highly heterogeneous disease entities that encompass mandibulofacial dysostoses (MFDs) with normal extremities and acrofacial dysostoses (AFDs) with limb anomalies in addition to craniofacial defects.

Methods: We examined 11 families with variable clinical symptoms of FDs, in most of which only one member was affected. We applied two custom gene panels—first comprising 37 genes related to the genetic disorders of craniofacial development such as FDs (On-Demand AmpliSeq Thermo Fisher Scientific gene panel with two primer pools) and second composed of 61 genes and 11 single nucleotide variants (SNVs) known to be involved in the development of skull malformations, mainly in the form of craniosynostoses (SureSelect Agilent Technologies). Targeted next-generation sequencing (NGS) was performed using the Ion Torrent S5 platform. To confirm the presence of each detected variant, we have analyzed a genomic region of interest using Sanger sequencing.

Results: In this paper, we summarized the results of custom targeted gene panel sequencing in the cohort of sixteen patients from 11 consecutive families affected by distinct forms of FDs. We have found three novel pathogenic variants in the *TCOF1* gene—c.2145_2148dupAAAG p.(Ser717Lys*fs**42), c.4370delA p.(Lys1457Arg*fs**118), c.83G>C p.(Arg28Pro) causing Treacher Collins syndrome type 1, two novel missense variants in the *EFTUD2* gene–c.491A>G p.(Asp164Gly) and c.779T>A p.(Ile260Asn) in two female patients affected by acrofacial dysostosis Guion-Almeida type, one previously reported–c.403C>T (p.Arg135Cys), as well as one novel missense variant–c.128C>T p.(Pro43Leu) in the *DHODH* gene in the male patient with Miller syndrome

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and finally one known pathogenic variant c.574G>T p.(Glu192*) in the *SF3B4* gene in the patient with Nager syndrome.

Conclusion: Our study confirms the efficiency and clinical utility of the targeted gene panel sequencing and shows that this strategy is suitable and efficient in the molecular screening of variable forms of FDs.

Keywords: facial dysostoses, mandibulofacial dysostoses, acrofacial dysostoses, pharyngeal arch, rare diseases, craniofacial development, targeted next-generation sequencing

BACKGROUND

The human skull composes of neuro- and viscerocranium that both develop independently from one another. Neurocranium mainly forms from the first five somites and the unsegmented somitomeres rostral to the first somite, whereas, viscerocranium, namely facial skeleton, forms from pharyngeal arches (Jin et al., 2016).

Defects concerning embryonic development of the first and second pharyngeal arches and their derivatives result in facial dysostoses (FDs), which encompass a group of rare and extremely variable clinical disease entities. FDs have been subdivided into mandibulofacial dysostoses (MFDs) with normal extremities and acrofacial dysostoses (AFDs) with limb anomalies in addition to craniofacial malformations (**Table 1**; Trainor and Andrews, 2013; Wieczorek, 2013). The characteristic facial features of FDs involve hypoplasia of the maxilla, mandible, zygomatic arches, various anomalies of the external and middle ear, and other facial defects including coloboma of the lower eyelid with partial to total absence of eyelashes or dysmorphic features, such as down-slanting palpebral fissures. Limb defects represent a broad spectrum of post- or preaxial abnormalities, including polydactyly, oligodactyly, and brachydactyly (Ogilvy-Stuart and Parsons, 1991; McDonald and Gorski, 1993; Wieczorek et al., 2009; Ng et al., 2010; Rainger et al., 2012; Czeschik et al., 2013; Trainor and Andrews, 2013; Wieczorek, 2013). Additional clinical signs, which may be present comprise microcephaly, cleft palate, oligodontia, defects of the internal organs, short stature, psychomotor delay, and intellectual disability (Guion-Almeida et al., 2006; Wieczorek, 2013).

Importantly, FDs present a high clinical heterogeneity, including a marked inter- and intra-familial variability of their phenotypic expression what in turn frequently impedes reaching an exact clinical diagnosis and its confirmation at a molecular level (Dixon et al., 2004; Vincent et al., 2016). Consequently, many patients affected by FDs remain molecularly undiagnosed and bereft of reliable genetic counseling, prenatal and preimplantation testing, but also of a proper and unequivocal clinical classification (Ng et al., 2010; Bernier et al., 2012; Lines et al., 2012; Fan et al., 2019).

MATERIALS AND METHODS

All procedures were performed under the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. We granted ethics approval from the Institutional Review Board of Poznan University of Medical Sciences (no. 741/17 and 742/17 obtained on 22nd June 2017). We examined 11 families with variable clinical symptoms of FDs, in most of which only one member was affected. We extracted genomic DNA from the peripheral blood lymphocytes using the MagCore HF16 Automated Nucleic Acid Extractor and quantified each gDNA using the Agilent Technologies TapeStation 2200 and Qubit.

Targeted Next-Generation Sequencing Facial Dysostoses Panel

We designed the custom On-Demand AmpliSeq (Thermo Fisher Scientific) gene panel with two primer pools and performed targeted sequencing of 37 genes related to the genetic disorders of craniofacial development, including FDs (patients 1.4, 1.6, 2–4, 6 and 7; **Supplementary Table 1**). The gene panel was 145.51 kb in size and comprised 761 amplicons, intervals sequenced for each gene were shown in **Supplementary Table 2**. The barcoded genomic DNA libraries were constructed according to the 'manufacturer's sample preparation protocol (Ion AmpliSeq Library Kit 2.0; On-Demand Panels). Pooled libraries were subjected to emulsion PCR on Ion Chef^{TM} instrument using the Ion 520^{TM} and 530^{TM}Kit, according to the standard protocol and sequenced on the Ion Torrent S5 platform.

Craniosynostosis Panel

The custom SureSelect (Agilent Technologies) gene panel comprised of 61 genes and 11 SNVs known to be involved in the development of craniofacial malformations, particularly craniosynostosis (CS), was designed (**Supplementary Table 3**). Intervals sequenced for each gene were shown in **Supplementary Table 4**. Targeted sequencing, encompassing 225.709 kb was performed for patient 5. We prepared the NGS library using hybridized capture-based target enrichment approach and sequenced it on the Ion Torrent S5 platform (Bukowska-Olech et al., 2020).

Next-Generation Sequencing Data Analysis

The present analysis follows the approach previously described in Bukowska-Olech et al. (2020). Briefly, the reads were cleaned and aligned to GrCh37 human reference sequence using the TorrentBrowser 5.0.4 software (Thermo Fisher Scientific). The alignments were further processed using IonReporter

TABLE 1 | Clinical classification of human facial dysostoses.

Type of facial dysostosis	Subtype of facial dysostosis	OMIM	Gene/locus	Inheritance
Mandibulofacial dysostosis	Bauru	604830	nd	nd
Mandibulofacial dysostosis	Burn-McKeown	608572	*TXNL4A*	AR
Mandibulofacial dysostosis	Hedera-Toriello-Petty	608257	nd	nd
Mandibulofacial dysostosis	Toriello	301950	nd	nd
Mandibulofacial dysostosis	Treacher Collins type 1	154500	*TCOF1*	AD
Mandibulofacial dysostosis	Treacher Collins type 2	613717	*POLR1D*	AD, AR
Mandibulofacial dysostosis	Treacher Collins type 3	248390	*POLR1C*	AD, AR
Mandibulofacial dysostosis	Verloes-Lesenfants	302562	nd	nd
Mandibulofacial dysostosis	Zhang	–	Duplication 1p36.33 Duplication 1q21.2-q22	nd
Acrofacial dysostosis	Arens	–	nd	nd
Acrofacial dysostosis	Bates	–	nd	nd
Acrofacial dysostosis	Catania	101805	nd	AD
Acrofacial dysostosis	Cincinnati	616462	*POLR1A*	AD
Acrofacial dysostosis	Guion-Almeida	610536	*EFTUD2*	AD
Acrofacial dysostosis	Karaman-Kavecci	–	nd	nd
Acrofacial dysostosis	Kennedy-Teebi	–	nd	AR
Acrofacial dysostosis	Kelly	–	nd	AR/XR
Acrofacial dysostosis	Macena Sobreira	–	nd	nd
Acrofacial dysostosis	Miller syndrome	263750	*DHODH*	AR
Acrofacial dysostosis	Nager syndrome	154400	*SF3B4*	AD
Acrofacial dysostosis	Patterson-Stevenson and Fontaine	183700	nd	nd
Acrofacial dysostosis	Palagonia	601829	nd	AD
Acrofacial dysostosis	Reynolds	–	*Microdeletion 16p13.3?*	nd
Acrofacial dysostosis	Richieri-Costa-Pereira	268305	*EIF4A3*	AR
Acrofacial dysostosis	Rodriguez	201170	nd	AR
Acrofacial dysostosis	Weyers	193530	*EVC, EVC2*	AD

AD, autosomal dominant; AR, autosomal recessive; nd, no data.

5.2 pipeline (Thermo Fisher Scientific), which incorporated variant calling with Torrent Variant Caller 5.4–11. Each variant was examined based on four criteria (read depth— $\geq$ 20, strandedness— $\leq$ 4:1, PHRED score $\geq$ 30 and variant frequency $\geq$ 0.15). The coverage of individual genes and exons was performed using bedtools 2.27.1 (*coverage* subcommand) against panel-specific BED files defining coding parts of canonical transcripts for each constituent gene (RefSeq mapped on UCSC hg19 reference; 5 bp padding around each exon included; **Supplementary Tables 5–8**).

Previous clinical annotation of called variants was obtained from Human Gene Mutation Database (HGMD) Professional (accessed on 14/02/2019), ClinVar (accessed on 27/07/2019) and dbSNP (accessed on 27/07/2019). The predictions for SIFT, PolyPhen, and PhyloP (46-way) tools were retrieved from IonReporter result files. Population allele frequencies were annotated by Ensembl/Variant Effect Predictor (software version 94, database version 94); additionally, gnomAD database was queried for homozygosity/heterozygosity of individual variants (version 2.1.1 of both exome and genome subsets; accessed on 27/07/2019 using tabix 1.5 software). The scores for Combined Annotation Dependent Depletion were obtained from CADD webserver (v1.4, accessed on 27/07/2019). MutationTaster results were obtained using the "query chromosomal position" option of the public webserver (accessed on 27/07/2019).

Sanger Sequencing

To confirm the presence of each pathogenic or likely pathogenic variant, as well as of variant of uncertain significance (VUS), we have analyzed the genomic region of interest using Sanger sequencing. The online available Primer3 tool was used to design specific primers for the amplification. The PCR reactions and PCR products purification were carried out following standard protocols. Sanger sequencing was performed using dye-terminator chemistry (kit v.3, ABI 3130XL) and run on automated sequencer Applied Biosystems Prism 3700 DNA Analyzer. Variants were annotated against the reference sequences: *DHODH*—NG_016271.1, NC_000016.10, NM_001361.4, NP_001352.2; *EFTUD2*—NG_032674.1, NC_000017.11, NM_004247.3, NP_004238.3; *SF3B4*—NG_032777.1, NC_000001.11 NM_005850.4, NP_005841.1, *TCOF1*—NG_011341.1, NC_000005.10, NM_001135243.1, NP_001128715.1 following the Human Genome Variation Society (HGVS) nomenclature guidelines (den Dunnen et al., 2016; **Table 2**).

TABLE 2 | Summary of pathogenic variants reported in this paper.

	Gene	HGVS genomic	HGVS chromosomal	HGVS coding	HGVS protein level	Location	ACMG Classification	CADD	SIFT	PolyPhen	MutationTaster
Family 1	*TCOF1*	NG_011341.1: g.18787_18790dup	NC_000005.10: g.150376425_ 150376428dup	NM_001135243.1: c.2145_2148dup	NP_001128715.1: p.(Ser717Lys*fs**42)	Exon 10	Pathogenic	15.57	n/a	n/a	Disease causing
Patient 2	*TCOF1*	NG_011341.1: g.191G>C	NC_000005.10: g.150357829G>C	NM_001135243.1: c.83G>C	NP_001128715.1: p.(Arg28Pro)	Exon 1	Variant of Uncertain Significance	33	n/a (0^)	0.998	Polymorphism
Patient 3	*TCOF1*	NG_011341.1: g.40740_40740delA	NC_000005.10: g.150398381delA	NM_001135243.1: c.4370delA	NP_001128715.1: p.(Lys1457Arg*fs**118)	Exon 25	Pathogenic	31	n/a	n/a	Disease causing
Patient 4	*SF3B4*	NG_032777.1: g.1837G>T	NC_000001.11: g.149926508C>A	NM_005850.4: c.574G>T[#]	NP_005841.1: p.(Glu192*)[#]	Exon 3	Pathogenic	38	n/a	n/a	Disease causing
Patient 5	*EFTUD2*	NG_032674.1: g.16569A>G	NC_000017.11: g.44883094T>C	NM_004247.3: c.491A>G	NP_004238.3: p.(Asp164Gly)	Exon 6	Likely Pathogenic	25	0.23	0.095	Disease causing
Patient 6	*EFTUD2*	NG_032674.1: g.23639T>A	NC_000017.11: g.44876024A>T	NM_004247.3: c.779T>A	NP_004238.3: p.(Ile260Asn)	Exon 10	Likely Pathogenic	27.2	0.0	0.972	Disease causing
Patient 7	*DHODH*	NG_016271.1: g.3569C>T	NC_000016.10: g.72012156C>T	NM_001361.4: c.128C>T	NP_001352.2: p.(Pro43Leu)	Exon 2	Variant of Uncertain Significance	26.1	1.0	0.0	Disease causing
	DHODH	NG_016271.1: g.6054C>T	NC_000016.10: g.72014641C>T	NM_001361.4: c.403C>T[#]	NP_001352.2: (p.Arg135Cys)[#]	Exon 3	Pathogenic	32	1.0	0.0	Disease causing

American College of Medical Genetics (ACMG) classification was obtained through VarSome online available tool (accessed on 23/08/2020). PolyPhen and SIFT scores were obtained through IonReporter pipeline and cross-referenced with Variant Effect Predictor (VEP) results. [#] *variant reported previously,* ∧ *score reported through Ensembl VEP.*

TABLE 3 | Summary of clinical data in reported patients with Treacher Collins syndrome (TCS).

	Patient 1.1 (Figures 1 A,B)	Patient 1.2 (Figures 1C,D)	Patient 1.3 (Figures 1E,F)	Patient 1.4 (Figures 1G,H)	Patient 1.5 (Figures 1I,J)	Patient 1.6 (Figure 1K)	Patient 2 (Figures 3A,B)	Patient 3 (Figures 3D,E)
Gender	F	M	F	M	F	F	F	M
Mandibular hypoplasia	+	+	+	+	+	+	+	+
Maxillary hypoplasia	+	+	+	+	+	+	+	+
Downward slanting palpebral fissures	+	+	+	+	+	+	+	+
Partial absence of eyelids	–	–	–	–	+	+	–	–
Microtia	–	–	–	–	+	+	–	+
Conductive hearing loss	+	+	+	+	+	+	+	+
Cleft lip/palate	–	–	–	–	+	–	–	+

RESULTS

Clinical Assessment

We recruited 11 consecutive families in whom 16 individuals were affected by various forms of FDs. Four families (no. 1–3, 6) received the clinical diagnosis of MFDs defined as hypoplastic maxilla, mandible, and zygomatic arches with down-slanting palpebral fissures. Six families (no. 4, 8–11) were clinically diagnosed with AFDs since the affected individuals showed not only craniofacial but also limb involvement. In one family (family 5), we did not recognize FD based on the dysmorphological assessment. The detailed clinical data of all screened patients is set forth in **Supplementary Data Sheet 1**.

Genetic Analyses

Targeted NGS allowed us to reach an exact diagnosis of FDs in seven out of eleven families (no. 1–7). Among them, we identified six novel and two previously reported variants in the following genes–*TCOF1*, *SF3B4*, *EFTUD2,* and *DHODH* (**Table 2**). The mean coverage for FDs panel ranged from 214 to 430 reads per gene, whereas for CS panel from 272 to 488 reads per gene (**Supplementary Tables 5**, **7**).

In families no 1–3, we found three novel heterozygous variants in the *TCOF1* that gave rise to the most well-characterized MFD, i.e., Treacher Collins syndrome (TCS) type 1 (TCS1, MIM:154500). The major clinical features of all affected individuals were summarized in **Table 3**. The rest of the diagnosed patients was affected by different types of AFDs. In a sporadic female case from family no 4, we identified the heterozygous variant in the *SF3B4* gene causing Nager syndrome (MIM:54400). Two other sporadic female cases from families no 5 and 6 harbored *de novo* heterozygous variants within the *EFTUD2* gene and presented with variable symptoms of acrofacial dysostosis Guion-Almeida type (AFDGA, MIM:610536). The comparison of their clinical features was shown in **Table 4**. Finally, in a male proband from family no 7, we diagnosed Miller syndrome (MIM:263750), as we found two biallelic variants in the *DHODH* gene. Importantly, none of the detected alterations was present in gnomAD or ExAC databases or found in 200 ethnically matched control alleles from our in-house, population-specific database. Segregation analysis of the causative variants performed in families 1–6 was consistent with the expected autosomal dominant inheritance pattern (**Figures 2**, **3C,F**, **4C**, **5D,H**), whereas in family 7 with autosomal recessive pattern (**Figure 6E**). The results of molecular screening and segregation analyses were shown in **Figures 1–6** and **Supplementary Figures 1A–I**.

TABLE 4 | Summary of clinical data in reported patients with acrofacial dysostoses Guion-Almeida type (AFDGA).

	Patient 5	Patient 6
Gender	F	F
Intellectual impairment	–	+
Delayed psychomotor development	–	+
Delayed speech development	–	+
Epilepsy	–	+
Microcephaly	–	+
Trigonocephaly	+	+
Midface hypoplasia	–	+
Malar hypoplasia	–	+
Micrognathia	–	+
Buccal tags	–	+
Microtia	–	–
Preauricular tag	–	+
Preauricular pit	–	+
Low-set ears	–	+
Dysplastic ears	–	+
Conductive hearing loss	–	+
Upslanting palpebral fissures	–	–
Downslanting palpebral fissures	–	+
Epicanthal folds	–	–
Choanal atresia	–	–
Upturned nose	+	–
Short nose	–	+
Cleft palate	–	–
Atrial septal defect	–	+
Breathing difficulties due to choanal atresia	–	–
Esophageal atresia	–	–
Preaxial polydactyly	+	–
Proximally placed thumbs	–	–

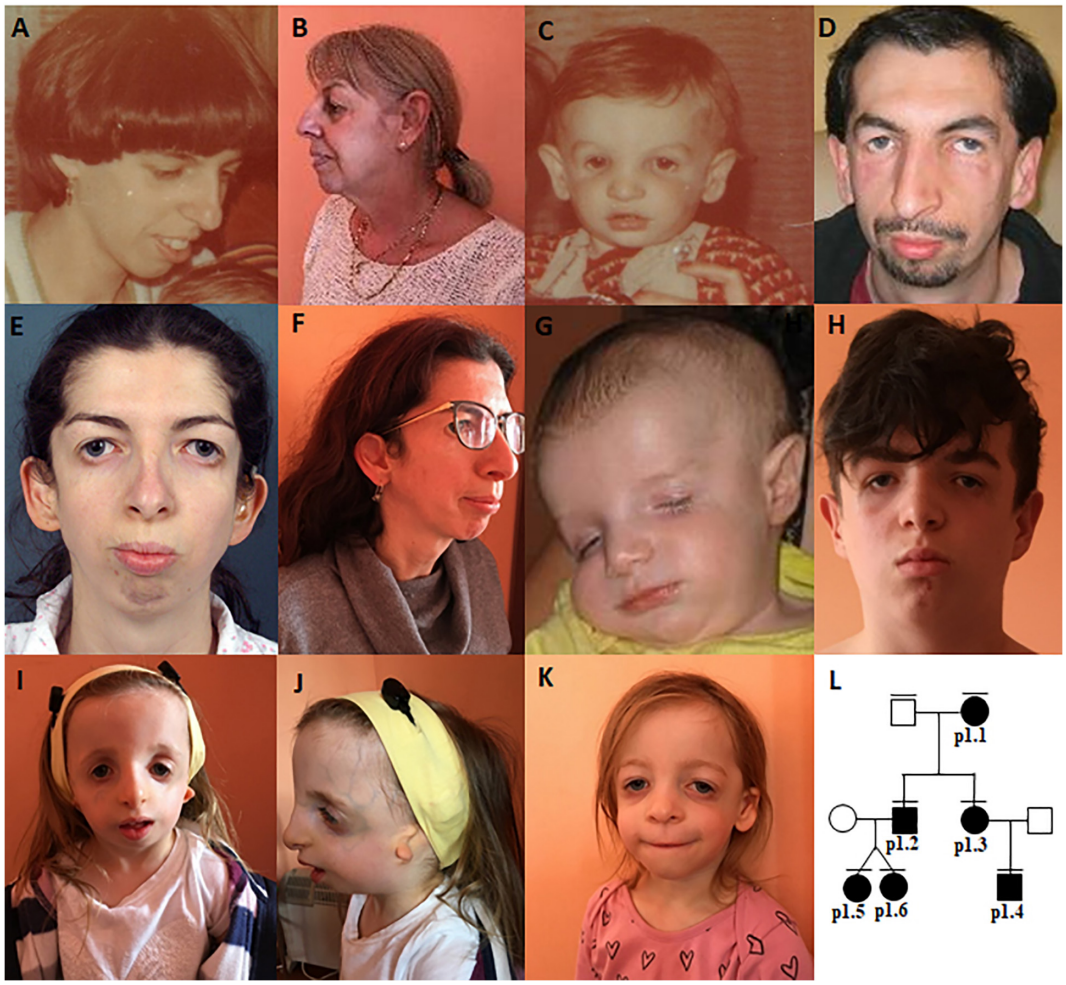


FIGURE 1 | Clinical characteristics of family 1–patient 1.1 **(A,B)**, patient 1.2 **(C,D)**, patient 1.3 **(E,F)**, patient 1.4 **(G,H)**, patient 1.5 **(I,J)**, patient 1.6 **(K)**, The pedigree **(L)**.

DISCUSSION

Before the advent of NGS, identification of the underlying causative genetic lesions in individuals affected by molecularly complex genetic syndromes such as FDs was often difficult or sometimes impossible (Fan et al., 2019). In an excellent review on human facial dysostoses, Wieczorek emphasized that the current classification of FDs bases mostly on the clinical characteristics of the facial features as well as the presence or absence of additional symptoms (**Table 1**; Wieczorek, 2013). The molecular confirmation of FDs is unquestionably crucial not only for reliable genetic counseling, prenatal and preimplantation testing, but also for a proper and unequivocal clinical classification.

In this paper, we summarized the results of the custom targeted gene panel sequencing in a group of 16 patients from 11 consecutive families affected by distinct forms of FDs that were consulted in our genetic clinic. In this cohort, we detected three novel heterozygous variants within the *TCOF1* gene, two of which were frameshifts: c.2145_2148dupAAAG p.(Ser717Lys*fs**42) (family 1; **Figure 2**), c.4370delA p.(Lys1457Arg*fs**118) (family 3; **Figure 3F**), while one was a missense alteration c.83G>C p.(Arg28Pro) (family 2; **Figure 3C**). *In silico* analyses predicted that frameshifts truncating Treacle protein at codons 717 and 1457 were pathogenic, whereas a missense variant p.(Arg28Pro) was initially classified as a VUS (**Table 2**). The results of segregation analysis performed in family 2 showed that the variant occurred *de novo*, suggesting its contribution to the patient's phenotype (**Figure 3C**). The rest of the diagnosed individuals was affected by various types of AFDs. Among them, we identified one previously reported nonsense heterozygous pathogenic variant in the *SF3B4* gene–c.574G>T p.(Glu192*) (family 4; **Figure 4C**), two novel missense likely pathogenic heterozygous variants in the *EFTUD2* gene, which occurred *de novo*–c.491A>G p.(Asp164Gly) (family 5; **Figure 5D**), c.779T>A p.(Ile260Asn) (family 6; **Figure 5H**), and one previously reported–c.403C>T (p.Arg135Cys)

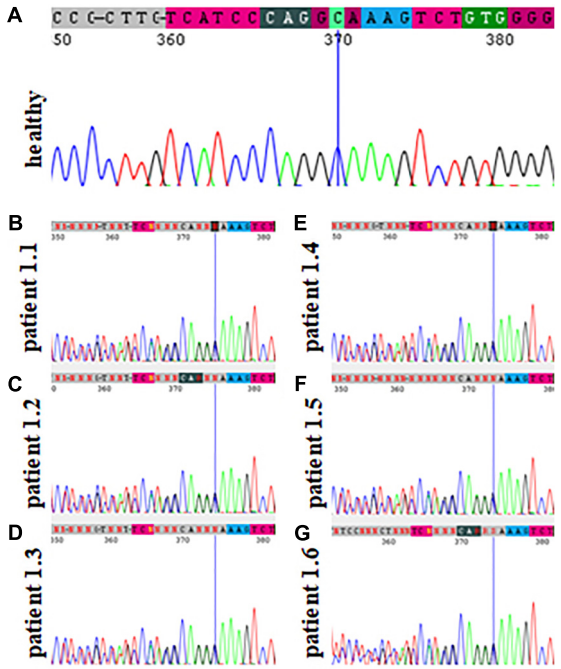


FIGURE 2 | Sanger sequencing results of family 1 showing the absence of the pathogenic variant c.2145_2148dupAAAG p.(Ser717Lys*fs**42) within the *TCOF1* gene in a healthy family member **(A)** and its presence in patients affected by Treacher Collins syndrome **(B–G)**.

(evaluated as pathogenic), as well as one novel—c.128C>T p.(Pro43Leu) missense variants (evaluated as VUS), both located in trans orientation in the *DHODH* gene (family 7; **Figure 6E**).

Pathogenic variants in the *TCOF1* but also the *POLR1D* and *POLR1C* genes have been previously associated with Treacher Collins syndrome (TCS) types 1–3, respectively (Dixon et al., 1996; Dauwerse et al., 2011; Schaefer et al., 2014). According to the current review of medical literature, in the majority of patients (i.e., 77–89%) causative alterations are found within the *TCOF1* gene (encoding Treacle protein). Consistently with these results, we detected all three causative variants in the *TCOF1* (families no 1–3), and not in other TCS-related genes (Dauwerse et al., 2011). TCS is characterized by malar hypoplasia, micrognathia, down-slanting palpebral fissures, atresia of the external auditory canals, anomalies of the middle ear ossicles, and bilateral conductive hearing loss (Stovin et al., 1960; Phelps et al., 1981; Trainor et al., 2009; Vincent et al., 2016). Nevertheless, the phenotype often varies not only between, but also within the affected families. We have confirmed that in cases with TCS1 there is no clear genotype-phenotype correlation and that the expressivity of the clinical symptoms shows considerable intra- and interfamilial variability suggesting the influence of additional contributors (**Table 3**). However, the presence of modifying factors, probably involved in the process of mRNA level regulation, requires further research.

In the vast majority of families affected by AFDs, we have suspected Nager syndrome, which surprisingly was proved genetically only in one individual (patient 4). The families 5 and 7 that were misdiagnosed with Nager syndrome turned out to represent AFDGA (family 6) and Miller syndrome (family 7). Nager syndrome, along with AFDGA and Miller syndrome, share similar facial and skeletal features, hence the unambiguous diagnosis based only on the clinical symptoms may be uncertain (Trainor and Andrews, 2013; Wieczorek, 2013). Variants in the *SF3B4* gene that encodes a splicing factor 3B subunit 4 are found in about half of the patients clinically diagnosed with Nager syndrome (Bernier et al., 2012; Czeschik et al., 2013). The main phenotypic features of this condition involve facial characteristics similar to TCS and preaxial limb defects such as hypoplasia or aplasia of the radius and thumbs, radioulnar synostosis or triphalangeal thumbs (Bowen and Harley, 1974; Meyerson et al., 1977; Opitz, 1987; McDonald and Gorski, 1993; Wieczorek, 2013). A nonsense alteration p.(Glu192*) within splicing factor 3B subunit 4, which occurred in patient 4, was firstly described by Czeschik et al. in a female patient with Nager syndrome, who manifested very similar symptoms to patient 4. Both individuals presented with extreme hypoplasia of the mandible, micrognathia, hypoplastic thumbs, bilateral limited elbow extension, hearing loss and delayed psychomotor development, with absent speech due to severe hypoplasia of the mandibulofacial structures (Czeschik et al., 2013). Additionally, patient 4 had an ectopic left kidney, which was located in the pelvis.

In families no. 5 and 6, we have found novel heterozygous variants in *EFTUD2,* the gene encoding elongation factor Tu GTP binding domain containing 2, resulting in AFDGA (Guion-Almeida et al., 2006; Lines et al., 2012). This entity was initially classified as mandibulofacial dysostosis Guion-Almeida type (MFDGA). Nevertheless, due to the occurrence of limb anomalies in some patients, it was reclassified to AFDs (Voigt et al., 2013). The clinical spectrum of AFDGA is broad and consists of microcephaly, postnatal-onset growth deficiency, choanal atresia, heart and gastrointestinal anomalies, preaxial polydactyly, slender fingers, and proximally placed thumbs (Guion-Almeida et al., 2006; Wieczorek et al., 2007, 2009; Lines et al., 2012; Gordon et al., 2015). Very recently, new craniofacial phenotypes, i.e., metopic craniosynostosis (CS) and cleft lip and palate, were reported in two individuals carrying heterozygous variants–c.2466+1G>A in intron 24 and c.2333C>A in exon 23 of *EFTUD2*. These cases were initially suspected of having an alternative craniofacial syndrome and were eventually diagnosed with MFDGA (Lacour et al., 2019). Similarly, patient 5 also presented with very unusual craniofacial features, including metopic synostosis and high forehead that were not suggestive for AFDGA.

Finally, in patient 7, we described compound heterozygous variants in *DHODH* gene. Biallelic variants in *DHODH* gene result in defects in pyrimidine biogenesis and give rise to Miller syndrome that has an autosomal recessive inheritance. Limb anomalies include postaxial abnormalities, such as the

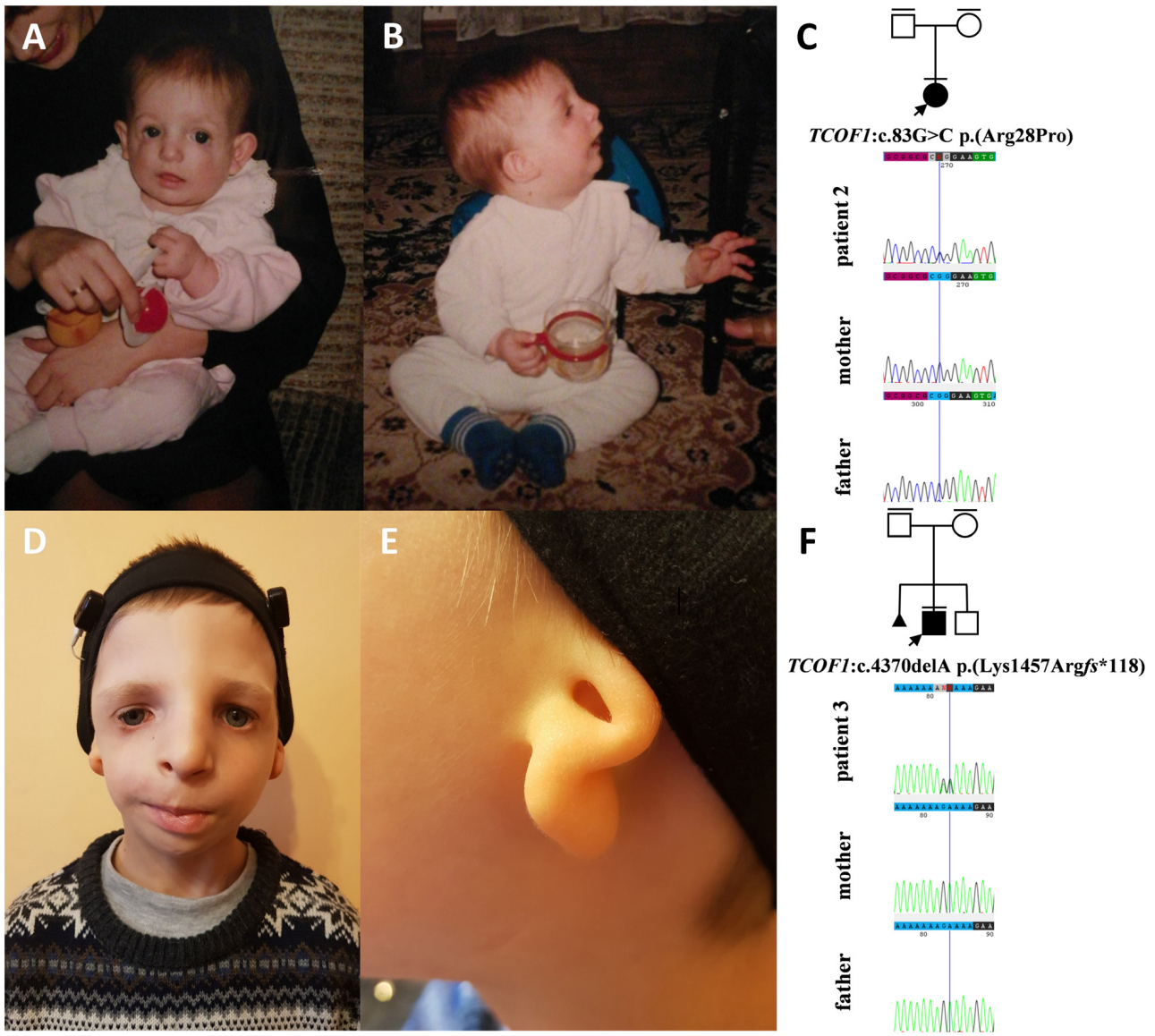


FIGURE 3 | Clinical characteristics and segregation results of patient 2 **(A–C)** and 3 **(D–F)** affected by Treacher Collins syndrome. Patient 2 carries a heterozygous variant c.83G>C p.(Arg28Pro) within the *TCOF1* gene, which occurred *de novo* **(C)** whereas patient 3 carries a heterozygous variant c.4370delA p.(Lys1457Argfs*118) within the *TCOF1* gene, which also occurred *de novo*.

absence of fifth digits with or without shortening and deviation of forearms with ulnar and radial hypoplasia, and syndactyly (Genee, 1969; Ogilvy-Stuart and Parsons, 1991; Chrzanowska and Fryns, 1993; Ng et al., 2010). The first pathogenic missense variant (p.Arg135Cys) has been reported previously in six individuals affected by Miller syndrome who were compound heterozygous for (p.Arg135Cys) in combination with one of the following alterations–c.155A>G (p.Glu52Gly), c.1036C>T (p.Arg346Trp), and c.1175A>G (p.Asp392Gly). All those patients, including patient 7 (**Figures 6A–D**), presented with strikingly similar features, encompassing severe micrognathia, cleft lip and/or palate, as well as hypoplasia or aplasia of the postaxial rays of the limbs (Ng et al., 2010; Al Kaissi et al., 2011; Rainger et al., 2012). The second missense variant c.128C>T p.(Pro43Leu) identified by us was novel.

The diagnostic success rate of the presented approach achieved 64%. Undiagnosed cases (families 8–11) may harbor other types of causative alterations that cannot be detected by targeted NGS, such as deep intronic or regulatory mutations affecting genes included in custom gene panels. Alternatively, causative mutations may be located elsewhere in the genome and not in the genes known to be involved in the pathogenesis of FDs. Therefore, in cases without the exact genetic diagnosis, another type of molecular investigation, including whole-genome sequencing (WGS) should be considered. Indeed, the

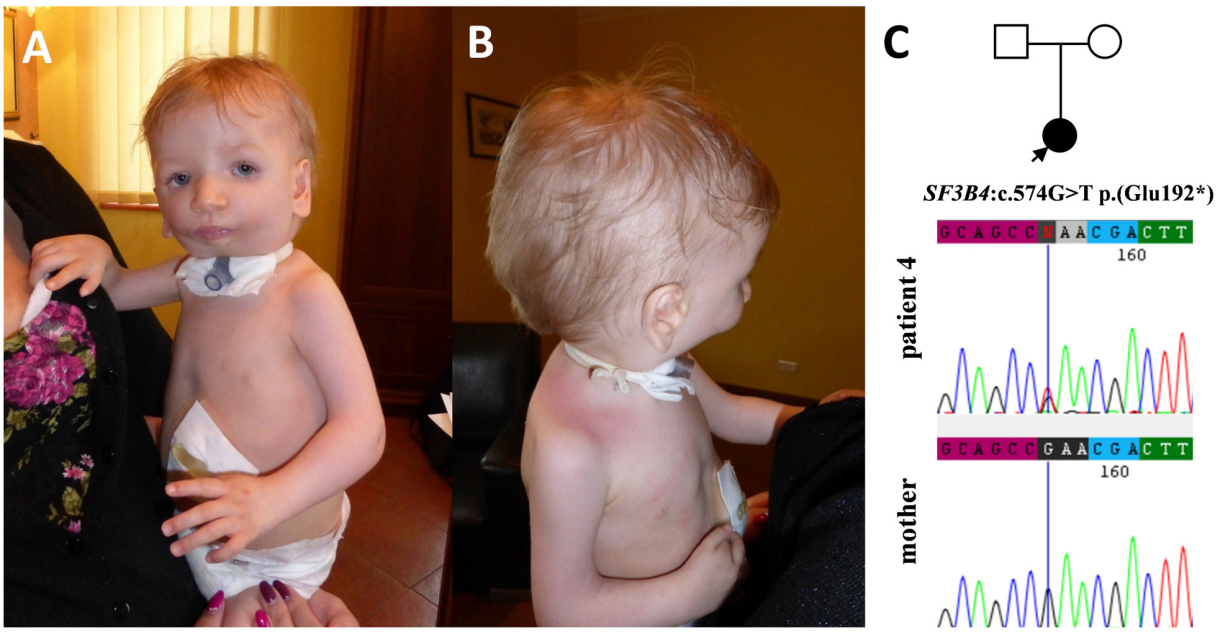


FIGURE 4 | Clinical characteristics of patient 4 affected by Nager syndrome **(A,B)**. Patient 4 carries a heterozygous variant c.574G>T p.(Glu192*) within the *SF3B4* gene, which was excluded in her healthy mother **(C)**.

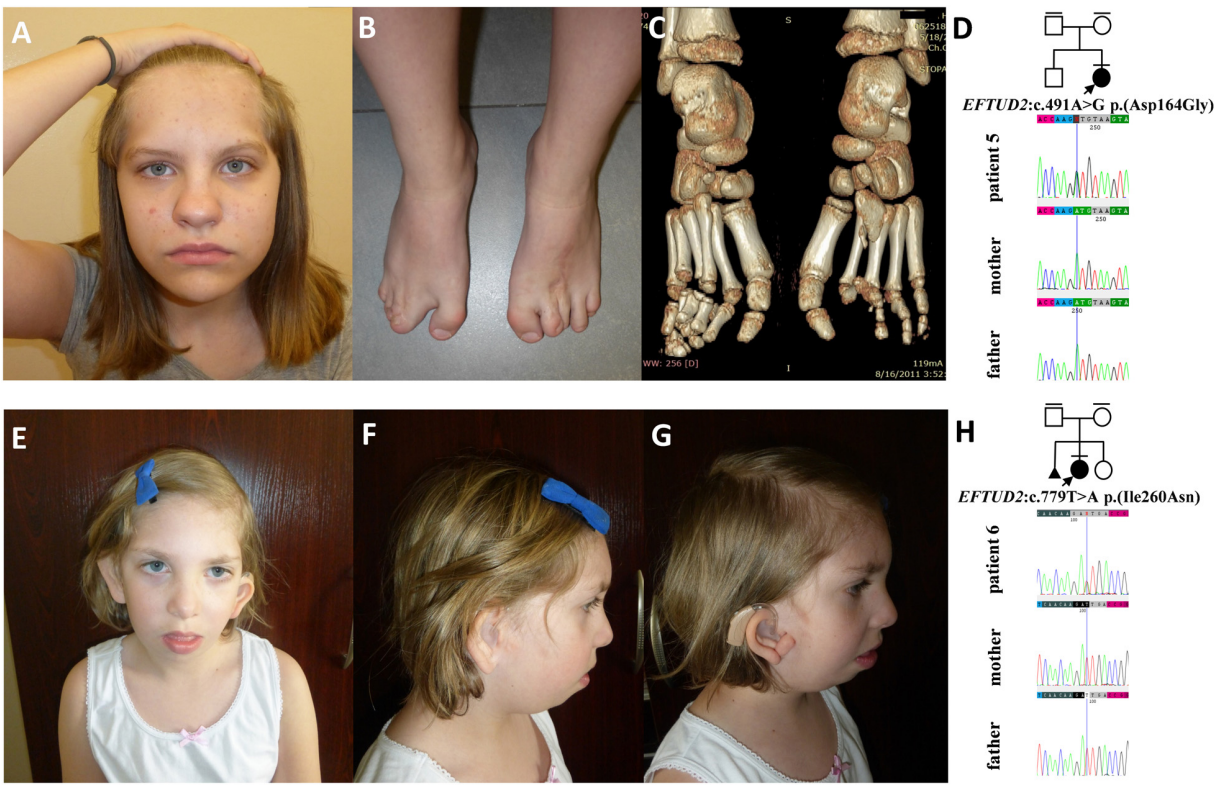


FIGURE 5 | Clinical characteristics and segregation results of patient 5 **(A–D)** and 6 **(E–H)** affected by acrofacial dysostosis Guion-Almeida type. Patient 5 carries a heterozygous variant c.491A>G p.(Asp164Gly) within the *EFTUD2* gene, which occurred *de novo* **(D)** whereas patient 6 carries a heterozygous variant c.779T>A p.(Ile260Asn) within the *EFTUD2* gene, which also occurred *de novo* **(H)**.

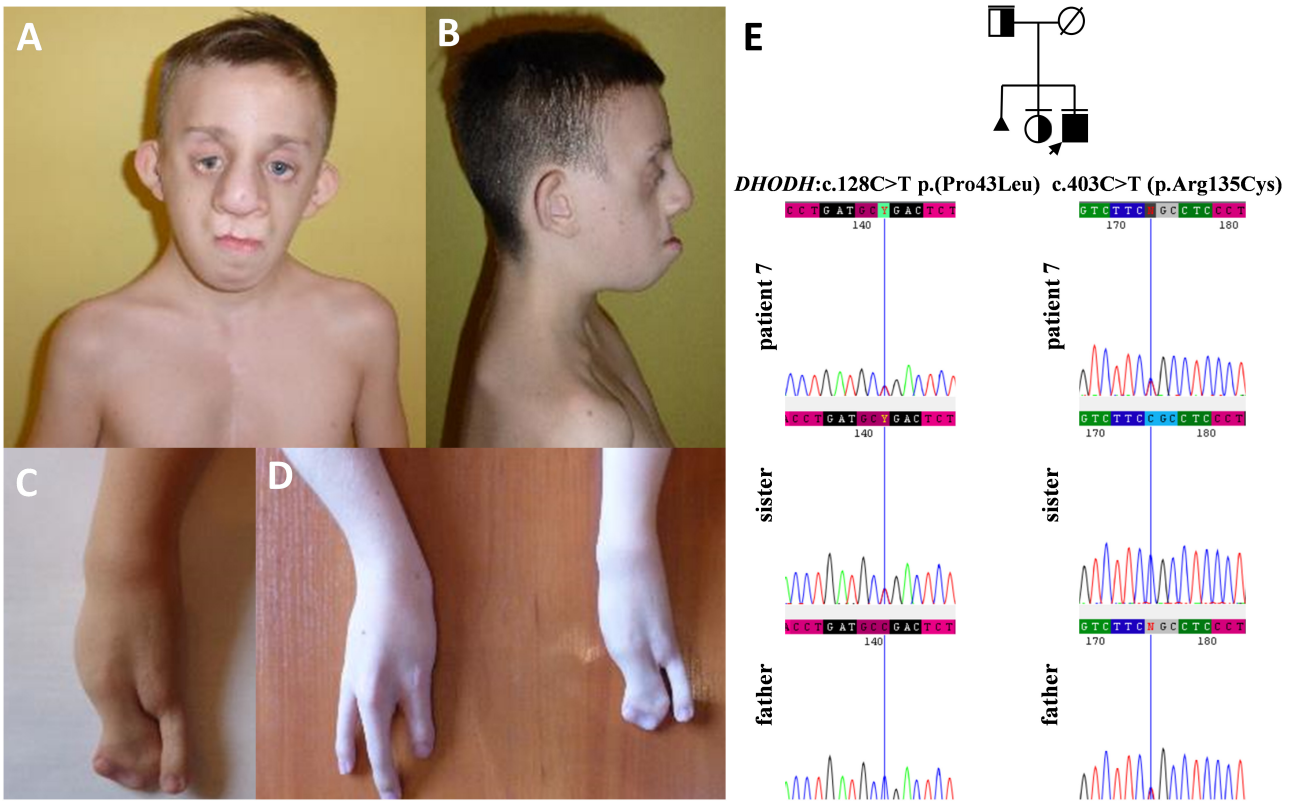


FIGURE 6 | Clinical characteristics **(A–D)** and segregation results **(E)** of patient 7 affected by Miller syndrome. Patient 7 carries c.403C>T (p.Arg135Cys) and c.128C>T p.(Pro43Leu) missense variants, both located in trans orientation in the *DHODH* gene **(E)**.

introduction of WGS in the molecular diagnostics of FDs requires advanced *in silico* and functional analyses, which are both necessary for the interpretation and proving of pathogenicity of genomic alterations (Smedley et al., 2016; Bodea et al., 2018; Posey, 2019). The proportion of molecularly undiagnosed patients, however, clearly suggests that some of the genetic lesions, causative for FDs, lie within non-coding DNA, and could be picked up only by WGS (Kindberg and Bush, 2019).

In conclusion, with this report, we demonstrated the clinical utility of NGS targeted gene panel approach as a valuable and cost-effective first-tier testing in the molecular analysis of patients presenting with FDs. We described the clinical phenotypes of seven families showing distinct forms of FDs and identified six novel and two previously described pathogenic variants. Consequently, we highlighted the need for molecular confirmation of FDs, which is crucial for a reliable genetic counseling, an early prenatal and preimplantation testing, but also a proper and unequivocal clinical classification.

DATA AVAILABILITY STATEMENT

Data generated during this study are included in this published article and its supplementary material. BAMs files were submitted on Sequence Read Archive (SRA) NCBI (SRA submission: PRJNA663125, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA663125.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethics Committee at Poznan University of Medical Sciences (no. 741/17 and 742/17) according to the Good Clinical Practice and Polish law. All patients and their parents agreed to participate in this study. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AJ, AM-K, and MB-S recruited and clinically diagnosed the patients. EB-O designed the study, performed, analyzed the molecular data, and wrote the manuscript. JW-S participated in data analysis. DP participated in a part of experimental procedures. GK participated in a part of data analysis. AD participated in a part of experimental procedures. AJ designed

the study and critically revised and corrected the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Pharmacodynamic Gene Testing in Prader-Willi Syndrome

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Prader-Willi syndrome (PWS) is a rare genetic disorder with a complex neurobehavioral phenotype associated with considerable psychiatric co-morbidity. This clinical case series, for the first time, describes the distribution and frequency of polymorphisms of pharmacodynamic genes (serotonin transporter, serotonin 2A and 2C receptors, catechol-*o*-methyltransferase, adrenergic receptor 2A, methylene tetrahydrofolate reductase, and human leucocytic antigens) across the two major molecular classes of PWS in a cohort of 33 referred patients who met medical criteria for testing. When results were pooled across PWS genetic subtypes, genotypic and allelic frequencies did not differ from normative population data. However, when the genetic subtype of PWS was examined, there were differences observed across all genes tested that may affect response to psychotropic medication. Due to small sample size, no statistical significance was found, but results suggest that pharmacodynamic gene testing should be considered before initiating pharmacotherapy in PWS. Larger scale studies are warranted.

Keywords: genetic testing, medication management, imprinting, Prader-Willi, pharmacogenetics

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INTRODUCTION

Pharmacogenomics is the study of how structural gene changes determine the function, regulation and production of gene products that affect the body's response to medication. Pharmacodynamic testing involves the assessment of genes that code for neurotransmitter receptors and transporters, antigens, and enzymes that have an impact on drug activity and response, usually in the brain, as a function of an individual's DNA pattern. Pharmacodynamic phenotypes may explain why certain classes of medications are not as effective in some people, beyond that predicted by the knowledge of drug pharmacokinetics and cytochrome P450 genes. Also, pharmacodynamic phenotypes may inform the risk of occurrence of side effects. Clinicians who are informed about the therapeutic value of pharmacodynamic testing appreciate and utilize this knowledge to evaluate potential side effects and variability of response to certain classes of psychotropic medication.

Using psychotropic medication to manage the multifaceted symptoms associated with Prader-Willi syndrome (PWS) requires a knowledge of evidence-based science, clinical expertise, and risk for potential side effects. PWS is a rare, complex, neurodevelopmental disorder best known for hypothalamic obesity, neuroendocrine disturbances, and psychiatric co-morbidity. PWS is an imprinting disorder resulting from absent paternal expression of the genes located in the chromosome 15q11-q13 region. The features of PWS include infantile hypotonia, a poor suck with

feeding difficulties, global developmental delay, hypogenitalism/hypogonadism and growth failure due to multiple hormone deficiencies (Butler et al., 2006). Short stature with small hands and feet are common findings, and food seeking with hyperphagia develops in early childhood leading to morbid obesity, if not controlled by dietary and environmental restriction and mandatory exercise. Disorders of sleep (central and obstructive sleep apnea) and wakefulness (excessive daytime sleepiness) are common. Mild intellectual disability, learning difficulties, and behavioral concerns are noted. Temper tantrums, stubbornness, emotional outbursts, and skin picking may be present along with excessively rigid and repetitive behavior that may appear obsessive-compulsive like. Mood disorder, psychosis, and symptoms of Autism spectrum disorder are more likely to correlate with specific PWS genetic subtypes (Butler et al., 2004, 2006; Weisensel et al., 2015, 2016; Manzardo et al., 2018). A positive DNA methylation test confirms the diagnosis of PWS in 99% of cases, but additional testing is required to determine the genetic subtype. The 15q11-q13 deletion (DEL) occurs in 60% of cases, maternal disomy 15 (UPD), in which one inherits both chromosome 15 s from the mother, occurs in 35% of cases, and the remaining persons have defects in the imprinting center that controls the activity of the imprinted genes in the 15q11-q13 region (Butler et al., 2019). The frequency of maternal disomy 15 appears to be increasing among older mothers and those who have had *in vitro* fertilization (Butler et al., 2019; Hattori et al., 2019).

The spectrum of clinical findings in PWS presents challenges for families, caregivers and providers alike to develop and implement various management strategies (Duis et al., 2019). Clinical care is most effectively directed by a multidisciplinary team that includes clinical geneticists, endocrinologists, dietitians, exercise physiologists, gastroenterologists, orthopedic specialists, and primary care physicians. The primary goal of clinical care is to manage weight gain, monitor growth, and treat associated comorbid conditions such as hormone deficiencies, scoliosis, gut mobility issues, sleep apnea, and skin picking. Ancillary disciplines of speech and language therapy, physical therapy, occupational therapy, and special educators are essential toward the achievement of developmental and vocational goals. Mental health experts manage behavioral symptoms through modified delivery of behavioral analysis techniques and cognitive behavior therapy.

Psychiatric evaluation elucidates the etiology of symptoms, including diagnosing co-morbid psychiatric disorders, and results in a multidimensional treatment plan that involves environmental modification, enhancing coping skills, behavior management and use of psychotropic medication, hopefully in that order. Too often psychotropic medications are used for crisis intervention, and the evidence-base for use of psychotropic medication to manage aspects of the behavioral phenotype is lacking. Psychotropic medication selection and dosage determination are important for optimal care. Clinical experience suggests that people with PWS as a group appear to be more sensitive to psychotropic medications, requiring lower than typical doses for clinical response. But there are individual variations, and this is a focus of investigation in this report.

In 2006 the National Institutes of Health (NIH) PWS Rare Disease Registry began recruiting persons with PWS for the largest naturalistic longitudinal study examining factors related to the cause, manifestations and treatment of this rare genetic disorder (Butler et al., 2018). Of the 355 registrants in the NIH Rare Disease PWS Registry, 265 of them received 483 psychotropic medications listed in **Table 1**. The frequency of use by medication class is found in **Table 2**.

The data from the NIH PWS Registry indicates that over the past 10 years, there has been a downward trend in the age that children with PWS first receive treatment with selective serotonin reuptake inhibitors (SSRIs). Nearly 50% of children age 5–12 year were receiving SSRIs, and this increased to 70% in the 12–21 years age group, often together with an atypical antipsychotic medication (Forster, 2019). Polypharmacy leads to drug-drug interactions, a significant problem contributing to medical and psychiatric co-morbidity (Olashore and Rukewe, 2017). Further, except for treatment of co-morbidities or specific augmentation strategies, the evidence base for using multiple medications simultaneously is lacking (Mrazek, 2010a). Therefore, there is a need for an informed and individualized approach to dose selection and medication management (Blasco-Fontecilla, 2019).

This clinical case series, for the first time in PWS, describes the clinical significance of the most commonly studied pharmacodynamic genes and their polymorphisms: serotonin transporter (SLC6A4), serotonin 2A receptor (HRT2A), serotonin 2C receptor (HRT2C), catechol-*o*-methyltransferase (COMT), adrenergic receptor 2A (ADRA2A), methylene tetrahydrofolate reductase (MTHFR), and human leucocytic antigens (HLA-A and B). None of the pharmacodynamic genes tested is located in the PWS chromosome 15q11-q13 region, which is deleted or not expressed in the majority of patients with PWS. To date, only serotonin 2C receptor activity and serotonin transporter activity have been identified as being affected in animal models and humans. There are epigenetic effects on the editing of serotonin 2C receptor believed to be caused by base pair complementarity with one of the sno-RNAs in the critical region, specifically *SNORD115* (Kishore and Stamm, 2006). However, the loss of *SNORD115* in animal models does not appear to be sufficient to cause the abnormal editing of HTR2C (Hebras et al., 2020). Falaleeva et al. (2015) has proposed a mutual interaction between SNORDs, where *SNORD116* is necessary to facilitate alternative splicing of RNAs by *SNORD115,* and *SNORD115* modulates the expression of genes by *SNORD116,* although none of those appear to be directly related to the pharmacodynamic genes in this case report (Falaleeva et al., 2015). Deficiency of *Necdin,* one of the imprinted genes not expressed in PWS, is related to an increase in serotonin transporter activity and altered neuroarchitecture of the serotonergic system in the *Necdin* knock-out mouse, specifically affecting the central respiratory mechanisms in the medulla (Zanella et al., 2008; Matarazzo et al., 2017). Also, decreased serotonin transporter has been identified in the brain stem of patients with PWS UPD vs. DEL using single photon emission tomography (SPECT) (Krishnadas et al., 2018). Similar imaging studies have noted a reduction in SERT in the brain stem and other brain regions of adults with depressive illness vs healthy controls (Kambeitz and Howes, 2015). This is relevant to PWS given the greater

TABLE 1 | Number, class and name of psychotropic medications prescribed to 265 PWS registrants in the National Institutes of Health Rare Disease Consortium for Prader-Willi Syndrome.

Antidepressant/ Anti-anxiety	*N*	Antipsychotic/Mood stabilizer	*N*	Anticonvulsant/Mood stabilizer	*N*	Stimulants/Non-stimulants	*N*
SSRI	**143**	**SGA**	**90**	**Anticonvulsant**	**76**	**Methyphenidate**	**36**
Fluoxetine	58	Risperidone	37	Topiramate	32	Ritalin*	21
Sertraline	37	Aripiprazole	26	Valproic acid	17	Concerta*	7
Citalopram	18	Quetiapine	15	Phenobarbital	7	Focalin*	6
Escitalopram	15	Ziprasidone	8	Carbamazepine	2	Metadate*	1
Paroxetine	12	Paliperidone	2	Oxcarbazepine	4	Daytrana*	1
Fluvoxamine	3	Olanzapine	1	R-etiracetam	4	**Amphetamine**	**11**
SNRI	**8**	**FGA**	**10**	Lamotrigine	4	Adderall*	5
Nefazodone	1	Haloperidol	3	Gabapentin	2	Dexedrine*	5
Venlafaxine	4	Loxapine	3	Tiagabine	2	Vyvanse*	1
Desvenlafaxine	1	Thioridazine	3	Zonisamide	1	**Nonstimulant**	**39**
Imipramine	1	Chlorpromazine	1	Phenytoin	1	Atomoxetine	5
Clomipramine	1			**Mood stabilizer**	**4**	Modafanil	34
Other				Lithium	4		
Bupropion	12						
Trazadone	1						
Anti-anxiety	**37**						
Benzodiazepine	18						
Clonidine	8						
Guanfacine	3						
Buspirone	4						
Hydroxyzine	4						

*SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin norepinephrine reuptake inhibitor; SGA, second generation antipsychotics; FGA, first generation antipsychotics. *Brand name used. The bolded numbers in the table are subtotals for medication classes.*

prevalence of affective psychosis among UPD than DEL (Singh et al., 2019a). Finally, MTHFR TT polymorphism, which is the low activity polymorphism, was higher in mothers who had a child with Angelman syndrome due to a maternal imprinting defect, a deficiency of the methylation process, but data from fathers did not reach statistical significance (Zogel et al., 2006). It should be noted that the American College of Medical Genetics does not endorse MTHFR testing because of population heterogeneity and minimal data for clinical utility in predicting risk for coronary artery disease or thromboembolism. The clinical utility of MTHFR polymorphism studies in psychiatry continues to be examined (Wan et al., 2018).

For the first time, the frequency of pharmacodynamic gene polymorphisms in this cohort of clinically referred patients with PWS is presented and compared with normative population data when possible. The capacity to use this knowledge to anticipate treatment efficacy and/or vulnerability to adverse effects of pharmacotherapy will be addressed. Finally, the potential for interactions between psychodynamic genes to affect psychiatric co-morbidity in genetic subtypes of PWS is discussed.

MATERIALS AND METHODS

In this clinical case series, 33 patients with genetically confirmed PWS were evaluated at one of three geographically distinct clinical centers in the United States (University of Kansas Medical Center, Vanderbilt University Medical Center and Pittsburgh Partnership, PA, United States). Pharmacogenomic testing was ordered as part of the clinical evaluation by the authors who are physicians treating patients with medications prescribed for behavioral or psychiatric problems. All patients in this clinically referred cohort met medical necessity criteria for testing. After a discussion with the physician about pharmacogenetics and its use in medication management, the guardians consented for genetic testing by signing order forms provided and approved by the three commercially licensed and CLIA accredited laboratories: Genesight (Mason, OH, United States), Genelex (Seattle, Washington), and Genomind (King of Prussia, PA,

TABLE 2 | Percentage of 265 persons from the NIH PWS registry receiving psychotropic medication by class.

Medication class	Frequency of use %
SSRI	54.0
SNRI + Other	7.9
Anti-anxiety	13.9
Atypical antipsychotics (SGA)	33.9
Typical antipsychotics (FGA)	3.8
Anticonvulsant/mood stabilizer	30.2
Stimulant	17.7
Non-stimulant	14.7

SSRI, selective serotonin reuptake inhibitor; NSRI, non-selective serotonin reuptake inhibitor; SGA, second generation antipsychotics; FGA, first generation antipsychotics.

United States). Buccal cells were collected from a total of 33 patients in the clinical setting and sent to the laboratories of Genesight (n = 27), Genelex (n = 5), and Genomind (n = 1) for DNA extraction and genotyping. The results were received by each clinician, deidentified with respect to age, gender, race and ethnicity, and added to the collective data set. Information about clinical treatment and response to psychotropic medication(s) in our PWS cohort was not included, although clinical necessity criteria for testing requires treatment failure due to inefficacy, treatment emergent side effects, or co-morbid conditions requiring multiple medications. Only the PWS genetic subtype was identified in the population. There were 14 persons with the 15q11-q13 deletion (DEL) and 14 persons with uniparental maternal disomy 15 (UPD). Genetic subtype was unknown in 5 persons whose diagnosis of PWS was confirmed previously with DNA methylation. The most commonly studied pharmacodynamic genes and their polymorphisms were analyzed: serotonin transporter (*SLC6A4*), serotonin 2A receptor (*HTR2A*), serotonin 2C receptor (*HTR2C*), *COMT*, adrenergic receptor 2A (*ADRA2A*), *MTHFR*, and human leucocytic antigens (*HLA-A* and *HLA-B*). Results were collated across PWS genetic subtypes, and a third category comprised of pooled data (DEL + UPD + PWS unspecified) was designated as "ALL." Genotype and allele frequencies were calculated and compared with normative population data. The frequency of pharmacodynamic gene polymorphisms has been based upon large population studies with ethno-geographic specificity. When possible, the data derived from our cohort of persons with PWS was compared to genotypic and allelic frequencies from populations designated as North American, European American, European, White American, or US Caucasian by chi-square test. The phenotype of each pharmacodynamic gene was inferred from the genotype. For example, it is well known that the s allele of the serotonin transporter promoter region has been associated with 50% less transcription than the L allele, so the homozygous form is designated as low activity. However, it is also acknowledged that there are other factors that may determine transcriptional activity (Mendlewicz et al., 2004).

Ethical review and approval were not required for this report. This article is a scholarly report of a clinical case series of patients with PWS who received pharmacogenomic testing as part of medical care at one of three specialty programs across the United States. This report will both inform and hopefully improve the quality of care of patients with PWS. The clinical care described in this article was not part of a research project. Prior to the collation and analysis of data, all pertinent private or protected health information about each patient was eliminated, except for the genetic subtype of PWS and the pharmacodynamic genotypes. Further, the results presented in this paper have been deidentified, will not affect the patient's clinical care, nor do they have the potential to cause the patient any harm. Therefore, IRB approval was not obtained for ethics oversight.

RESULTS

This is the first attempt to ascertain the frequency of polymorphisms of pharmacodynamic genes in persons with PWS. From the frequency data presented in the combined cohort of "ALL" PWS, polymorphisms of pharmacodynamic genes tested were similar to normative population data. However, differences were observed between DEL and UPD genetic subtypes. In summary, those with UPD displayed an increased frequency of the L allele of the serotonin transporter and an increased frequency of VAL/VAL polymorphism of *COMT* gene. Those with DEL displayed increased frequency of HRT2A polymorphisms associated with risk of autonomic and respiratory dysfunction in infancy and sleep apnea and metabolic syndrome with age. Among patients of both subtypes, there was an increased frequency of serotonin 2C polymorphism resulting in potential risk for side effects associated with pharmacotherapy with SSRIs and atypical neuroleptics. There were genetic subtype differences for the ADRA2A gene suggesting increased risk of side effects in DEL and decreased efficacy in UPD during treatment with alpha-adrenergic agonists. MTHFR results indicate that both PWS genetic subtypes have an increased frequency of diminished function alleles, as well as increased frequency of compound genotypes associated with potential risk of developing psychosis. In summary, when analyzed by genetic subtype, there are pharmacodynamic gene differences in this referred population that may contribute to response and/or the emergence of adverse effects with treatment. Although the above findings showed differences, they were not significantly different by chi-square test ($p < 0.05$) due to small sample size. The complete results of the pharmacodynamic genes tested (*SLC6A4, HTR2A, HTRT2C, ADRA2A, COMT*, and *MTHFR*) in our referred cohort clustered by genetic subtype and testing lab are found in **Table 3**.

Serotonin Transporter

One of the best studied pharmacodynamic genes is the serotonin transporter (5-HTT, SERT, or SLC6A4) that has both short and long polymorphisms of the gene promoter allele. The SERT gene is located at Ch17q11.1-q12. The serotonin transporter shuttles through the synaptic cleft from the serotonergic neuron to the post-synaptic receptor, collecting and recycling unbound serotonin. The homozygous form of the short allele (s/s) is associated with reduced efficiency of transcription, lower gene expression, and decreased serotonin binding affinity (Lesch et al., 1996). Both human and animal studies have shown that this genotype is associated with increased stress sensitivity to adverse life events, although a positive outcome can be expected with environmental support (Kumsta et al., 2010). The ss genotype is associated with anxious, neurotic and somatic personality styles (Caspi et al., 2003), not unlike some of the personality characteristics seen in PWS. The s allele appears to modulate the impact of environmental stress on depression (Daws and Gould, 2011). Studies have demonstrated an additive effect of the number of s alleles contributing to low emotional resilience and distress intolerance (Stein et al., 2009; Amstadter et al., 2012). Schepers and Markus (2017a,b) found that BMI was positively correlated with a ruminative cognitive style among s allele carriers, and that in this group, stress increased attention bias for high calorie, savory foods identified by an eye-tracking task. The homozygous form of the long allele (L/L) is associated with high gene expression, and this genotype confers stress

TABLE 3 | Selected results of pharmacodynamic genotypes and phenotypes among the referred cohort of patients with PWS assorted by genetic subtype and pharmacogenomic testing lab.

	SLC6A4	*HTR2A*			*HTR2C*	*ADRA2A*	*COMT*		*MTHFR*	
Lab		**1438G > A**	**−998G > A**	**614-2211T > C**	**−759C > T**	**−1291G > C**	**VAL^{158}MET**	**472G > A**	**677C > T**	**1298A > C**
	"PWS"									
GS	L/s	GA t								
GS	s/s	GA i				CC d	VAL/MET i		CT i	
GS	L/L	GG r				GC t	VAL/MET i			
GS	L/L	AA d				CC d	MET/MET d		CC t	
GS	s/s	GA t				GC t	VAL/MET i		CT i	
	DEL									
GL			GA i	CC d	CC r	CC d		AA d	CT i	AC i
GS	L/s	GG r				GC t	VAL/MET i			
GL			GG r	CC d	CC r	GC t		AA d	CT i	CC d
GL			AA d	TC t	CC r	GC t		AA d	CT i	AA t
GS	s/s	GG r				CC d	VAL/MET i			
GS	L/s	GA t				GC t	VAL/MET i			
GS	L/s	GA t				GC t	MET/MET d			
GS	s/s	AA d				CC d	MET/MET d		CC t	
GL	LA/sA		AA d	CC d	CC r	GC t		AA d	TT d	AA t
GS	s/s	GA t				GC t	VAL/MET i			
GS	L/L	AA d				CC d	VAL/MET i			
GS	L/L	GG r				GG t	VAL/VAL h			
GS	s/s	GA t				CC d	VAL/VAL h			
GS	L/s	GA t				GC t	MET/MET d			
	UPD									
GL			GA i	TC t	CC r	GC t		GA i	CC t	AC i
GS	L/L	GA t				CC d	VAL/VAL h			
GS	L/s	GA t				GC t	MET/MET d		CC t	
GS	L/s	GA i				GC t	VAL/VAL h		CT i	
GS	L/s	GA i				CC d	VAL/VAL h		CT i	
GS	L/s	GA t				CC d	VAL/MET i		CT i	
GS	L/L	GA t				CC d	VAL/MET i		CC t	
GS	L/L	GA i				GC t	VAL/VAL h		CC t	
GS	L/s	GA t				CC d	VAL/MET i			
GM	L/L	GA t								
GS	L/L	GG r				GC t	MET/MET d			
GS	L/L	GA t				CC d	VAL/VAL h			
GS	L/L	GG r				CC d	VAL/VAL h			
GS	L/s	GA t				CC d	VAL/MET l			

Lab: GS, GeneSight; GL, Genelex; GM, Genomind. PWS Genetics: DEL, Deletion; UPD, Uniparental disomy. "PWS" – Methylation positive, subtype unspecified. Phenotypes: d, decreased activity; i, intermediate activity; t, typical activity; h, high activity; r, at risk.

resilience (Stein et al., 2009). Medications of the selective serotonin reuptake inhibitor (SSRI) type bind with variable affinity to the transporter, displacing serotonin into the cleft for increased availability at the post-synaptic receptor. This is the mechanism for producing the antidepressant response from these medications. The ss genotype is associated with decreased response to SSRI medications, and the LL genotype is associated with increased response. Although the genotype is subject to Mendelian inheritance, there is variability of gene frequency across ethno-geographic groups as discussed by Mrazek (2010b). This case series infers the phenotype from the genotype, although there are epigenetic factors that ultimately determine gene expression such as nutrition, stress, immune activation, age, and pharmacological effects as discussed by Daws and Gould (2011).

The distribution of serotonin transporter genotype among the 29 patients with PWS in our cohort compared to the Caucasian American population is shown in **Figure 1**. The alleles in the combined cohort "ALL" show some skewing toward the L/L genotype comparable to the predicted cohort.

Table 4 shows the allelic frequencies for the combined cohort of PWS (ALL), which are 59% for the L allele and 41% for the s allele. These frequencies compare favorably with the allelic frequencies reported in the Caucasian American population, which are 60% for the L allele and 40% for the s allele

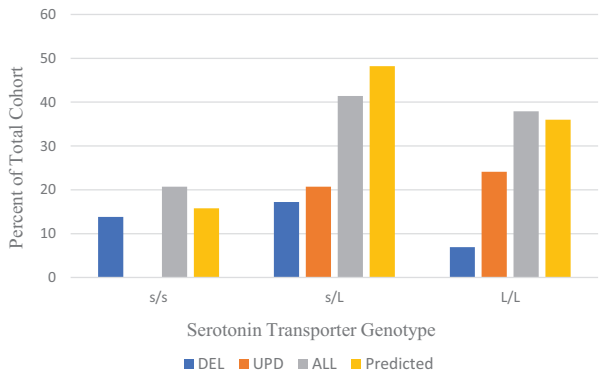


FIGURE 1 | Distribution of serotonin transporter genotypes by PWS genetic subtype ALL (n = 29), DEL (n = 11), UPD (n = 13) compared to a predicted Caucasian American population (n = 114) (Poland et al., 2013). DEL, deletion; UPD, uniparental disomy; ALL, combined cohort of PWS.

TABLE 4 | Distribution of serotonin transporter genotype and allele frequencies among PWS DEL, UPD and ALL cohort (n = 29) compared to a predicted Caucasian American population (n = 114); both number and within group (%) are reported.

Cohort	*N*	s/s (%)	s/L(%)	L/L(%)	s Allele	L Allele
Caucasian American*	114	18(15.8)	55(48.2)	41(36)	0.40	0.60
Current: ALL PWS	29	6(20.7)	12(41.4)	11(37.9)	0.41	0.59
DEL subtype	11	4(36.4)	5(45.5)	2(18.2)	0.59	0.41
UPD subtype	13	0	6(46.2)	7(53.8)	0.23	0.77

**Poland et al., 2013.*

(Poland et al., 2013). However, the data is different when the genetic subtype of PWS is considered. For the UPD subtype, the distribution is skewed toward the L/L genotype, and no one with UPD had the s/s genotype. The frequency of the L allele among our UPD cohort is 76.9%. This is higher than the expected 60% ascertained for the Caucasian American population (Poland et al., 2013). This finding may indicate increased sensitivity to SSRIs and greater possibility of mood activation. Results for the deletion cohort are skewed toward the s/s genotype. The frequency of s allele is 59.1% among the deletion subgroup, higher than the 40% expected for the Caucasian American population (Poland et al., 2013). This finding is consistent with what might be expected for persons with PWS, who show traits of stress sensitivity, neuroticism and somatization and have a better outcome in a structured, socially supported environment.

Serotonin Receptors

Serotonin receptors 2A (*HTR2A*) and 2C (*HTR2C*) are most relevant to understanding the action and efficacy of antidepressant medication. They are G-protein coupled, post-synaptic receptors with expression developmentally regulated in the prefrontal cortex (PFC) where two serotonin receptors appear have opposing effects. Serotonin 2A receptors are located on excitatory glutamate neurons while HTR2C are located on inhibitory GABA interneurons. Lambe et al. (2011) studied gene expression (mRNA) from *HTR2A* and *HTR2C* in the PFC

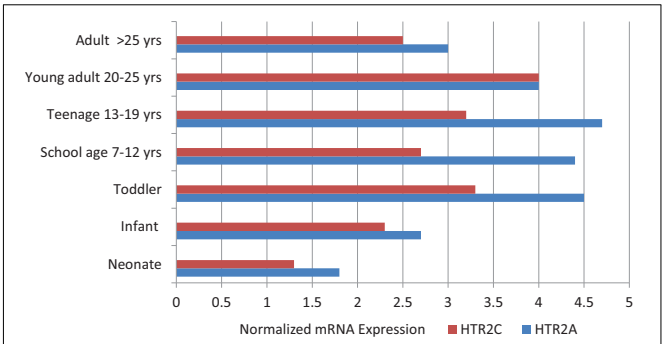


FIGURE 2 | Normalized mRNA expression of serotonin receptors in human prefrontal cortex obtained from post-mortem cases of different ages, as adapted from Lambe et al. (2011).

of post-mortem brains sampled at various ages across the life cycle (**Figure 2**).

Expression of the mRNA from *HTR2A*, the excitatory serotonin receptor, increases from infancy through the toddler and school age years, peaks during adolescence, and then declines in the young adult years until reaching adulthood levels. Expression of the mRNA from *HTR2C*, the inhibitory serotonin receptor, follows a similar pattern of upregulation at a level two thirds of *HTR2A* expression until parity is achieved in the young adult and later adult years. The greatest discrepancy in expression occurs in adolescence. From this mRNA data in the human prefrontal cortex, it appears that the activating form of *HTR2A* expression predominates from the school age years through adolescence. It is well known that the phenomenon of mood and behavioral activation associated with the use of SSRIs is more likely to occur in younger individuals and adolescents (Luft et al., 2018). This is the reason for the black box warning about suicide potential among children and adolescents started on SSRIs and some atypical antipsychotics. Further, Pandey et al. (2002) found an increase in the serotonin 2A receptors in the PFC of post-mortem brains of adolescents who completed suicide, and Schmauss (2003) found abnormal editing of the serotonin 2C receptor in the PFC of depressed suicide victims.

This information is particularly relevant to persons with PWS. Located in the imprinted region of chromosome 15q11-q13 is a small nucleolar RNA (HBII-52 or SNORD115) that is required for proper editing of the serotonin 2C receptor (Kishore and Stamm, 2006). The clinical implication for PWS is that the serotonin 2C receptor is produced, but its overall efficacy is diminished due to improper editing. Therefore, it is likely that persons with PWS have a serotonin 2C receptor with deficient function. With a typical expression of *HTR2A* throughout development and lower functional expression of *HTR2C* in PWS leading to less inhibitory effect, there is greater potential for clinical response to lower doses of SSRIs as well as adverse effects of mood activation at typical doses (Durette et al., 2012; Gourash et al., 2015).

Serotonin 2A Receptor

The serotonin receptor 2A (*HTR2A*) gene is located on chromosome 13q14-21. Only the rs6311 polymorphism in the

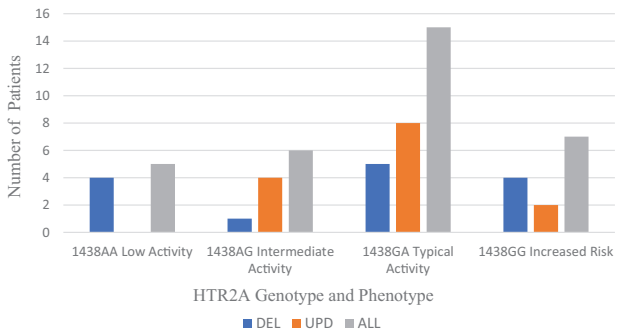


FIGURE 3 | Frequency of HTR2A genotype and phenotype per PWS genetic subtype cohort: DEL (n = 14), UPD (n = 14), and ALL (n = 33). DEL, deletion; UPD, uniparental disomy; ALL, combined cohort of PWS.

TABLE 5 | Distribution of HTR2A genotypes and allele frequencies among PWS and European cohorts; both number and within group (%) are reported.

Cohort	N	AA(%)	GA(%)	GG(%)	A	G
European*	286	50(17.5)	144(50.3)	92 (32.2)	0.43	0.57
Current: ALL PWS	33	5(15.2)	21(63.6)	7(21.2)	0.47	0.53
DEL subtype	14	4(28.6)	6(42.9)	4(28.6)	0.50	0.50
UPD subtype	14	0	12(86.7)	2(14.3)	0.43	0.57

Gorwood et al., 2002.

promoter (−1438G > A) of *HTR2A* is tested on the Genesight panel. The −998G > A polymorphism on the Genelex panel is the same as −1438G > A. The phenotypic activity of the −1438GA allele has been reported as typical or intermediate by GeneSight and Genelex. The frequencies of the genotypes and phenotypes reported in the PWS genetic subgroups are displayed in **Figure 3**.

Across the total PWS cohort, the typical phenotype of −1438G > A is expressed more frequently (45.5%) than the intermediate phenotype (18.26%). The −1438AA genotype, a low activity phenotype, is reported in 15.2% of the total cohort. In several meta-analyses, the AA genotype carries the greatest risk for obstructive sleep apnea and hypopnea (Zhao et al., 2013; Xu et al., 2014). The -1438GG genotype occurs in 21.2% of the total cohort, and this phenotype carries the greatest risk for increased waist circumference associated with metabolic syndrome and hypertension (Halder et al., 2007).

Among 286 individuals of European ancestry, the prevalence of −1438G > A polymorphisms was 50 AA (17.5%), 144 GA (50.3%), and 92 GG (32.2%); the frequency of the G allele was 0.57 and the frequency of the A allele was 0.43 (Gorwood et al., 2002). Among the total PWS cohort, the frequency of the G allele was 0.53, and the frequency of A allele was 0.47, similar to that reported by Gorwood et al. (2002).

Table 5 displays the genotypic frequencies among the PWS genetic subtypes and compares them to the typical European population described above. There was a more normal distribution of -1438G > A gene polymorphisms in the DEL subtype: AA (28.6%), GA (42.9%), and GG (28.6%) where the frequency of both A and G alleles was 0.50. In the UPD subtype, the distribution of polymorphisms was skewed: AA (0), GA (86.7%), and GG (14.3%) with the frequency of the G allele being 0.57 and the A allele 0.43, remarkably similar to that reported by Gorwood et al. (2002) in the typical European population. So, compared to UPD, the deletion subtype in our cohort appears to be at greater risk for adverse effects of SSRIs, sleep apnea and metabolic syndrome.

In addition to the *HTR2A* -998G > A polymorphism, the Genelex panel also tests for the 614-2211T > C allele, also known as rs7997012 or −1178G > A. In the STAR*D study, people with depression who had the adenosine substitution (AA) were more likely to respond to citalopram than those who had the GA or GG genotype (McMahon et al., 2006). Of the five patients tested using the Genelex panel in our case series, three had the low activity phenotype and two had typical activity.

In addition to phenotypic differences in functional activity determined by the polymorphism, binding affinity varies across drugs. Among the atypical antipsychotic medications, risperidone and ziprasidone have the highest affinity for HTR2A followed by olanzapine and aripiprazole. Increased risk for movement disorder, such as tardive dyskinesia, which is an adverse effect associated with the use of antipsychotic medications, is increased among those with the G allele of −1438G > A (rs6311) and the C allele of 102T/C (rs6313) (Lattuada et al., 2004). These two polymorphisms are in strong linkage disequilibrium with rs1928040, another polymorphism of *HTR2A* associated with the orofacial type of tardive dyskinesia particularly among females (Pozhidaev et al., 2020).

The promoter region of *HTR2A* is subject to epigenetic modification by methylation during fetal development, and these effects have been associated with neurobehavioral problems during infancy, psychiatric disorders in young adulthood, and chronic fatigue syndrome in adults as discussed by Paquette and Marsit (2014). Stress has an ongoing role in epigenetic regulation of *HTR2A*, and there are both glucocorticoid as well as cortisol binding sites in the promoter region (Paquette and Marsit, 2014).

Serotonin Receptor 2C

The Serotonin 2C receptor gene (*HTR2C*) is located on the X chromosome (Xq24). So males, who by definition have only one X chromosome, are maternally hemizygous for the *HTR2C* gene alleles. The serotonin 2C receptor is a site of action of most antidepressants and atypical antipsychotic medications. All these agents have variable affinity for the receptor. Polymorphisms of the *HTR2C* are associated with increased risk of side effects of weight gain and movement disorder with the use of atypical antipsychotics (MacNeil and Müller, 2016). Only Genelex tests for polymorphisms of the serotonin 2C receptor gene, so the data from our PWS cohort is limited. Of the five cases receiving Genelex testing of the *HTR2C* -759 C > T polymorphism, all of them had the CC phenotype suggesting greater risk of side effects of weight gain and movement disorder associated with the use of atypical antipsychotic medications. As previously described, the function of the serotonin 2C receptor is likely to be abnormal in PWS.

Alpha-2A Adrenergic Receptor

The alpha-2A adrenergic receptor is a G-protein coupled receptor that primarily provides presynaptic feedback inhibition on norepinephrine (NE) release by central and peripheral

sympathetic neurons. This results in reduced peripheral vasoconstriction and blood pressure. The gene is located at Ch10q25.2. The -1291C/G promoter polymorphism in *ADRA2A* has been associated with vascular reactivity to stress, and vasoconstriction increases linearly with the number of copies of the G allele (Flordellis et al., 2004). Specifically, there is an association between this genetic variation and cardiovascular reactivity in young African Americans (Kelsey et al., 2012). Also, the adrenergic receptor 2A modulates response to methylphenidate and alpha-adrenergic agonists. There is a linear relationship between the number of G substitutions in the −1291C/G promoter polymorphism and the response to methylphenidate targeting inattentive symptoms in ADHD (Schmitz et al., 2006; Polanczyk et al., 2007; Cheon et al., 2009). However, more recent studies have implicated a more robust pharmacodynamic association between a diagnosis of ADHD and serotonin transporter, dopamine transporter, dopamine receptor, *COMT* and other pertinent genes (McGough et al., 2009; Joensen et al., 2017).

ADRA2A (−1291CG) and other adrenergic receptor SNPs are also involved in metabolism and central obesity (Halder et al., 2007). Alpha-2A adrenergic receptors are located in pancreatic islet β-cells, and there is an association between stress and hyperglycemia associated with stress, especially among Caucasians (Adefurin et al., 2016). Insulin activates *ADRA2A*, and expression of *ADRA2A* inhibits lipolysis, contributing to weight gain (Stich et al., 1999, 2003). Garenc et al. (2002) found an association between some SNPs of *ADRA2A* with the accumulation of abdominal fat unrelated to overall body mass among African Americans. Although *ADRA2A* alone has not been associated with obesity, there is an interactive effect with other SNPs (Lima et al., 2007). Some atypical antipsychotics such as risperidone have alpha-adrenergic agonist effects. Given that hyperglycemia and an accumulation of abdominal fat is associated with adrenergic agonist effects, it is possible that the weight gain and metabolic syndrome occurring during treatment with second generation antipsychotics may be related, in part, to their adrenergic effects (Riordan et al., 2011).

Figure 4 shows the frequency of the -1291C/G promoter polymorphism in *ADRA2A* in our cohort of patients with PWS. Overall, the frequency of polymorphisms is almost evenly distributed between the low affinity and typical affinity phenotypes; there was only one person with the *ADRA2A*−1291GG allele, which also confers the typical affinity phenotype. When examining the results for the genetic subtypes of PWS, there is a tendency toward the typical affinity phenotype among the deletion group and the low affinity phenotype among the UPD group.

Population studies show a variation in the distribution of −1291C/G polymorphism with race and ethnicity, as reviewed by Karahalil et al. (2008). The genotype and allelic frequencies among White Americans (Lima et al., 2007) are compared to the data from our PWS cohort in **Table 6**.

The frequency of the G allele is increased among the DEL subgroup. An increased dose of G allele has been associated with increased vascular reactivity with stress and improved response to methylphenidate and alpha-adrenergic agonists. There is an increase in the C allele among the UPD subgroup that may contribute to lesser efficacy of alpha-adrenergic agonists in treatment trials. The alpha-2 adrenergic receptor agonist guanfacine has been used successfully in patients with PWS to target symptoms of aggression, skin picking, and ADHD (Singh et al., 2019b), but it was not effective in managing the severe psychopathology associated with PWS, which is usually found in UPD.

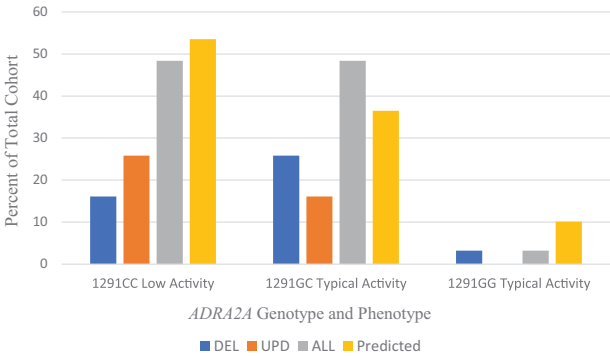


FIGURE 4 | Frequencies of 1291C > G *ADRA2A* genotype and phenotype among PWS genetic subtypes DEL, UPD, and ALL (n = 31) compared to a predicted cohort of White Americans (n = 232) (Lima et al., 2007). DEL, deletion; UPD, uniparental disomy; ALL, combined cohort of PWS.

TABLE 6 | Distribution of 1291C > G *ADRA2A* genotypes and allele frequencies among PWS and White American cohorts; both number and within group (%) are reported.

Cohort	*N*	CC(%)	CG(%)	GG(%)	C	G
White Americans*	232	85(53.5)	58(36.5)	16(10.1)	0.72	0.28
Current: ALL PWS	31	15(48.4)	15(48.4)	1(3.2)	0.73	0.26
DEL subtype	14	5(35.7)	8(57.1)	1(7.1)	0.64	0.36
UPD subtype	13	8(61.5)	5(38.5)	0	0.81	0.19

**Lima et al., 2007.*

Catechol-*O*-Methyltransferase

The COMT enzyme is responsible for clearing dopamine and other monoamine neurotransmitters from the synaptic cleft. *COMT* polymorphism is a major determinant of dopamine function in the prefrontal cortex, limbic system and reward centers of the brain (Schacht, 2016). Dopamine tone in the prefrontal cortex determines cognitive functions along a U-shaped continuum; either too much or too little dopamine can impair executive function (Meyer-Lindenberg and Weinberger, 2006). Bilder et al. (2004) described an inverse relationship between cortical and subcortical activation associated with *COMT* polymorphism, dopamine tone, and psychosis susceptibility. Further, Mier et al. (2010) performed a meta-analysis of fMRI studies that supported a *COMT* polymorphism-related bias for cognitive vs emotional processing as a function of activation of the prefrontal cortex. In another meta-analysis, Taylor (2018) reviewed the association between *COMT* polymorphisms and major psychiatric disorders, such

as schizophrenia, schizoaffective disorder, bipolar disorder, depression, obsessive compulsive disorder and ADHD. Many studies have explored how specific behavioral attributes (endophenotypes), such as stress sensitivity, reward response, extraversion, neuroticism and aggression are related to *COMT* polymorphisms (Hoth et al., 2006; Collip et al., 2011; Lancaster et al., 2012; Lee and Prescott, 2014; Qayyum et al., 2015; Millenet et al., 2018). Some studies have focused on the association of age across development and *COMT* expression (Bowers et al., 2020).

The COMT gene is located on chromosome 22q11.21. The rs4680 or 472G > A gene variant is the most widely studied. It occurs as a VAL/MET polymorphism and appears in 3 combinations (VAL/VAL, VAL/MET, and MET/MET) depending on a substitution of methionine for valine. The frequency of occurrence of the MET allele differs across geographical ancestry as reviewed by Mrazek (2010b). Also, there are racial differences with African Americans displaying a decreased frequency of MET/MET polymorphism (Fiocco et al., 2010).

COMT (VAL/VAL) is associated with high enzyme activity resulting in lower dopamine levels in the synaptic cleft. This has been associated with greater cognitive flexibility and a positive response to environmental support, but a greater likelihood of distractibility; it predicts a typical response to psychostimulants (He et al., 2012).

COMT (MET/MET) is associated with low enzyme activity (as much as 2–4 times lower than VAL/VAL) and higher dopamine levels in the synapse (Chen et al., 2004) of neurons in the PFC. It is associated with reward focused behavior and response (Lancaster et al., 2012) as well as stress sensitivity (He et al., 2012; Boecker-Schlier et al., 2016). MET/MET carriers are more likely to respond to stressful life events occurring in childhood and adolescence (Crum et al., 2018). This endophenotype may result in stress sensitivity throughout life and may predispose to addiction (Lovallo et al., 2017; Buchanan and Lovallo, 2019). Collip et al. (2011) found that the persons with the MET/MET phenotype were more emotionally reactive to stress, displayed more negative affect, and were more likely to experience "momentary psychosis" (most often with delusional thinking) in response to a stress. However, in a stress-is-enhancing mindset manipulation, MET/MET carriers were more likely than VAL/VAL carriers to reappraise the effect of stress on their lives and respond with improved cognitive function and positive affect (Crum et al., 2018). Also, MET/MET carriers who have schizophrenia or schizoaffective disorder are more likely to respond to treatment with atypical antipsychotic medications affecting dopamine receptors (D2) resulting in a reduction in positive symptoms (Huang et al., 2016; Schacht, 2016).

Our cohort of 33 patients with PWS was sampled from clinics in the United States. The distribution of *COMT* polymorphisms is found in **Figure 5** and compared to a normal Caucasian population (Lee and Prescott, 2014).

Overall, the MET/MET genotype was found in 32.3% of our collective cohort, which is higher than predicted (28.3%), and the VAL/VAL genotype was found in 25.8% of the collective cohort, also higher than predicted (22%) from a meta-analysis of studies yielding a normal Caucasian population of 13,399 people (Lee and Prescott, 2014). These data sets are comparable. When examining the results from the PWS genetic subtypes, however, the distribution is skewed toward MET/MET among those with DEL subtype and VAL/VAL among those with UPD.

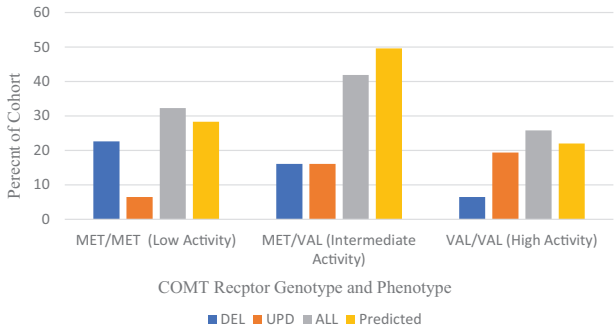


FIGURE 5 | Frequency of COMT receptor phenotypes and genotypes among PWS genetic subtypes DEL, UPD, and ALL (*N* = 31) compared to a predicted Caucasian Cohort (*N* = 13,399) (Lee and Prescott, 2014). DEL, deletion; UPD, uniparental disomy; ALL, combined cohort of PWS.

Table 7 compares the *COMT* genotype and MET and VAL allelic frequencies of the PWS genetic subtypes with a normative Caucasian population. The frequency of MET allele among PWS with deletion is 0.68, which is higher than expected compared to the frequency among Caucasians, which is 0.53 (Lee and Prescott, 2014). The frequency of MET allele among PWS with UPD was 0.35, which is lower than Caucasian norms. Karayiorgou et al. (1997) looked at *COMT* polymorphisms among a large cohort of people with obsessive compulsive disorder (OCD) and found that among male subjects, the Met allele frequency was 0.68. The Type I deletion in PWS is more likely to be associated with obsessional thoughts and compulsive behaviors compared to Type II deletion or UPD (Manzardo et al., 2018). In the cognitive dimension, MET carriers have sustained dopamine activation in neural networks that promotes attention fixation and protect from distraction (Lee and Prescott, 2014). VAL carriers are better able to switch sets, update neural networks with new information, and extinguish patterns associated with rewards (Lee and Prescott, 2014). Further studies are required to ascertain the association between *COMT* polymorphisms and the unique neuropsychiatric phenotype associated with the PWS genetic subtypes.

Methylene Tetrahydrofolate Reductase

Methylene tetrahydrofolate reductase is an enzyme that converts dietary folate into L-methyl folate, the building block of brain monoamine neurotransmitters and the catalyst of brain energy processes. This biochemical process is responsible for the methylation of homocysteine to produce methionine and eventually *S*-adenosylmethionine (SAMe). This epigenetic process of DNA methylation is the one-carbon metabolic pathway essential for DNA and neurotransmitter synthesis. When MTHFR function is impaired, abnormalities of the methylation cycle lead to oxidative stress (Fryar-Williams, 2016). As a result, serum folate may decrease, and serum homocysteine may increase. This contributes to a variety of illnesses such

TABLE 7 | Comparison of COMT genotype and allele frequencies in PWS cohort (*n* = 31) with Caucasians (*N* = 13,399); both number and within group (%) are reported.

Cohort	*N*	MET/MET	MET/VAL	VAL/VAL	MET	VAL
Caucasians*	13,399	3,796 (28.3%)	6,650(49.6%)	2,953(22.0%)	0.53	0.47
Current: ALL PWS	31	10 (32.3%)	13 (41.9%)	8 (25.8%)	0.53	0.47
DEL subtype	14	7 (50.0%)	5 (35.7%)	2 (14.3%)	0.68	0.32
UPD subtype	13	2 (15.4%)	5 (38.5%)	6 (46%)	0.35	0.65

**Lee and Prescott, 2014.*

as cardiovascular disease, recurrent pregnancy loss, neural tube defects, cancer, leukemia, venous thrombosis and stroke, which require genetic counseling (Levin and Varga, 2016). *MTHFR* polymorphisms have been associated with Autism spectrum disorder (Rai, 2016), ADHD (Gokcen et al., 2011; Baykal et al., 2019), and migraine (Liu et al., 2019). Their association with neuropsychiatric disorders, such as depression, bipolar disorder and schizophrenia, has been well studied as reviewed by Gilbody et al. (2007), Peerbooms et al. (2011), Kevere et al. (2012), Hu et al. (2015), and Wan et al. (2018). Also, *MTHFR* polymorphisms have been associated with severity of illness, longer duration of symptoms, diminished response to medication as well as risk of side effects. In particular, the low activity *MTHFR* alleles have been found to contribute to symptoms of metabolic syndrome in patients receiving atypical neuroleptics (Ellingrod et al., 2012; Misiak et al., 2017). L-methyl folate has been accepted as a method to augment treatment of depression and bipolar disorder (Coppen and Bolander-Gouaille, 2005; Lewis et al., 2012; Baek et al., 2013).

The MTHFR gene is located on chromosome 1p36.22. The two most common single nucleotide polymorphisms are 677C > T and 1298A > C, both of which result in a proportional decrease in enzyme function across allelic frequencies. The 677C > T polymorphism alleles have been associated with a reduction in folate levels in erythrocytes (Molloy et al., 1997). Population studies in the USA have noted the association of the 677C > T polymorphism alleles (CC, CT, and TT) with race (Wilcken et al., 2003). Further, there is an ethno-geographical association with 677C > T polymorphism that varies across the world according to the distance from the equator (Wilcken et al., 2003; Castro et al., 2004; Mischoulon et al., 2012). When comparing results of *MTHFR* polymorphism studies race, ethnicity and geography should be considered.

Studies suggest a relationship between the *MTHFR* 677C > T mutation and increased susceptibility for depression (Lewis et al., 2006, 2012), and risk of depression has been found to increase with environmental stress and the dosage of T allele (Lok et al., 2013). Low serum folate, vitamin B12 and elevated serum homocysteine have variously been indicated (Bjelland et al., 2003). *MTHFR* polymorphisms have been reported to be associated with treatment resistant depression (Pan et al., 2017). Mischoulon et al. (2012) found that serum folate was negatively associated with treatment response in a study of adults with major depressive disorder (MDD). Supplementation with folate and vitamin B12 has been considered as an adjunctive treatment for depression (Coppen and Bolander-Gouaille, 2005). In direct treatment trials, there is evidence to suggest that individuals with refractory depression respond to antidepressant augmentation with L-methylfolate, and that higher doses (15 mg/d) are more effective than lower doses (1–5 mg/d) (Papakostas et al., 2012; Venkatasubramanian et al., 2013). There is evidence to suggest that *MTHFR* polymorphism status is associated with antidepressant treatment response, specifically in males receiving serotonin-norepinephrine re-uptake inhibitors (SNRIs) (Sun et al., 2013).

There appears to be a shared epigenetic vulnerability for schizophrenia, bipolar disorder (BPD) and MDD Peerbooms et al. (2011). Kevere et al. (2012) studied the association of serum homocysteine, severe mental illness and *MTHFR* polymorphisms. The highest serum levels of homocysteine were associated with the *MTHFR* 677TT genotype, and the most severely impaired individuals had cyclic episodes of psychosis with an affective component. Homocysteine levels were twice as high among heterozygotes who developed schizophrenia, and the course of their illness was more severe with recurring episodes and more affective symptoms (Kevere et al., 2014). Some studies have indicated that abnormal serum levels of vitamin B12 and folate, associated with cognitive impairments, psychotic and mood symptoms, were unrelated to *MTHFR* 677TT status (Moorthy et al., 2012).

The other polymorphism is the *MTHFR* 1298A > C form; it does not show as much ethnic or geographical variance. It is a less efficient form of the enzyme. In its homozygous CC form, 1298A > C is associated with twice the prevalence of major depression in a Slovak population (Evinova et al., 2012). There is also an association with bipolar disorder Peerbooms et al. (2011) and schizophrenia (Lajin et al., 2012). The 1298A > C genotype (but not the 677C > T genotype) may be associated with increased rates of ADD/ADHD (Gokcen et al., 2011; Baykal et al., 2019). Some studies indicate an association between the MTHFR gene mutations and increased risk of autism spectrum disorder. Case-control comparisons revealed significantly higher frequency of homozygosity as well as heterozygosity for both the 677C > T and 1298A > C genotypes among autistic versus non-autistic children (Liu et al., 2011; Rai, 2016). Also, migraine headache is associated with *MTHFR* polymorphisms. The 677TT polymorphism is more frequent among those with migraines, but the 1298CC polymorphism is more frequent among those with tension headaches and migraine with aura (Liu et al., 2019). Migraine has also been associated with higher homocysteine levels (Pizza et al., 2012; Rainero et al., 2013).

Weisensel et al. (2016) studied 30 patients with PWS residing in a specialized residential setting (Residential PWS). There was no statistical relationship between *MTHFR* 677C > T or 1298A > C polymorphisms, PWS genetic subtype, psychiatric diagnosis, or the severity of mental illness as determined by

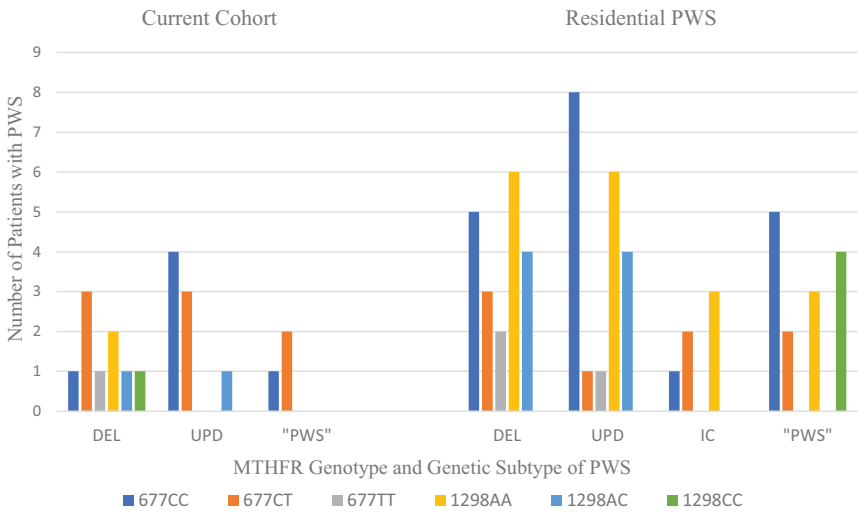


FIGURE 6 | Distribution of MTHFR 677C > T and 1298A > C polymorphisms among PWS genetic subtypes from current cohort compared to residential PWS (Weisensel et al., 2016). DEL, deletion; UPD, uniparental disomy; IC, imprinting center defect; "PWS," methylation positive, subtype undetermined.

a combination of the frequency of behavioral outbursts and the number of psychiatric medications needed to stabilize. Serum folate was within normal limits in all but one person who had a mild elevation at 35.1 (7–31.4) and who also had the 677CT/1298AA genotype. Homocysteine was marginally elevated 15.19 (5–15) in one person with 677CT/1298AA. Vitamin B12 levels were elevated in one third of the cohort and were higher among PWS deletion subtype with 677CT and 677TT alleles. The data from our cohort of PWS persons is limited to fifteen cases for the 677C > T polymorphism and 5 cases with the 1298A > C polymorphism. **Figure 6** displays the data from our cohort with the data from the Residential PWS study by Weisensel et al. (2016).

In **Table 8** the results from the current cohort and the Residential PWS study are compared with the frequencies of 677C > T and 1298A > C polymorphisms in a typical Caucasian population residing in the US, as reviewed in Nefic et al. (2018). Over half of our PWS cohort of referred patients had the intermediate metabolizer phenotype of *MTHFR* 677CT, which exceeds frequencies for both the Residential PWS and US Caucasian population. Another 20% have the poor metabolizer phenotype of 1298CC, which exceeds population norms. Unfortunately, our small sample size limits statistical analysis.

Compound genotypes have a clinically additive effect across several medical and psychiatric conditions. The frequency of compound genotypes of 677C > T and 1298A > C found in the current cohort is compared with the Residential PWS data and published norms (Ogino and Wilson, 2003) in **Table 9**.

Data from Weisensel et al. (2016) indicates greater numbers of the following compound genotypes in Residential PWS compared to the typical population: CC/AA, CC/CC, and CT/AA. The frequency of CC/AC is comparable to the typical population, and the frequencies of CT/AC and TT/AA are less than expected. Analysis of the data from the Residential PWS study did not find any correlations between psychiatric diagnosis and single mutations in *MTHFR*, but the frequency of compound phenotypes among those with a diagnosis of psychosis was not ascertained. There is an increased incidence of psychosis in PWS (Sinnema et al., 2011; Manzardo et al., 2018) and association between compound genotypes and schizophrenia has been identified (Sazci et al., 2005; Foroughmand et al., 2015). Sazci et al. (2005) found that the risk of schizophrenia in Turkish male patients compared to healthy controls was associated with the 677TT and 1298CC genotypes and the 677TT/1298AA and 677CC/1298CC compound genotypes. Comparing patients with controls, the risk of schizophrenia in Iran was found to be associated with the following compound genotypes in descending frequency: 677CC/1298CC > 677TT/1298AA > 677CT/1298AA > 677CT/1298CC > 677CT/1298AC (Foroughmand et al., 2015). Compared to the typical US population, the Residential PWS cohort displayed increased frequencies of two of the at-risk compound genotypes (CC/CC and CT/AA) identified in the Middle Eastern studies of susceptibility to schizophrenia.

Other studies have explored the frequency of *MTHFR* compound genotypes among those who had ischemic or hemorrhagic embolism (Sazci et al., 2006). Among typical persons with venous thromboembolism (VTE), Liu et al. (2017) determined that twice as many had *MTHFR* compound genotypes compared to single mutations of *MTHFR*, and this finding was not associated with elevated serum homocysteine levels. This is of relevance to PWS as there is an increased risk of VTE (Butler et al., 2020).

MTHFR deficiency predisposes to major psychiatric disorders and life-threatening medical conditions. It is accepted clinically that supplementation with L-methyl folate is indicated in typical patients who have treatment resistant depression (Zajecka et al., 2016; Jain et al., 2019) and bipolar disorder (Nierenberg et al., 2017). Further, mood stabilizers such

TABLE 8 | MTHFR genotype frequency of 677C > T and 1298A > C gene polymorphisms in the current cohort, US Residential PWS, and US Caucasian population displayed by group and within group n/%.

	MTHFR 677C > T				*MTHFR 1298A > C*			
Cohort	***N***	**677CC**	**677CT**	**677TT**	***N***	**1298AA**	**1298AC**	**1298CC**
US Caucasian†	564	236/41.8%	246/43.6%	82/14.5%	574	265/46.2%	250/43.6%	59/10.3%
US Residential PWS*	30	19/63.3%	8/26.7%	3/10%	30	18/60%	8/26.7%	4/13.3%
Current: ALL PWS	15	6/40%	8/53.3%	1/6.7%	5	2/40%	2/40%	1/20%

†*Adapted from Nefic et al. (2018). *Weisensel et al. (2016).*

TABLE 9 | Frequency of compound genotypes of MTHFR 677C > T and 1298A > C in typical US population[a] residential PWS[b] and the current cohort[c].

	Compound Genotypes MTHFR 677C > T/MTHFR 1298A > C								
N	**CC/AA**	**CC/AC**	**CC/CC**	**CT/AA**	**CT/AC**	**CT/CC**	**TT/AA**	**TT/AC**	**TT/CC**
7389[a]	15.6%	22.7%	9.1%	20.8%	20.3%	0.15%	11.2%	0.19%	0%
30[b]	26.6%	23.3%	13.3%	23.3%	3.3%	0	10%	0	0
5[c]	0	1	0	1	1	1	1	0	0

[a] *Typical US population (Ogino and Wilson, 2003).*
[b] *Residential PWS (Weisensel et al., 2016).*
[c] *Current PWS cohort.*

as lithium, valproic acid and lamotrigine may interfere with MTHFR function over time, and supplementation with oral L-methyl folate is recommended (Coppen and Bolander-Gouaille, 2005; Baek et al., 2013). Given the frequency of mood disorders in the PWS population, and the fact that more than half of individuals tested have the intermediate or poor phenotype, MTHFR testing or an empirical trial of the supplement L-methyl folate is recommended, especially in patients whose symptoms have been refractory to treatment.

Human Leucocytic Antigens: HLA-A and HLA-B

One of the best recognized pharmacodynamic tests are alleles for the human leucocyte antigens (*HLA-A* and *HLA-B*) that determine cytotoxic T-cell function in the immune system. These surface receptors are heterodimers consisting of a major protein with multiple polymorphisms located on Ch6p21.3 and a minor invariant microglobulin located on Ch15q22, outside the PWS critical region of Ch15q11-q13. HLA-B*1502 determines risk for Stevens-Johnson rash or toxic epidermal necrolysis. This severe, life threatening skin reaction is associated with the use of carbamazepine, lamotrigine, phenytoin, olanzapine, modafinil, and allopurinol in some individuals. This HLA test is now recognized and recommended by the FDA prior to starting carbamazepine, especially among Southeast Asian populations, where the odds ratio is reported to be 10 to 1 compared to near 0 for Europeans, Hispanics and Africans (Wang et al., 2017). Of the 33 persons with PWS referred for testing, only one of them carried the polymorphism for this cutaneous risk sensitivity, and this occurrence is compatible with statistical prediction of occurrence in the general population.

DISCUSSION

In this report, for the first time, the results of pharmacodynamic gene testing in a cohort of outpatients with PWS are presented with specific attention to the PWS genetic subtype. This combined cohort represents a referred population of patients with PWS whose pharmacogenomic testing was deemed medically necessary. In this clinically referred population, the number of patients with DEL was equal to UPD. The higher number of patients with UPD may be consistent with a referral bias due to the greater psychiatric co-morbidity among those who have this molecular subtype. Given the high likelihood of psychiatric co-morbidity in PWS with possible genetic subtype specificity, pharmacodynamic gene testing may be considered as an additional tool to inform psychotropic medication management.

When the results of pharmacodynamic testing for our cohort of 33 patients are pooled without regard to PWS genetic subtype, the distribution of most of the pharmacodynamic polymorphisms is comparable to population norms. However, differences were noted for PWS genetic subtypes for the polymorphism of the serotonin transporter promotor (increased frequency of the L allele in UPD) and *COMT* (increased frequency of VAL/VAL polymorphism in UPD and increased MET/MET in DEL). Serotonin 2A receptor gene testing revealed increased distribution of alleles in DEL with increased risk for side effects as well as diminished efficacy. Epigenetic effects on the efficiency of the serotonin 2C receptor in PWS are not expected to have genetic subtype specificity, and gene testing on 5 patients indicated the presence of the at-risk allele contributing to side effects. For *ADRA2A*, the frequency of the G allele is increased among the DEL subgroup, which may correlate with both treatment efficacy and adverse events, and there is an increase in the C allele among the UPD subgroup that may contribute

to lesser efficacy of alpha-adrenergic agonists in treatment trials. MTHFR results indicate that both PWS genetic subtypes have an increased frequency of diminished function alleles, as well as increased frequency of compound genotypes associated with the risk of developing psychosis. Although limited by a small number in this clinical case series, polymorphisms of pharmacodynamic genes associated with PWS genetic subtype may contribute to disparities in treatment response and emergence of adverse effects. Further investigation of pharmacodynamic gene-gene interactions in the PWS population is recommended in a larger cohort.

Although there is no reason to suspect a difference in the frequency of serotonin transporter polymorphisms in PWS, there is a predominance of the L/L genotype among our cohort of persons with UPD subtype. The L allele of the serotonin transporter is well recognized as conferring resilience to stress. Although we typically consider PWS persons with both deletion and UPD to be equally susceptible to stress, we find more severe psychopathology among those persons with UPD subtype. It is possible that these findings indicate that people with UPD and PWS appear to tolerate rising levels of stress until their coping strategies fail precipitously, and then they present with symptoms of severe mental illness. It is also recognized that higher serotonin transporter activity is associated with greater sensitivity to the use of SSRIs. This may make those with UPD more susceptible to mood and behavioral activation, which clinically may appear similar to affective psychosis.

Among persons with PWS, it is suspected that the function of the serotonin 2C receptor is faulty due to the absence of function of the snoRNA gene HBII 52 (SNORD 115), which is located in the critical region and results in the failure to transcribe the antisense protein essential for proper editing of serotonin 2C receptor (Kishore and Stamm, 2006; Kishore et al., 2010). Further, the difference in expression of mRNA from *5HTRA* (excitatory) and *5HTRC* (inhibitory) results in an imbalance of serotonergic action in the prefrontal lobe resulting in mood and behavioral activation, especially during the childhood and adolescent years (Lambe et al., 2011). This has been reported to occur in up to one-third of juveniles and adolescents with PWS treated with SSRIs and some atypical antipsychotic medications (Durette et al., 2012; Gourash et al., 2015). In the current case series, up to 18% of persons with PWS carry the at-risk *HTR2A* alleles associated with sleep apnea and symptoms of metabolic syndrome. Although only 5 cases were tested for polymorphisms of the serotonin 2C receptor, all of them carried the CC allele placing them at greater risk for weight gain and movement disorder when treated with atypical neuroleptics. Currently, there are no studies examining the incidence of side effects of psychotropic medications, especially the SSRIs and atypical neuroleptics, that are so widely used in the PWS population of all ages. For example, even though most people with PWS live in circumstances with dietary management and food control in the environment, some individuals receiving atypical neuroleptics display increased food seeking, weight gain, and increased abdominal girth. Going forward, it would be important to ascertain if *HTR2A* and *HTR2C* polymorphisms are contributing factors.

With respect to the adrenergic receptor gene, *ADRA2A*, nearly 50% of those in our PWS cohort carried the low activity allele. Although this is typical in comparison with population normative data, there was some selectivity toward the UPD genotype in our data. Decreased receptor affinity may result in decreased efficacy of alpha-2 adrenergic agonists, which are currently being used to manage ADHD, skin picking, and aggressive/disruptive behavior in children and adolescents with PWS (Singh et al., 2019b). There is an increased dose of G allele among the deletion group compared to normative population data, indicating a tendency toward increased vascular reactivity with stress.

In our cohort, the distribution of *COMT* polymorphisms appeared to display genotypic specificity, with a greater number of persons with PWS DEL having the low activity MET allele and a greater number of persons with PWS and UPD and having the high activity VAL allele. COMT has a major effect on dopamine activity in the prefrontal cortex and plays a role in the etiology of the affective-psychotic spectrum of disorders. The homozygous VAL/VAL genotype has the lowest dopamine level and greatest risk for psychosis (Schacht, 2016). Further, dopamine level in the PFC decreases with age. It is tempting to associate the higher frequency of VAL/VAL genotype in our small PWS cohort to the larger group of persons with PWS and UPD who are at increased risk for affective psychosis with age. A linear association between low dopamine and psychosis has been abandoned in favor of a "U-shaped" hypothesis, suggesting that either extreme of dopamine availability in the PFC is likely to result in significant psychopathology. This would help to explain the extreme stress sensitivity among people with PWS as well as the increased risk for psychosis in both deletion and UPD subtypes. With respect to the behavioral phenotype of PWS, diminished working memory, selective attention, and impaired cognitive flexibility are several executive dysfunctions associated with low dopamine levels in the PFC (Fallon et al., 2012). Of interest, *COMT* gene expression has also been implicated in the placebo response, which is widely known anecdotally to occur in persons with PWS (Hall et al., 2019).

The results of *MTHFR* testing in our PWS cohort did not inform any PWS subtype specificity, but the frequency of 677TT was elevated compared to Residential PWS or US Caucasian. About one-third of the combined PWS cohort had diminished *MTHFR* 677CT phenotypic activity. The presence of the T allele increases stress response and predisposes to depression (Lok et al., 2013). There is evidence to suggest that those individuals who have been diagnosed with mood disorder may benefit from supplementation with L-methyl folate. Further, given the increased frequency of use of mood stabilizers (lithium, valproate, lamotrigine) in PWS, and evidence that these medications can interfere with MTHFR function, L-methyl folate supplementation is recommended. Analysis of compound genotypes is consistent with studies indicating an increased risk for schizophrenia. Additional studies are needed to explore the occurrence of these at-risk compound genotypes among those persons with PWS who have been diagnosed with psychosis. A larger cohort of subjects may identify statistically significant differences.

Finally, it was beyond the scope of this report with its small sample size to consider issues of gene-gene interactions. However, it is not unreasonable to expect that gene interactions might be-contributing to the risk of co-morbid psychiatric disorder in the PWS population. This is especially true of the interaction between serotonin transporter and serotonin 2A receptor in predicting response to treatment of depression and anxiety disorders with SSRIs and SNRIs (Lohoff et al., 2013). Further, Peerbooms et al. (2012) found that the number of T alleles of *MTHFR* 677CT in combination with the MET/MET polymorphism of *COMT* increased the risk of psychosis in response to environmental stress. Fryar-Williams (2016) discussed the impact of MTHFR deficiency on deficits in the methylation cycle related to the etiology of psychosis. Rahimi et al. (2016) have identified a synergistic interaction between the VAL allele of *COMT* and the T allele of the *MTHFR* 677CT polymorphism, which increased the incidence of Bipolar I disorder by a factor of 2.58 ($p < 003$). On the other hand, Wang et al. (2015) found that the VAL/VAL genotype of COMT in combination with the *MTHFR* 677TT genotype had a protective effect on the development of Bipolar II disorder among Han Chinese. Larger scale studies of pharmacodynamic factors in PWS are recommended in the future with a larger sample size to further address these preliminary observations. Pharmacodynamic gene testing can inform our understanding of the PWS genetic phenotype, enhance our knowledge of medication efficacy in treating psychiatric co-morbidity, and improve our awareness of potential side effects in the clinical setting.

LIMITATIONS

There were three recognized limitations of this case series. The small number of patients did not allow us to attain sufficient power to perform statistical analysis. Patient data were deidentified for HIPPA compliance, so factors of age, gender, race and ethnic origin were not available for association. Psychiatric and behavioral history informing past experience with psychotropic medications, including doses and adverse effects, was not detailed. Although all patients met criteria for medical necessity, their psychiatric diagnosis and psychotropic medication history were not available for clinical correlation of testing results. In this case series, the phenotypic activity of the pharmacodynamic genes tested was inferred from the genotype. There are other factors that impact pharmacodynamic gene expression, such as epigenetic effects related to stress and effects of pharmacological treatment. Further, the results of cytochrome P450 gene polymorphisms were not part of this clinical report, and interactions between pharmacokinetic and pharmacodynamic genotypes are necessary to inform the clinical phenotype for each patient. Finally, our testing results came from three different pharmacogenomic companies whose panels differed in some respects as expected for an emerging field of medical care.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval were not required for this report. This article is a scholarly description of a clinical case series of patients with PWS whose guardians consented for them to receive pharmacogenomic testing as part of their medical care at one of three specialty programs across the United States. All pertinent private or protected health information was eliminated, except for the genetic subtype of PWS and the pharmacodynamic gene results.

AUTHOR CONTRIBUTIONS

JF designed the format for this report and drafted the original manuscript while JD and MB revised and added to the manuscript. JF, JD, and MB contributed data from PWS individuals for the case series. All the authors agreed to the final version of the manuscript.

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Genetic Testing in Neurodevelopmental Disorders

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Neurodevelopmental disorders are the most prevalent chronic medical conditions encountered in pediatric primary care. In addition to identifying appropriate descriptive diagnoses and guiding families to evidence-based treatments and supports, comprehensive care for individuals with neurodevelopmental disorders includes a search for an underlying etiologic diagnosis, primarily through a genetic evaluation. Identification of an underlying genetic etiology can inform prognosis, clarify recurrence risk, shape clinical management, and direct patients and families to condition-specific resources and supports. Here we review the utility of genetic testing in patients with neurodevelopmental disorders and describe the three major testing modalities and their yields – chromosomal microarray, exome sequencing (with/without copy number variant calling), and *FMR1* CGG repeat analysis for fragile X syndrome. Given the diagnostic yield of genetic testing and the potential for clinical and personal utility, there is consensus that genetic testing should be offered to all patients with global developmental delay, intellectual disability, and/or autism spectrum disorder. Despite this recommendation, data suggest that a minority of children with autism spectrum disorder and intellectual disability have undergone genetic testing. To address this gap in care, we describe a structured but flexible approach to facilitate integration of genetic testing into clinical practice across pediatric specialties and discuss future considerations for genetic testing in neurodevelopmental disorders to prepare pediatric providers to care for patients with such diagnoses today and tomorrow.

Keywords: autism, intellectual disability, global developmental delay, genetic testing, neurodevelopment, exome sequencing, chromosomal microarray, fragile x

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NEURODEVELOPMENTAL DISORDERS

With a combined prevalence of ∼17% among 3- to 17-year-old children in the United States, neurodevelopmental disabilities are the most prevalent chronic medical conditions encountered in pediatric primary care (1). The vast majority of these individuals have diagnoses captured within the *Diagnostic and Statistical Manual of Mental Disorders, 5th Edition* (DSM-5) *neurodevelopmental disorders* category that includes intellectual disability (ID), global developmental delay (GDD), communication disorders (language disorder, speech sound disorder, childhood onset fluency disorder, and social/pragmatic communication disorder), autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD), specific learning disorder (involving reading, written expression, and/or mathematics), and motor disorders (developmental coordination

disorder, stereotypic movement disorder, and tic disorders) (2). Broader conceptualizations of neurodevelopmental disorders include conditions outside of the realm of the DSM-5, such as cerebral palsy (CP) and epilepsy, and sometimes neuropsychiatric disorders for which there is strong clinical and biological evidence of developmental origins, such as schizophrenia (3–6).

Neurodevelopmental disorders are characterized by developmental deficits in cognition, language, behavior, and/or motor skills that cause impairment of personal, social, academic, and/or occupational functioning (2, 7). These clinically and etiologically heterogeneous disorders represent manifestations of altered neural development and, as such, are typically diagnosed during infancy, childhood, or adolescence. Although subject to maturational changes, neurodevelopmental disorders are non-progressive and tend to follow a relatively steady trajectory rather than a pattern of remitting and relapsing (7). Tic disorders are an exception to the latter, since there is typically waxing and waning of core symptoms rather than a steady course. Because there is insufficient information to allow systematic classification of neurodevelopmental conditions based on etiology and pathophysiology, a descriptive (phenomenological) categorical nosology based on groups of signs and symptoms that define disorders or syndromes (e.g., ASD, ADHD, CP, etc.), has been adopted (8, 9). Descriptive categorical diagnoses are useful heuristics for improving interrater reliability and enhancing information exchange, but their limited alignment with many clinical and biological findings is well-described (8, 10–12). Pearn (9) noted that "syndromic diagnosis is a concise shorthand for describing a constellation of clinical symptoms and signs - but is an acknowledgment of causal ignorance, which in turn demands differential reappraisal as new biochemical, genetic, or ultimately molecular causes of the syndrome are defined." Comprehensive developmental care, in addition to identifying appropriate descriptive diagnoses and guiding families to evidence-based treatments and supports, includes a search for an underlying etiologic diagnosis (13–16).

The scope of this review is limited to the genetic etiologic evaluation of children and adolescents with neurodevelopmental disorders. The focus is on individuals with diagnoses including GDD, ID, or ASD, for which there are existing guidelines for genetic testing, but we discuss the prospect of future genetic etiologic testing across broader neurodevelopmental disorders. Because of the focus on determining etiology using diagnostic genetic testing, the review does not cover pharmacogenomics, carrier testing, newborn screening, or pre-symptomatic/predictive testing. We provide an overview of the value of clinical genetic testing; the most commonly performed tests and the yield of these tests for determining a genetic diagnosis (focusing on established indications, ID/GDD and ASD); approaches to service delivery and implementation of genetic testing in clinical practice; and future directions, including emerging indications for genetic testing and forthcoming technologies.

UTILITY OF GENETIC TESTING IN INDIVIDUALS WITH NEURODEVELOPMENTAL DISORDERS

Identification of an underlying genetic etiology for a child's categorical neurodevelopmental diagnosis (or diagnoses) can provide both clinical and personal utility to patients and their families. **Table 1** provides several examples of genetic causes of neurodevelopmental disorders and the potential utility of identifying such a diagnosis. Establishing a genetic basis for a child's neurodevelopmental phenotype can provide additional information about their prognosis and enable caretakers to understand potential areas of need and opportunities for increased support (39). For example, a genetic diagnosis, and the prognostic information it provides, may facilitate acquisition of educational, disability, and employment services (40–45). Although currently infrequent in the case of neurodevelopmental disorders, a genetic diagnosis might also provide access to etiology-specific treatments (45–49). Given the number of treatments that are currently being explored in animal models and clinical trials, such gene-specific therapies are likely to become more ubiquitous (50). In the interim, as new therapies are being developed, a genetic diagnosis can provide access to condition-specific research protocols enrolling human subjects (51, 52). It is also important to note that elucidating etiologies and pathophysiologic processes is a necessary step toward developing animal models and human cell lines to use in pathogenesis studies, clinical trials, and, ultimately, mechanism-based targeted treatments for neurodevelopmental disorders (13, 53). In addition to facilitating medical management and access to services, a genetic diagnosis can also enable families to avoid unnecessary diagnostic tests (42, 45, 49, 52, 54) and, with an etiologic diagnosis in hand, families may be more empowered to avoid therapeutic interventions that are based on unfounded etiologic theories and are potentially harmful (e.g., chelation therapy) (55). Additionally, genetic testing can afford patients and their caregivers the ability to identify, treat, and/or prevent medical comorbidities at the time of diagnosis, as well as conditions that may develop later in life (42, 45, 49, 52, 56–65).

Furthermore, genetic testing allows healthcare providers to refine recurrence risk counseling for the family to inform reproductive decision making (39, 42, 45, 49, 52, 58, 59, 63, 65–67). When an etiology is determined, the risk of recurrence for an individual family varies depending on the specific genomic variant(s) identified. For parents of a child with a neurodevelopmental disorder, recurrence may be 50%, for example, in the case of an inherited, maternally-derived chromosome 15q11–q13 interstitial duplication, 25% in the case of an autosomal recessive disorder such as Smith-Lemli-Opitz syndrome, or approximately the prevalence rate of the particular disorder in the general population (e.g., ∼1.5% for ASD) if the child has a *de novo* explanatory variant. This refined recurrence risk information offers patients, parents, and family members an understanding of their reproductive risks and informs reproductive decision making, restores reproductive confidence, and enables prenatal diagnosis (45, 48, 68). Furthermore, an

TABLE 1 | Examples of clinical utility of specific genetic findings.

Gene/copy number variant	Characteristics pertinent to clinical utility	Clinical utility
15q11.2-q13.1 (BP2-BP3) interstitial duplication (17)	• Dup 15q syndrome, interstitial type • Dup 15q syndrome is caused by presence of at least one extra maternally derived copy of the Prader-Willi/Angelman critical region (PWACR) within chromosome 15q11.2-q13.1. • Approximately 20% of cases of dup 15q syndrome are due to an interstitial duplication on the maternally derived chromosome resulting in one extra copy of 15q11.2-q13.1 and, therefore, trisomy of this region. • Interstitial duplications are inherited from the mother in ∼15% of cases and *de novo* in the other ∼85%. • If the mother of a proband inherited a 15q interstitial duplication from her father, she will not have dup15q syndrome; she may appear to be unaffected or have milder features associated with paternal duplications.	• Genetic Counseling: Autosomal dominant inheritance with phenotype impacted by parent of origin due to methylation. Determining if the interstitial duplication was inherited or *de novo* can inform reproductive risks. Mothers who carry a 15q11.2-q13.1 interstitial duplication have a 50% chance of passing it on to each of their offspring.
17q12 deletion (18)	• 17q12 recurrent deletion syndrome; renal cysts and diabetes (RCAD) syndrome • Features include structural (e.g., renal cystic dysplasia), functional (e.g., tubulointerstitial disease) abnormalities of the kidneys and urinary tract, and maturity-onset diabetes of the young type 5 (MODY5).	• Genetic Counseling: Autosomal dominant inheritance and variants are usually *de novo*. Inheritance from a parent has been reported. Determining if the variant was *de novo* can provide information regarding reproductive risks for the family. • Periodic renal ultrasound, renal function tests, monitoring for tubulointerstitial disease, consultation with a nephrologist, and monitoring for MODY (including HgbA1C testing) are recommended.
ALDH5A1 biallelic variants (19–21)	• Succinic semialdehyde dehydrogenase deficiency (SSADH deficiency) is a condition caused by biallelic pathogenic, loss of function variants in the *ALDH5A1* gene. • Biochemical phenotype includes elevated gamma-amino butyric acid (GABA) and gamma hydroxybutyric acid (GHB). • Features include age-dependent worsening of epilepsy severity, behavioral disturbances, sleep disturbances, and high risk of sudden unexpected death in epilepsy (SUDEP). Therapeutics that rescue a mouse model from premature lethality include $GABA_B$ and GHB receptor antagonists, taurine, vigabatrin, ketogenic diet, and rapalog agents such as Torin 2 (an mTOR inhibitor).	• Genetic counseling: Autosomal recessive inheritance with recurrence risk of 25% for parents of an affected child. • Valproate is avoided when possible because of inhibition of potential residual enzymatic activity. • SGS742, a $GABA_BR$ antagonist, was the subject of a recently completed human clinical trial (results pending). • Additional opportunities for participation in clinical trials are anticipated.
CACNA1C (22)	• Timothy syndrome is caused by heterozygous, gain of function variants in the *CACNA1C* gene. • Features include long QT interval, other ECG abnormalities, congenital heart defects, frequent infections because of altered immune response, and intermittent hypoglycemia.	• Genetic counseling: Autosomal dominant and variants are typically *de novo*. Parental germline mosaicism has been observed. Determining if the variant was *de novo* can provide information regarding reproductive risks for the family. • Evaluation and treatment related to long QTc (beta-blocker, pacemaker, implantable defibrillator, close cardiac monitoring during anesthesia) and avoidance of drugs reported to prolong QT interval are recommended. • Avoidance/treatment of hypoglycemia is recommended.
DHCR7 biallelic variants (23, 24)	• Smith-Lemli-Opitz syndrome (SLOS) is caused by biallelic pathogenic, loss of function variants in the *DHCR7* gene, resulting in a deficiency of the enzyme 7-dehydrocholesterol reductase. • Biochemical phenotype includes elevated serum 7-dehydrocholesterol (7DHC) and, in most cases, low serum cholesterol. • Features include cleft palate, postaxial polydactyly, congenital heart defects, renal anomalies, cataracts, strabismus, photosensitivity, behavioral disturbances, and hypospadias in males.	• Genetic counseling: Autosomal recessive inheritance with recurrence risk of 25% for parents of an affected child. • Currently, most SLOS patients are treated with dietary cholesterol supplementation, despite limited evidence of efficacy. Efficacy of cholesterol supplementation is thought to be limited by the inability of dietary cholesterol to cross the blood-brain barrier, and a small controlled clinical trial did not observe differences in hyperactivity or problem behaviors between placebo and treatment phases. • Treatment with simvastatin can be considered. In a randomized, double-blind, placebo-controlled trial, treatment with simvastatin was associated with significant decreases in plasma dehydrocholesterol levels and improvement in irritability. • Treatment with haloperidol and perhaps other antipsychotic medications may exacerbate the biochemical sterol abnormalities in individuals with SLOS and cause an increase in symptoms, so they should be used with caution and avoided if possible. • Associated features require investigation or monitoring (e.g., cardiovascular and renal anomalies, cataracts, anticipatory guidance of photosensitivity).

(Continued)

TABLE 1 | Continued

Gene/copy number variant	Characteristics pertinent to clinical utility	Clinical utility
FMR1 (25–28)	• Fragile X syndrome is caused by loss of function of the *FMR1* gene. • 99% percent of cases of fragile X syndrome are caused by an expansion of the unstable CGG repeat sequence in the 5′ untranslated region (UTR) of the FMR1 gene. • Features include strabismus, seizures, musculoskeletal abnormalities (pes planus, pectus excavatum, scoliosis), cardiovascular phenotypes (mitral valve anomalies, aortic root dilatation), and behavioral disturbances.	• Genetic counseling: X-linked inheritance. All mothers of individuals with a *FMR1* full mutation are carriers of a premutation or full mutation. Reproductive risks are dependent on the mother's expansion size and, if the mother has a premutation, AGG interruptions within the CGG tract • Counseling and testing should be offered to extended family. In addition to reproductive risks, premutation carriers are at risk for fragile X-associated tremor/ataxia syndrome (FXTAS) and, in female premutation carriers, *FMR1*-related primary ovarian insufficiency. • Associated features require investigation or monitoring (e.g., scoliosis, cardiovascular phenotypes, seizures). • Several targeted pharmacologic therapies are under investigation (e.g., metformin, minocycline and lovastatin, sertraline).
MECP2 (29)	• In females, *MECP2* disorders due to heterozygous, loss of function variants include Rett syndrome, variant Rett syndrome, and broader clinical phenotypes including mild intellectual disability. • In males, *MECP2* disorders due to hemizygous, loss of function variants include severe neonatal encephalopathy, pyramidal signs, parkinsonism, and macroorchidism (PPM-X) syndrome, as well as broader clinical phenotypes including syndromic and non-syndromic intellectual disability. • Features of individuals with *MECP2* disorders are variable but can include long QT interval, seizures, behavioral disturbances, breathing abnormalities, and scoliosis.	• Genetic counseling: X-linked inheritance and the majority of variants are *de novo*. Inheritance from a mildly or unaffected mother has been reported. Determining if the variant was *de novo* can provide information regarding reproductive risks for the family. • Evaluation and treatment related to long QTc (beta-blocker, pacemaker, implantable defibrillator, close cardiac monitoring during anesthesia) and avoidance of drugs reported to prolong QT interval are recommended. • Other associated features require investigation or monitoring (e.g., scoliosis, seizures, breathing abnormalities).
PTEN (30–32)	• *PTEN* hamartoma tumor syndrome is caused by heterozygous, loss of function variants in the *PTEN* gene. • Features include benign tumors (e.g., skin hamartomas, gastrointestinal polyps, thyroid nodules/goiter, fibrocystic breast disease, cerebellar dysplastic gangliocytoma), and increased risk for certain cancers (e.g., breast, thyroid, renal cell, endometrial, colorectal, and melanoma). • Expressivity is highly variable between and within families.	• Genetic counseling: Autosomal dominant inheritance with ∼50–90% of pathogenic variants being inherited. • Determining if the variant was *de novo* or inherited can provide information regarding reproductive risks, cancer risks, and need for surveillance/management in family members. • Associated features require investigation or monitoring (e.g., breast, thyroid, renal cell, endometrial, colorectal, and skin cancers).
SLC2A1 (33, 34)	• Glucose Transporter Type 1 Deficiency (GLUT1) deficiency is caused by heterozygous, loss of function variants in in the *SLC2A1* gene. • Features include epilepsy that tends to be unresponsive to antiepileptic mediations, acquired microcephaly, complex movement disorder. Milder phenotypes include the absence of seizures and paroxysmal events triggered by exercise, exertion, or fasting.	• Genetic counseling: Autosomal dominant in the majority of cases, with variants being *de novo* in 90% of cases. Rarely, autosomal recessive inheritance has been reported. Determining the inheritance within the family can provide information regarding reproductive risk and risk for phenotypic manifestations in family members. • Ketogenic diet to control seizures and improve gait disturbance.
SYNGAP1 (35)	• *SYNGAP1*-Related Intellectual Disability is caused by heterozygous, loss of function variants in *SYNGAP1*. • Features include epilepsy, strabismus, musculoskeletal disorders (hip dysplasia, kyphoscoliosis, pes planus), constipation, and behavioral disturbances.	• Genetic counseling: Autosomal dominant and variants are typically *de novo*. Parental mosaicism (somatic and germline) has been reported. Determining if the variant was *de novo* can provide information regarding reproductive risks for the family. • Associated features requiring investigation or monitoring (seizures, kyphoscoliosis, hip dysplasia, constipation requiring intervention).
TSC2/TSC1 (36–38)	• Tuberous sclerosis is caused by heterozygous, loss of function variants in *TSC1* or *TSC2*. Both genes are important for regulation of cell growth through the mTOR pathway. • Features include skin abnormalities (hypopigmented macules, angiofibromas, shagreen patches (connective tissue nevi), ungual fibromas), retinal hamartomas, renal manifestations (angiomyolipomas, cysts, renal cell carcinomas), subependymal nodules, cortical dysplasias, seizures, subependymal giant cell astrocytomas (SEGAs), rhabdomyomas, arrhythmias, lymphangioleiomyomatosis, multifocal micronodular pneumonocyte hyperplasia, and behavioral disturbances.	• Genetic counseling: Autosomal dominant inheritance and variants *de novo* in 2/3 of cases. Determining if the variant was *de novo* or inherited can provide information regarding reproductive risk, risk for phenotypic manifestations, and need for surveillance in family members. • Associated features requiring investigation or monitoring (e.g., CNS tumors, seizures, renal angiolyolipomas or cysts, cardiac rhabdomyomas, and arrhythmias). • Potential role for targeted pharmacologic therapy (mTOR inhibitors). Clinical trials examining the efficacy of mTOR inhibitors have shown evidence to support use of these therapies for SEGAs, renal angiomyolipoma, skin manifestations, and epilepsy.

understanding of recurrence risk in subsequent children and generations can enable families and their medical providers to identify neurodevelopmental disorders and initiate beneficial behavioral treatments and therapies at earlier ages (69). In the case of inherited etiologies, identifying a genetic diagnosis for the proband may also provide diagnoses to other family members with a history of a neurodevelopmental disorder (70).

In addition to the clinical utility conferred through a genetic diagnosis, identifying an etiology can also provide psychosocial benefit to families. A genetic diagnosis can provide families with an explanation for their child's developmental history and, therefore, bring an end to the child's "diagnostic odyssey" that may have included years of uncertainty, anxiety, and evaluations (49, 58, 60, 71), and can also guide patients and families to condition-specific resources and supports (45). Receiving a diagnosis has also been shown to increase knowledge, provide a sense of empowerment (45), result in peace of mind (41), increase parental quality of life (72), decrease parental guilt (59, 73), and foster increased acceptance (74).

GENETIC TESTING FOR INDIVIDUALS WITH NEURODEVELOPMENTAL DISORDERS

In addition to wanting to know their child's clinical diagnosis and prognosis, families typically want to know the cause of the child's developmental disability. In the last two decades, rapid advances in the development of genetic testing technologies and application of these technologies to well-characterized patient cohorts have revolutionized our ability to make specific genetic diagnoses in patients presenting with neurodevelopmental disorders. New genes are being implicated in neurodevelopment at a rapid pace. Online resources such as the Geisinger Developmental Brain Disorder Genes Database (https://dbd.geisingeradmi.org/), the Clinical Genome Resource's Gene Validity Curations and Dosage Sensitivity Map (https://search.clinicalgenome.org/kb/gene-validity/; https://dosage.clinicalgenome.org/), and DECIPHER's Development Disorder Genotype - Phenotype Database (https://decipher.sanger.ac.uk/ddd#ddgenes) can provide up to date information about genes' and genomic variants' relationship to neurodevelopmental disorders (75–78).

Genetic testing is now the standard of care for several neurodevelopmental disorders and the indications for testing will almost certainly broaden in the future. In practice, a genetic etiologic diagnosis may be suspected clinically and confirmed by gene-specific genetic testing or, more commonly, it may be revealed by chromosomal microarray (CMA) analysis, exome sequencing (ES), or *FMR1* CGG repeat analysis for fragile X syndrome completed as a routine part of the evaluation of a patient with ID/GDD or ASD in the absence of a clinically recognizable syndrome. It is important for pediatric clinicians to understand these common tests and their role in the care of children with neurodevelopmental disorders.

Chromosomal Microarray

Genome-wide CMA has been endorsed as a first-tier test for several indications including in patients with ASD, ID, GDD, and/or multiple congenital anomalies (79, 80). CMA technologies (Comparative Genomic Hybridization (CGH) array CGH and Single Nucleotide Polymorphism (SNP)-based testing) detect copy number variants (CNVs) – gains or losses of chromosomal material (81). In some cases, such gains or losses affect gene function and impact health and development. The resolution, or size of gains and losses that can be detected by CMA, varies and is determined by the specific technology used and the genomic distance between DNA probes.

CNVs detected through CMA should be categorized into the following categories in accordance with the American College of Medical Genetics and Genomics (ACMG) and Clinical Genome Resource (ClinGen) guidelines (82).

1) *Pathogenic* – CNVs that are thought to be associated with disease. Pathogenic CNVs may include those that explain the patient's phenotype, those that are associated with carrier status for a recessive condition, and those that indicate disease risk for an unrelated phenotype. Pathogenic CNVs that explain a patient's neurodevelopmental history can include recurrent deletions or duplications such as 22q11.2 deletion syndrome or the 17p11.2 recurrent microdeletion that causes Smith-Magenis syndrome (83, 84), or novel gains or losses that impact dosage sensitive gene(s).
2) *Likely pathogenic* – CNVs that have considerable evidence to suggest that they are associated with disease but where additional evidence could further clarify the variant's pathogenicity.
3) *Uncertain Significance* – CNVs that do not have enough information to determine if they are pathogenic or benign. When a CNV with uncertain significance is identified, parental studies might be used to provide additional information to clarify pathogenicity.
4) *Likely Benign* – CNVs that have considerable evidence to suggest that they are not associated with disease but where additional evidence could further clarify this.
5) *Benign* – CNVs that are not thought to be associated with disease. These are often present in phenotypically normal individuals or in the general population.

Interpretations may change over time as new evidence emerges informing a variants' pathogenicity. Because CMA employs genome-wide testing, incidental findings, or those that are unrelated to the primary indication, may be identified (82). CNVs often include multiple genes and, in rare instances, a CNV might explain a child's neurodevelopmental history, but given the gene content, may also confer risk for an unrelated condition (61). In other cases, a CNV that is unrelated to a patient's phenotype but that has implications for care may be identified and reported through testing (85).

In addition to CNVs, laboratories that employ SNP-based CMA can also detect regions of homozygosity (chromosomal segments that are identical to one another). Identification of regions of homozygosity enables the potential detection of conditions that can be caused by uniparental isodisomy (UPD)

such as Silver-Russell (maternal UPD chromosome 7), Angelman (paternal UPD chromosome 15), and Prader-Willi syndromes (maternal UPD chromosome 15) (86). In other instances, regions of homozygosity may be indicative of ancestral homozygosity or parental consanguinity (86–88). Regions of homozygosity can also be indicative of an increased risk for an autosomal recessive condition due to the potential for homozygous variation in single gene (88, 89).

Although CMA is able to detect CNVs, this technology has limited ability to detect balanced chromosomal rearrangements (translocations, inversions), trinucleotide repeat expansions, imbalances in the mitochondrial genome, epigenetic abnormalities (e.g., methylation abnormalities), sequence level variants, or low-level mosaicism for CNVs (**Table 2**) (65, 90). As stated above, the size of deletions and/or duplications that can be detected by CMA varies. Current clinical CMA platforms can detect CNVs ∼400 kb in size (80) with many laboratories detecting those >250 kilobases. Certain regions may be more specifically targeted enabling even smaller CNV detection (**Table 2**).

Additional testing discussed in this review can aid in detection of some variants that CMA is unable to detect, including *FMR1* CGG repeat analysis for the trinucleotide repeat expansion that causes fragile X syndrome and ES for detection of exonic sequence variants. Cytogenetic testing, including G-banded karyotype and/or florescence *in situ* hybridization (FISH) as well as methylation testing, can detect additional variants or clarify results from CMA. As reviewed elsewhere, such testing should be considered on a case by case basis (e.g., for detection of balanced rearrangements and other complex rearrangements or mosaicism for partial or whole chromosome aneuploidy, or for clarification of the location of a duplication) (90).

Diagnostic Yield of CMA in ID/GDD and ASD

Authors reporting CMA yield use variable nomenclature to describe variants' pathogenicity and diverse interpretation practices, thus limiting between-study comparisons and pooling of data across studies of CMA diagnostic yield. The diagnostic yield of CMA is the proportion of tests performed that identify a variant that is considered causative for a patient's phenotype. Taken together, a causative result can be identified by CMA in 15–20% of individuals with ID/DD, ASD, and/or multiple congenital anomalies (54, 79, 80). For the purpose of trying to capture yield specific to neurodevelopmental disorders, one can examine 19 studies that report on the yield of CMA in more than 150 individuals with ID/GDD where the diagnostic yield ranged from 4.5 to 28.0% (median 13.7%) (91–109). Additionally, one can look to 11 studies limited to patients with ASD (each with a sample size ≥50), in which CMA identified a causative variant in 1.5 to 20.5% of subjects (median 8.1%) (96, 98, 99, 104, 109–115). Because karyotype was the standard etiologic approach before the clinical integration of CMA, many publications summarizing diagnostic yields of CMA excluded patients with an abnormal karyotype. Since the majority of pathogenic results identified by karyotype would be detected by CMA, a number of publications may be reporting yields ∼3.7% percent lower, on average, than if CMA had been applied as a first-tier evaluation (116). Overall, the diagnostic yield of CMA in patients with ID, GDD, and/or ASD suggests that CNVs comprise a substantial proportion of underlying genetic etiologies.

Although the diagnostic yield of CMA has been well-examined in the literature, there are few studies exploring the frequency that incidental findings are identified with this testing technology. In two studies, 0.15–0.48% of patients had a CNV that included a gene associated with hereditary cancer predisposition (117, 118), and in a third study, 1.2% had a CNV that included a gene associated with an adult-onset condition (119) The pathogenicity of these CNVs were not reported by the study authors, so the true rate of pathogenic incidental findings in these study populations is somewhat unclear (117–119). Taken together, however, these studies suggest that clinically significant incidental findings likely occur in <1% of patients undergoing CMA.

Exome Sequencing

In the last decade, the arrival of high-throughput sequencing technologies, collectively referred to as next generation sequencing (NGS) or massively parallel sequencing, has reduced the cost and increased the speed of sequencing, enabling laboratories to sequence large amounts of DNA. Rather than testing a single gene or several genes, laboratories can now offer sequencing of extensive gene panels, the exome, or the genome.

In ES, protein coding regions, or exons, of the genome are sequenced. The exome makes up ∼1.5–2% of the genome, but the vast majority of alleles underlying Mendelian disorders impact coding sequences (120). ES allows detection of sequence-level, single gene variants across almost all of the exome and a small number of intronic nucleotides at the boundaries of each exon. In addition to sequence-level variant detection, several clinical laboratories are now incorporating CNV calling into ES and are able to detect multi-exon deletions and duplications (65, 121, 122) and more laboratories will likely add this to their exome analyses moving forward. Shorter CNVs including those that involve one to two exons are less reliabily detected using ES (121). Given the breadth of information that is generated using ES, parental samples are recommended for analysis (ideally trio analysis or duo analysis if only one parent is available) since this can reduce the number of candidate variants that require review and facilitate variant interpretation (78, 123).

As with CMA, variants detected by ES are categorized as pathogenic, likely pathogenic, uncertain significance, likely benign, or benign in accordance with ACMG/ Association for Molecular Pathology (AMP) sequence variant interpretation guidelines (123). Due to the rapid increase in our understanding of genes and genomic variants, improved variant annotation and filtering, and evolving patient phenotypes (65, 124–129), an iterative reanalysis of ES data may enable additional diagnoses. Over time, a variant's pathogenicity may be updated as new information emerges. ES also includes analysis of genes not yet associated with a specific phenotype. As a result, iterative reanalysis might also identify a novel variant.

ES, like CMA, may identify results that are clinically relevant but unrelated to the patient's neurodevelopmental disorder. ES includes almost all of the coding portions of known

TABLE 2 | Genetic tests commonly used in evaluation of neurodevelopmental disorders.

Test	Results/variants detected	Detection limitations
Chromosomal Microarray (CMA)	• Copy number variants (generally >250 kb but could be smaller if region is specifically targeted) • Regions of homozygosity*	• Repetitive DNA sequences including trinucleotide repeat expansions (e.g., *FMR1* repeat expansion) • Balanced chromosomal rearrangements • Sequence level variants in the exome/genome • Mitochondrial variants • Epigenetics alterations (e.g., Methylation abnormalities, uniparental heterodisomy) • Low-level mosaicism
Exome Sequencing (ES)	• Sequence level variants in the coding region (exome) • Copy number variants**	• Repetitive DNA sequences including trinucleotide repeat expansions (e.g., *FMR1* repeat expansion) • Balanced chromosomal rearrangements • Smaller copy number variants including deletions/duplications involving one to two exons • Mitochondrial variants • Epigenetics alterations (e.g., Methylation abnormalities) • Intronic/non-coding variants • Variants in regions of the exome that are not well-covered by sequencing
FMR1 CGG Repeat Testing	• CGG repeat number in the *FMR1* gene	• Sequence level variants in *FMR1* or elsewhere in the exome/genome • Copy number variants • Balanced chromosomal rearrangements • Exon-level deletions/duplications • Mitochondrial variants • Epigenetics alterations (e.g., Methylation abnormalities)

** - SNP Based CMA; ** - Several laboratories are now calling CNVs as a routine part of ES and this trend will continue to expand.*
Sources: Miller et al. (80); Srivastava et al. (65); Monaghan et al. (25); Collins et al. (26, 27).

genes, providing an opportunity to examine sequence data for pathogenic/likely pathogenic variants in medically actionable genes that are unrelated to the indication for testing (secondary findings). The ACMG has recommended that clinicians notify their patient if a variant known or expected to increase disease risk was identified in a list of, currently 59, clinically actionable genes (130, 131). ACMG has specified that patients and families have the ability to opt out of such findings (132) and will continue to update this list of clinically actionable genes as additional evidence emerges (130). In addition to secondary findings, the use of parental samples in ES can result in incidental identification of possible misattributed parentage; reporting and disclosure of this information varies between laboratories and clinicians (133, 134).

Although ES captures the majority of the exome, the exome is not covered in its entirety and coverage may differ across platforms and laboratories (135). As a result, ES may not detect all coding, sequence variants. ES also has lower sensitivity for detection of mosaicism and exon-level deletions and/or duplications compared to gene panels that include deletion/duplication analysis (65). An increasing number of laboratories are calling CNVs from ES data; laboratories that are detecting and reporting CNVs have limited ability to detect deletions or duplications involving only one to two exons, however (**Table 2**) (121). Furthermore, ES is unable to detect repetitive DNA sequences including trinucleotide repeats (e.g., the CGG repeat expansion that causes fragile X syndrome), intronic/non-coding variants, mitochondrial variants, epigenetic variants (e.g., methylation abnormalities), or balanced chromosomal rearrangements.

Diagnostic Yield of ES in ID/GDD and ASD

The diagnostic yield is the proportion of exome analyses with variants that are determined to be pathogenic or likely pathogenic and explain the patient's phenotype. A recent meta-analysis of 21 ES studies that focused on isolated neurodevelopmental disorders (GDD, ID, and/or ASD; $n = 3{,}173$) identified a diagnostic yield of 31% (95% CI 25–38%) (65). When nine additional studies of individuals with these neurodevelopmental disorders plus associated neurological or syndromic conditions or clinical characteristics were included, increasing the total number of participants to 3,350, the yield was 36% (95% CI 30–43%). In a large, laboratory-based study not included in the meta-analysis because the cohort included a potentially broader group of neurodevelopmental phenotypes, the diagnostic yield among individuals undergoing ES due to neurodevelopmental disorders (ID, ASD, developmental delay, or speech delay) with or without involvement of other organ systems was 25.4% (425 of 1,673) (136).

Delineation of the diagnostic yield of ES for cohorts ascertained for ID/GDD vs. ASD is limited by the phenotype data reported in the literature. Srivastava et al. (65) reported in their meta-analysis that among studies including individuals with primarily ID ($n = 10$), the diagnostic yield was 39% (95% CI 29–50%), whereas studies including individuals with primarily ASD ($n = 5$) had a yield of 16% (95% CI 11–24%) and those with a more heterogenous mix of ID and/or ASD ($n = 6$) identified a diagnosis in 37% of participants (95% CI 29–46%) (65). Among seven ES studies each involving more than 50 individuals with ID/GDD, a diagnosis was established in 34% of patients (range 28–43%) (128, 137–142). Studies restricted to

clinically ascertained samples of patients with ASD and analyses of ASD subgroups within the large, laboratory-based samples have reported lower yields of 8–26% (median 15%) (139, 140, 143–146). There is substantial variability among these studies regarding the information provided about cognitive status and the stringency of ASD diagnosis. In most cases the patients included had previously had genetic testing including fragile X analysis and CMA that did not result in a molecular diagnosis. ES reveals two or, rarely, three molecular diagnoses in ∼1% of individuals undergoing clinical testing (136, 140). When this occurs, the patient typically has a "blended phenotype," with features that are accounted for by each of the pathogenic variants and not by a single molecular diagnosis.

Several studies suggest that the diagnostic yield of ES is higher when trios (probands and parents) are tested than when only the DNA of probands is sequenced (139, 140, 147). Because of the rapid advances in gene and variant curation and factors such as evolution of the phenotype in an individual over time, periodic reanalysis of ES data may enhance the diagnostic yield considerably (48, 124, 127, 138, 148, 149). There is wide variability in the rate of secondary findings among individuals who undergo ES; several recent large studies have reported rates of 2–6% (136, 140, 150, 151). These reportable secondary findings, which are most commonly related to cardiomyopathies, cardiac conduction disorders, hereditary cancer pre-disposition, or familial hypercholesterolemia, often result in additional testing and/or surveillance of the proband and relatives (140).

Exome Sequencing Compared to More Targeted Sequencing

In addition to ES, single gene testing and targeted next-generation sequencing panels have been used in evaluation of patients with neurodevelopmental disorders including ID, GDD, and ASD historically, and a number of clinical laboratories currently offer such testing. Compared to ES, more targeted sequencing allows for increased read depth and sequence coverage thus increasing detection of mosaicism (152). Moreover, single gene testing and gene panels are better able to detect indels and those that include deletion/duplication analysis are better able to detect exon-level deletions and/or duplications (65, 153). While ES identifies 100–200 potentially deleterious sequence variants on average, more targeted analysis identifies a smaller number of variants requiring less analysis and reducing the potential for variants of uncertain significance (81, 120, 154).

Although there are some advantages to more targeted testing and clinical scenarios that warrant such testing, the genetic heterogeneity of ID, GDD, and ASD and frequency of causative *de novo* variants, ES is an appropriate approach for many patients with these diagnoses (129). Studies have found that ES can detect more than 98% of pathogenic variants identified on gene panels (155). Additionally, the gene content of targeted gene panels vary significantly between laboratories meaning that diagnostic yields also vary. In Hoang et al. (156), comparison of ASD gene panels across 21 laboratories found that the number of genes included on ASD-related panels ranged from 11–2,562 and only a single gene (*MECP2*) was included on all panels. In a simulation study comparing ES to gene panels, providers were asked to choose a commercially available gene panel as an alternative when ordering ES for a patient; of the patients receiving a diagnosis through ES, 23% would not have had their variant identified through the provider-chosen gene panel (157). Additionally, as an increasing number of genes are being implicated in Mendelian conditions (158) including neurodevelopmental disorders, panels quickly can become out of date and updating panels can be a time-consuming process (159). After undergoing gene panel testing, 11% of panel-negative cases in one cohort received a diagnosis via ES; many of these diagnoses were attributed to a gene-disease relationship being identified after the panel assay was established (154). In addition to being able to more readily report recently described genes implicated in neurodevelopmental disorders, ES also enables increasing diagnoses over time due to reanalysis. Unlike gene panels, ES includes analysis of genes not yet associated with a specific phenotype. As a result, iterative reanalysis enables reporting of novel variants recently implicated in neurodevelopment and can allow for diagnoses into the future while gene panels typically only enable reanalysis of the sequenced genes. Finally, more targeted testing has been viewed as a less expensive testing option; however, more recent publications have suggested that ES is cost-effective (142, 157, 160–163).

Variants of Uncertain Significance (VUS) From CMA and ES

Although ES and CMA have revolutionized our ability to detect genomic variants, there are limitations in our ability to interpret such variants and determine their impact on health and development. Copy number and sequence variants identified through CMA and ES might be interpreted as variants of uncertain significance (VUS) based on available evidence. VUS include variants in known disease genes that have insufficient evidence to be classified as benign or pathogenic as well as sequence and copy number variants that involve genes that are not yet associated with a disease or phenotype (also referred to as genes of uncertain significance/ candidate genes) (82, 123). VUS in patients undergoing CMA due to a history of developmental delay, ID, ASD, and/or multiple congenital anomalies have been reported to carry at least one VUS 7.9–19% of the time (94–96, 104, 105, 107, 164). The frequency of VUS in patients undergoing ES depends on a number of factors including the reporting laboratory's reporting practices (165), phenotypic features provided by the ordering clinician (166), and inclusion of parental samples. Several studies examined VUS rates among patients undergoing ES for a variety of indications and found VUS rates of 25.3–86% (63, 166, 167).

Uncertain results, although not unique to genetics (168), can pose challenges for patients and providers alike. Compared to other potential results, providers are least comfortable explaining VUS results to families and report that additional preparation is required for such results (169–171). Non-genetics providers express a need for additional education and access to genetics professionals' expertise to facilitate understanding and disclosure of such results (169). Despite expressing less comfort with VUS results and calls for more support and education, non-genetics,

pediatric providers that regularly order testing appear to be able to understand and interpret VUS results accurately (172).

Studies of parents have suggested that they are interested in receiving uncertain results and those that receive such results post-natally see the them as important, often demonstrate an understanding that the result is uncertain, and acknowledge that future advancements can increase understanding (73, 171, 173–178). Although parents seem to accurately recall the concept of uncertainty, parents report difficulty understanding how the result impacts them and their child, and some over interpret the variant as being causative (73, 170, 173, 174). Emotional reactions to VUS vary between families and over time (174, 176).

Given the potential for misunderstandings and variable emotional reactions, providers need to be equipped to discuss uncertain results or refer on to providers with genetics expertise (73). The option for in-person, timely discussions with empathic and honest providers and access to supplemental information including documentation of the result have been suggested to improve understanding of VUS (73, 173, 176, 177). In contrast, internet searches have been shown to increase uncertainty; therefore, anticipatory guidance about misleading or irrelevant online information should be provided to families receiving VUS results (73). In addition to providing families with appropriate information, support, and resources to facilitate understanding of uncertain results, providers returning VUS should consider if additional evaluations that could inform the pathogenicity of the variant are indicated such as parental or familial testing, imaging, or specialist referrals (82, 123, 137, 179). Furthermore, knowledge of genomic variants and their relationship to health and development will continue to improve. As such, VUS will be updated to benign or pathogenic over time. For example, in one study of 2,250 patients undergoing ES reanalysis, 23 had variants initially reported as VUS upgraded to diagnostic (125). Patients receiving VUS results need to be informed of the potential for interpretation updates and ordering providers should discuss the process for reassessing variants.

FMR1 CGG Repeat Analysis

Fragile X syndrome, caused by loss of function of the *FMR1* gene, is thought to be one of the most common inherited genetic causes of ID and ASD (54, 180, 181). Ninety-nine percent of cases of fragile X syndrome are caused by an expansion of the unstable CGG repeat sequence in the 5′ untranslated region (UTR) of the *FMR1* gene (25–27). Most individuals in the general population have 44 or fewer CGG repeats, while more than 200 CGG repeats in the *FMR1* gene results in hypermethylation and, consequently, transcriptional silencing of the gene (182, 183). The *FMR1* gene is located on the X chromosome (Xq27.3) and, consequently, mutations can cause variable phenotypes in males and females (54, 184–186). *FMR1* CGG repeat analysis is usually completed using polymerase chain reaction (PCR) analysis and Southern Blot Analysis (25). *FMR1* CGG repeat analysis can yield four main categories of results (25):

1) *Normal:* ≤44 repeats.
2) *Intermediate (Inconclusive, Borderline, Gray Zone*): 45–54 repeats. Alleles with 45–54 repeats have not been observed to expand to a full mutation in one generation. Because minor increases or decreases in repeat size can occur, alleles of this size could be associated with fragile X syndrome in future generations.
3) *Pre-mutation:* 55–200 repeats. Expansions in *FMR1* of this size are unstably passed from parent to child and, when passed from the mother, expansion from the pre-mutation to a full mutation may occur. The risk for expansion is greatest in those with larger repeat sizes (187). Furthermore, the presence of AGG interruptions within the CGG repeat tract is associated with decreased risk for expansion (187–189).
4) *Full Mutation*: >200 repeats (typically several hundred to several thousand repeats).

In addition to the risk of expansion to a full mutation in offspring, female pre-mutation carriers are at risk for hypergonadotropic hypogonadism (fragile X pre-mature ovarian insufficiency) before age 40 years. Although more prevalent in males, both male and female pre-mutation carriers are also at an increased risk for fragile X-Associated Tremor/Ataxia Syndrome (FXTAS), a late-onset neurodegenerative condition characterized by cerebellar ataxia, intention tremor, cognitive impairment with increased penetrance observed in males (190). When offering *FMR1* CGG repeat testing, pre-mutation carrier status may be identified in the proband and/or relatives and incidentally identify risks for these adult-onset conditions (181).

FMR1 CGG repeat testing cannot detect other variants causing loss of function of the *FMR1* gene (e.g., deletions, sequence variants that result in protein truncation), which are rare. Testing does not detect other genomic causes of neurodevelopmental disorders (e.g., sequence variants in other genes, copy number variants, epigenetic abnormalities) (**Table 2**).

Diagnostic Yield of *FMR1* CGG Repeat Analysis in ID/GDD and ASD

The yield of fragile X testing among individuals with ID/GDD and/or ASD varies widely based on study design and characteristics of the population being tested, such as severity of cognitive impairment, whether both males and females are included, and whether clinical judgement or phenotypic feature checklists were used to exclude some potential participants (191, 192). For example, the diagnostic yield varied from 0.5 to 6% among 14 studies reviewed by Peprah (192) that included at least 200 individuals with ID who were ascertained either through clinical referral or special needs service utilization (192). Hunter et al. (191) identified 15 studies that estimated the frequency of the full mutation in populations with ID before extrapolating to the total population, with the goal of including them in a meta-analysis, but it was not possible to combine the data and calculate a valid mean prevalence due to variability in study methods and lack of reported measures of uncertainty (191).

Several studies that attempted to capture populations of males with neurodevelopmental disorders for assessment of the rate of fragile X syndrome reported full mutations in 8/611 (1.3%) with unexplained ID, 20/3,738 (0.5%) with special education needs related to cognitive deficiencies, and 7/2,471 (0.28%) receiving

TABLE 3 | Genetic testing guidelines for ID/GDD and/or ASD.

Organization	Recommendation(s) for genetic testing
Autism and Intellectual Disability Committee of the American Academy of Child and Adolescent Psychiatry (AACAP) (15)	• CMA in all individuals with ID/GDD and/or ASD • *FMR1* repeat analysis in males and females with ID or a family history of ID • Depending on history and physical examination, consider: ○ *PTEN* testing if head circumference (HCM) is more than 2.5 SD above the mean for age in a child with ID/GDD and/or ASD ○ *MECP2* testing for Rett syndrome in females with severe ID ○ Karyotype if a chromosomal syndrome is suspected • If other investigations do not provide an etiology and there are unresolved clinical findings, consider ES and mitochondrial DNA testing.
American Academy of Pediatrics (AAP) (13, 14)	• If a comprehensive history and exam are indicative of a specific syndrome or disorder, proceed with specific testing in patients with ASD and/or ID/GDD. • In all individuals with ASD and ID/GDD without specific findings, consider CMA and *FMR1* CGG repeat analysis. • In females with ASD and/or ID/GDD without specific findings, consider *MECP2* testing. • In males with ID/GDD without specific findings, consider an X-linked ID panel (XLID). • If an etiology is not identified, consider a referral to genetics for additional work-up including possible ES.
International Standard Cytogenomic Array (ISCA) Consortium (80)	• CMA is the first-tier genetic test in patients with GDD/ID, ASD, and/or multiple congenital anomalies.
American College of Medical Genetics (ACMG) (54)	• After a detailed family history and physical examination, proceed with specific testing for patients with ASD if a syndrome is suspected or if features are suggestive of a mitochondrial or metabolic condition. • If family history and physical exam are not suggestive of a specific diagnosis, metabolic, or mitochondrial condition, proceed with CMA for all patients with ASD and *FMR1* repeat analysis for all males with ASD. • If CMA (in males and females) and *FMR1* repeat analysis (in males) are not diagnostic, consider: ○ *MECP2* sequencing in all females with ASD ○ *MECP2* duplication testing in males with ASD if phenotype is suggestive ○ *PTEN* testing in patients with ASD if HCM is more than 2.5 SD above the mean for age ○ *FMR1* repeat analysis in females with ASD and additional features suggestive of fragile X (e.g., family history and phenotype)
American College of Medical Genetics (ACMG) (79)	• CMA is the first-tier genetic test in patients with multiple congenital anomalies that are not indicative of a specific genetic syndrome and those with non-syndromic ID/GDD, and ASD.
Multidisciplinary Expert Consensus Panel (65)	• ES in all individuals with ID and/or ASD • If ES is non-diagnostic and does not including copy number variant analysis, proceed with CMA. • If ES (and CMA if needed) is non-diagnostic, reanalysis of data from testing should be undertaken periodically.
American Academy of Neurology (AAN) and Child Neurology Society (CNS) (213, 215)	• High resolution karyotype and *FMR1* repeat analysis for patients with ASD that also have ID, family history of fragile X and/or ID, or dysmorphic features. • After obtaining a detailed medical, developmental, and family history for patients with ID/GDD, if a specific etiology is considered, perform appropriate testing such as single gene testing, metabolic testing, or XLID panel. • If a specific etiology is not suspected, perform CMA (or, if not possible, karyotype and subtelomeric FISH) for all individuals with ID/GDD, *MECP2* testing for females with moderate to severe ID/GDD, and *FMR1* repeat analysis in all individuals with mild ID/GDD. • If these and other etiologic work-ups are negative, consider a referral to genetics.

special education services (excluding isolated speech therapy or gifted services) (193–195). An epidemiological study of a representative sample of 3,313 people with ID (56% male) in the Netherlands included 1,143 individuals (55% male) who were not eligible for testing because they had other etiologic diagnoses, including fragile X syndrome (30 males and 2 females; 4.75 and 0.39%, respectively), or had previous negative clinical testing, and 2,170 (57% male) who were eligible for testing as part of the study (196). Among those who were eligible, 1,520 individuals (57% male) consented and were tested. Full mutations were identified in 9/866 males (1.0%) and 2/654 females (0.31%) (196). Allowing the unknown diagnostic yield for those who were eligible but did not consent to testing to vary from 0.5 to 2.0 times the sex-specific diagnostic yield of those who were tested and including the ineligible individuals, the overall prevalence estimates are 2.2–2.5% for males with ID and 1.3–1.6% for females with ID. The rates of full *FMR1* mutations identified among clinical ASD cohorts are generally lower than those identified in clinical ID cohorts. Although early studies suggested higher rates, the larger studies published in the last decade ($n = 142$ to $n = 861$) have identified fragile X full mutations in only 0.23 to 1.2% of individuals ascertained for ASD diagnosis (111, 197–201). The combined yield of these studies was nine full mutations in 1,984 individuals tested (0.45%), including seven males and two females. Fragile X syndrome has been identified in females with ASD with and without ID (202–204).

The diagnostic yield of fragile X testing has also been described by several clinical laboratories, typically with little information available about phenotype. For example, a large-volume commercial diagnostic laboratory reported *FMR1* full mutation alleles in 1.4% of 59,707 males and 0.61% of 59,525 females tested post-natally over a 14-year period (1992–2006) (205). More recently, two university hospital diagnostic laboratories reported yields of 0% (0/654) and 0.9% (11/1,177) in males under age 22 and 19 years, respectively (206, 207). A similar yield of 0.8% (43/5,401) was reported by another hospital-based genetics laboratory that did not describe the diagnostic rate in males and females separately (208). Borch et al. (209) found a diagnostic yield of 1.2% (30/2,486) among pediatric patients who had fragile X testing, including 1.3% of males (25/1,919) and 0.9% of females (5/567). The vast majority, 96% (29/30),

had clinical features and/or family history that were suggestive of fragile X syndrome.

Additional Factors Influencing the Diagnostic Yield of CMA, ES, and *FMR1* CGG Repeat Analysis

Although studies vary in the level of phenotypic detail provided, several authors reporting on yields of CMA, ES, and *FMR1* CGG repeat analysis in patients with neurodevelopmental disorders suggest that specific characteristics and additional diagnoses are associated with increased diagnostic yields (95, 111, 113, 140, 146, 198, 201, 210, 211). Lower IQ, dysmorphic features, and congenital anomalies have been found to be associated with higher diagnostic yield of CMA and ES in some studies examining yield in patients with ASD (111, 113, 146, 198, 201). Similarly, in Fan et al. (95), co-occurring congenital heart defects, facial dysmorphisms, microcephaly, and hypotonia were associated with increased diagnostic yield of CMA in patients with developmental delay or ID (95). A higher diagnostic yield has also been reported for moderate to severe ID than for mild ID. Among individuals with severe ID, it is estimated that more than 60% harbor causative CNVs or exonic sequence variants (211, 212). Finally, in a meta-analysis of published studies using clinical checklists among patients with ID undergoing testing for fragile X, soft and velvety skin on the palms with redundancy on the dorsum of the hand, large prominent ears, pale blue eyes, family history of ID, autistic behavior, flat feet, and plantar crease all are correlated with an increased likelihood of identifying a *FMR1* full mutation (210). Although specific features and additional diagnoses have been correlated with increased diagnostic yield, the yield in patients with isolated ID/GDD and/or ASD is high enough to warrant an etiologic genetic investigation following the application of these categorical diagnoses.

Summary of Diagnostic Yield

Although heterogeneous study designs and methodologies limit conclusions that can be drawn regarding diagnosis-specific diagnostic yields, current evidence suggests that among patients with ID or GDD, the diagnostic yields are at least 15% for CMA, 35% for ES, and 1% (in females) to 2% (in males) for *FMR1* CGG repeat analysis. For those with primarily ASD, the diagnostic yields are at least 10% for CMA and 15% for ES, and 0.5% or less for *FMR1* CGG repeat analysis. Across all of these tests and clinical diagnoses, the diagnostic yield rates are higher in the presence of lower IQ, neurological comorbidities, dysmorphic features, and congenital anomalies. The cumulative diagnostic yield for all three tests, therefore, is over 50% for individuals with ID/GDD and over 25% for individuals with primarily ASD.

APPROACH TO GENETIC ETIOLOGIC EVALUATION

Due to the yields of genetic testing and corresponding potential for clinical and personal utility, there is consensus that genetic testing should be offered to patients with GDD, ID, and/or ASD diagnoses (13–15, 65, 80, 213). The specific testing algorithm varies between guidelines and consensus statements (214), and many recommendations predate the broad integration of exome sequencing into clinical care (**Table 3**).

Despite widespread recommendations for genetic testing for neurodevelopmental disorders in pediatric patients, surveys, interviews (69, 214, 216–221) and retrospective studies of clinical cohorts (111, 198) suggest that that a minority of children with ASD (16.5%-45%) and ID (43%) in the United States have undergone any genetic testing. Furthermore, a study examining pediatric providers' use of genetic testing in the care of simulated patients with ID, GDD, and/or ASD suggest that genetic testing is underutilized (222). In Europe, rates of testing vary from country to country with 61.7% French parents reporting that their child with ASD underwent some diagnostic genetic test compared to 13% of children with ASD in Spain (216, 223). Although there is consensus agreement that a genetic evaluation should be offered to individuals with GDD, ID, and/or ASD, this does not appear to be occurring in practice. Pediatric providers caring for patients with neurodevelopmental disorders should work to address this gap in care.

Directly Consent for and Order Testing

Pediatric providers, from general practitioners to neurodevelopmental pediatricians, are in a position to empower themselves to consent for and order genetic testing. Given the increasing availability of genetic testing and limited number of genetics professionals, genetics providers cannot and need not be involved in all instances of consent for genetic testing and disclosure of results (224–227). Genetic testing for patients with neurodevelopmental disorders has shifted to be within the purview of non-genetics providers and is something that pediatric providers can be equipped to undertake (214, 228–233). Below we describe a structured but flexible approach to facilitate integration of genetic testing into clinical practice across pediatric specialties (**Table 4**).

In most cases of patients with ID, GDD, and/or ASD, developmental, medical, and family histories do not point to a specific genetic etiology. In these cases, the approach to genetic testing should include broad analysis for copy number and exonic sequence variants (**Table 4**). This can be achieved by ordering a single genetic test - ES with CNV analysis (65). Diagnostic yields of ES and emerging evidence that ES is cost-efficient in the etiologic evaluation of children with neurodevelopmental disorders (142, 160–163) support this assertion that ES is the most appropriate first test to pursue (65). Payers are even altering their policies to support increasing efficiency through such testing algorithms (234).

It is important to note that, at present, some genetic testing laboratories may not include CNV analysis as part of their ES and some payers may require completion of CMA prior to coverage of ES. As a result, ES (without CNV analysis) and CMA may need to be pursued in a stepwise manner (65, 141). In cases where both CMA and ES are pursued independently, the order of testing will be dictated by individual factors such as insurance requirements (e.g., payer may only cover ES after a negative CMA) and additional phenotypic features (e.g., non-specific

TABLE 4 | Approach to genetic etiologic evaluation in GDD, ID, and/or ASD.

1. **Complete physical examination and collect developmental, medical, and family history.**
   - If a specific etiology is suspected, pursue the appropriate specific genetic testing (e.g., *PTEN* sequencing and deletion/duplication analysis, *MECP2* sequencing and deletion/duplication analysis, *FMR1* CGG repeat analysis)
   - If a specific etiology is not suspected, proceed to step 2.
2. **Pursue broad examination for causal exonic sequence variants and copy number variants.**
   - This is most efficiently achieved through ES with CNV calling.
   - If insurance restrictions or laboratory offerings dictate a stepwise approach to CNV analysis and ES, CMA and ES can be pursued individually. ES should be pursued as the initial evaluation when possible.
   - ES should be performed using a trio analysis when possible to decrease the number of candidate variants and inform variant classification.
   - If an etiology is not determined, proceed to step 3.
3. **Complete periodic reanalysis of reported variants and exome data; consider additional genetic testing (e.g., genome sequencing, *FMR1* CGG repeat analysis if not already done, cytogenetic testing, if warranted, based on ES/CMA results and clinical findings) and/or referral for medical genetics evaluation if indicated.**

epilepsy phenotype in addition to ASD increasing the likelihood of a sequence variant) (235).

Less often, a patient presenting with ID, GDD, and/or ASD may have a constellation of features and/or history that are suggestive of a particular genetic etiology. In these instances, if a specific disorder is suspected, specific genetic evaluation(s) should be pursued (**Table 4**). For example, in a male patient with developmental delay, head circumference that is more than 2.5 standard deviations above the mean for age, and penile freckling, *PTEN* gene analysis may be pursued as an initial evaluation given that these features are highly suggestive of *PTEN* hamartoma tumor syndrome (13, 30). Similarly, in the case of a female with severe developmental delay, acquired microcephaly, seizures, and stereotypic hand movements, *MECP2* gene analysis may be pursued given that these features are suggestive of Rett syndrome (13, 79).

Recommendations for when to pursue *FMR1* repeat analysis are diverse with some publications recommending that all individuals with ASD and/or ID/GDD undergo testing (13, 14, 213) while others make a distinction based on sex and recommend testing for all males (54). Finally, some authors suggest ordering *FMR1* repeat analysis as a second-tier test (209) or restricting testing to those with the highest likelihood of testing positive for an *FMR1* full mutation based on specific clinical findings could reduce the number of individuals tested while maintaining high sensitivity (236–238). With the relatively low cost of *FMR1* repeat analysis and reproductive risks for family members should a child have fragile X, there should be a low threshold for offering this testing if testing is not offered to all patients with ID, GDD. and/or ASD.

Once a testing approach is determined, the ordering provider should engage families in a conversation prior to testing so they understand the nature and scope of the test(s) (e.g., purpose and potential results including uncertain results), benefits, limitations (e.g., does not detect all genetic causes of neurodevelopmental disorders), risks (e.g., uncertain results and familial implications), and costs (228, 233). Following receipt of results, providers will need to review any variants identified and put them in the context of their patient's clinical features and what is known about the gene, communicate results to the family, establish a follow-up plan, and collaborate with and refer to subspecialists (e.g., medical geneticist) as needed (233).

Integrate a Genetic Counselor Into Your Clinic

Some non-genetics providers express a lack of knowledge and/or discomfort in ordering and interpreting genetic testing (239–243), but genomics education (244), relationships with genetic testing laboratory staff (245), and the ability to refer to genetics (241) once results are received can afford providers with knowledge and support to integrate genomic medicine into their practice. In addition to consenting for and ordering testing independently, pediatric providers can integrate genetic counselors into their clinics for additional support and expertise and a multidisciplinary approach to care. Genetic counselors provide care in at least 28 countries and are typically graduate level providers that have training in both genetics and counseling enabling them to interpret genetic test results and support patients and families in their care (246–249). Increasingly, genetic counselors are working as experts in genetic testing alongside non-genetics providers in various clinical settings, including pediatric neurology and neurodevelopmental clinics (250–252). With the ability to foster and maintain interdisciplinary relationships included in their code of ethics and practice-based competencies, genetic counselors can educate other providers about genetic testing and the genetics of neurodevelopmental disorders (249, 253–256). Furthermore, genetic counselors' expertise makes them well-suited to work alongside pediatric providers to obtain consent for genetic testing, aid in ordering of appropriate testing, interpret genetic test results, and facilitate a family's understanding of and adjustment to genetic information (179, 249, 253–259). Genetic counselors' skill sets can enable them to be important members of a multidisciplinary neurodevelopmental care team along with other specialists such as neurodevelopmental pediatricians (16).

Refer to Genetics Provider

Pediatric healthcare providers may also elect to refer a subset of patients with neurodevelopmental disorders on to an external genetics provider (260). Medical geneticists have expertise in genetic diagnostics, management of genetic conditions, and counseling services (261). Pediatric healthcare providers might choose to refer to a genetics provider in cases where a specific genetic condition is suspected, additional etiological work-up is indicated after non-diagnostic testing, the provider is not equipped to facilitate discussion and ordering of specific testing, or where insurance requires a genetics evaluation before coverage of genetic testing (137, 234). Following receipt of results, pediatric healthcare providers could also refer to medical

geneticists given that medical geneticists are well-poised to facilitate clinical correlation of genomic variants, evaluation of VUS, and management of patients with genetic diagnoses requiring ongoing surveillance and care (14, 137, 224, 262, 263).

Approach to Genetic Testing Around the Globe

Given the diagnostic yields and implications of genetic test results, genetic testing should be considered for any patient with ID, GDD, or ASD across the globe (**Table 4**). With that said, approaches to testing might be influenced by country and/or regional-specific factors. The healthcare system (universal health coverage vs. primarily private, for profit insurers) (216), recognition of genetic professionals (223, 247), access to testing due to availability and cost (247, 264–266), and genetics education for non-genetics professionals (265–267) all affect integration of genetics into the care of patients with neurodevelopmental disorders.

ADDITIONAL CONSIDERATIONS FOR TODAY AND THE FUTURE

Although the majority of the literature concerning genetic testing for patients with neurodevelopmental disorders focuses on genetic testing for three neurodevelopmental conditions – ASD, ID, and GDD; the rapid evolution of genomic medicine and expansion of the literature will continue to change the landscape of genetic testing shifting who is offered genetic testing, the test offerings available, and our understanding of genomic variants.

Expanded Indications for Clinical Testing

In addition to ASD, ID, and GDD, increasing evidence supports consideration of genetic testing for other neurodevelopmental disorders. In epilepsy, between 4 and 78% of selected patients have genomic variants that are probable or definitive explanations for their epilepsy phenotypes (235). In a recent meta-analysis, of studies reporting on the yield of CMA, epilepsy gene panels, and ES, the diagnostic yield was 8, 23, and 45%, respectively (235). Specific epilepsy phenotypes, such as epileptic encephalopathies and comorbid ID have been associated with increased yields of testing (268, 269). Importantly, an increasing number of genetic conditions associated with seizure phenotypes influence therapeutic decision making; therefore, identification of a genetic etiology increasingly can enable personalized care (270–274). Despite genetic testing yields similar to those if ID, GDD, and ASD, no formal practice guidelines regarding genetic testing for individuals with epilepsy have been published by major medical societies; although various approaches to etiologic genetic testing in this patient population have been suggested by independent authors (273, 275).

In CP, studies have suggested a yield of 9.6–31.0% from CMA (276–279) and 10.6–65.0% using ES (280–283). In addition to emerging evidence that CP has a sizable genetic underpinning, genetic testing in CP has already resulted in changes to care (282, 284). Although a practice parameter published more than 15 years ago, the 2004 joint practice parameter from the American Academy of Neurology and Child Neurology Society, indicates that genetic testing should not be pursued in children with CP (285), no guidelines have been published since the emergence of data that demonstrates a significant diagnostic yield of genetic testing in CP patients.

Additional neurodevelopmental disorders, including speech disorders (286–290), Tourette syndrome (291–294) ADHD (295–298), and schizophrenia (299) have emerging evidence that genetic testing can provide an etiology to a portion of patients. Further studies are needed to understand the diagnostic yields and inform testing algorithms for these conditions.

The overlapping symptoms (300, 301), shared genetic underpinnings (4, 81, 211, 229, 302), and comorbidity of neurodevelopmental and neuropsychiatric diagnoses (303–307), have prompted many to advocate that neurodevelopmental and neuropsychiatric conditions collectively be considered as a continuum of developmental brain dysfunction (4, 229, 308). Familial aggregation and genetic studies have further supported the heterogenous nature of neurodevelopment and neuropsychiatric disorders (309–314). Given this and the emerging evidence of utility of genetic testing in a number of neurodevelopmental disorders, in the future, evidence may support broad implementation of genetic testing across neurodevelopmental and neuropsychiatric conditions.

Advances in Clinical Testing

In addition to considering genetic testing for additional neurodevelopmental diagnoses, current testing technology will continue to improve, and new testing offerings will emerge. For example, ES analysis did not routinely detect copy number variants and uniparental disomy, but now some laboratories are identifying and reporting these potentially causative variants (121, 140). With the rapid improvement of testing technologies and bioinformatics pipelines, ES may one day be able to detect additional genetic causes of neurodevelopmental disorders, including trinucleotide repeat expansions (315).

Not only will existing testing continue to improve, the clinical integration of other testing modalities such as genome sequencing, that increases coverage of exons, improves detection of structural variants, and interrogates non-coding regions; RNA-sequencing that analyzes gene expression and mRNA splicing; and genome-wide methylation studies that assesses epigenetic modification of the genome that can impact gene expression will increase diagnostic yield (212, 316–319). It is estimated that up to 3% of all patients with negative exomes could be explained by variants in the non-coding, regulatory regions that would be detectable by genome sequencing (316) and could be further assessed through technologies such as RNA-sequencing (317) and genome-wide methylation studies (319, 320). In addition to informing pathogenicity of non-coding variants, RNA-sequencing and genome-wide methylation studies could also detect epigenetic changes that impact gene expression but do not alter the DNA sequence and are thus undetectable through sequencing (319, 321).

Furthermore, genome sequencing and single-cell sequencing will also enable increased detection of somatic mosaicism that, using current technologies, appear to constitute 5–10% of *de*

novo variants (211, 322). Because somatic variants are not always detectable in the blood, future testing that includes additional cell types will also increase the identification of genetic etiologies for patients and families (211). Availability of relevant tissues, such as single neurons, may be limited, however.

In addition to advancements in testing technology, the understanding of genes and variants and their relationships to disease will continue to improve. Currently, only a fraction of the 20,000 human coding genes have an established disease relationship, and variants in known disease genes may be uncertain given the current knowledge base. As reference databases are bolstered (317), data sharing of variants and candidate genes increases (317, 323, 324), and additional technologies such RNA-sequencing and genome-wide methylation studies are integrated into testing algorithms (317, 319), our understanding of genes' relationship to neurodevelopment disorders and variants' pathogenicity will improve, thus increasing diagnostic yield of testing.

As new tests emerge and previous test results are reanalyzed or updated, recontacting of patients should be a shared responsibility among health-care providers, the clinical testing laboratories, and the patients (325, 326). As such, pediatric providers ordering genetic testing and those with an ongoing relationship with the patient and family, should remain informed of advancements that might provide additional information about a reported variant or indicate that additional testing or evaluation is warranted (82, 325, 327). Providers should bear in mind that, even if an individual has had genetic testing, there may be additional testing (e.g., exome reanalysis, CMA if ES does not include adequate CNV detection, genome sequencing, and novel testing such as genome sequencing) to consider over time and it is important that clinicians continue to consider the utility of genetic testing in individuals with neurodevelopmental diagnoses.

Additional Genetic Factors

Currently, diagnostic genetic testing in neurodevelopmental disorders is focused on identifying rare variants of large effect size. As our ability to detect and interpret genetic causes of NDD increases, including those of more modest effect size, more families will be afforded the clinical and personal utility of genetic diagnoses. Many genetic underpinnings of NDD are characterized by variable expressivity, however, often leaving families with questions about recurrence and prognosis. In addition to the important impact of environmental and stochastic variation, recent advances suggest that the observed variability in phenotypic expression of large-effect rare variants may be explained, in part, by the additive effects of additional high-impact variants (328, 329) and background polygenic risk conferred by a large number of common variants of small individual effect size (330–336). Larger sample sizes and improved study methodologies are expected to lead to identification of rare variants of smaller effect size and elucidate the potential clinical role for polygenic scores in the near future. Increased understanding of additional genetic and other factors that influence phenotypes will enable tailored counseling and care for families receiving a genetic etiology for their child's NDD (17, 334).

CONCLUSIONS

Neurodevelopmental disorders are common, and a significant proportion are caused by rare copy number and exonic sequence variants of large effect size that can be identified by genetic testing. Identifying a genetic etiologic diagnosis can allow clinicians to provide more accurate prognostication and recurrence risk counseling, identify and treat or prevent medical comorbidities, guide patients and families to condition-specific resources and supports and, in some cases, refine treatment options. Genetic testing is now the standard of care for individuals with ID/GDD and/or ASD, and the indications for testing will almost certainly broaden to include other neurodevelopmental disorders in the future. Most genetic etiologic diagnoses in patients with ID/GDD and/or ASD can be made with broad examination of copy number and exonic sequence variants that is most efficiently achieved using ES with CNV calling. It is important for clinicians who provide healthcare to children and adolescents with neurodevelopmental disorders to gain an understanding of these common tests and their role in providing the best medical care for patients and to work to facilitate the genetic etiologic evaluation of these patients by ordering testing or partnering with genetic providers.

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REVIEW
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Genetic and Non-genetic Workup for Pediatric Congenital Hearing Loss

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Hearing loss is one of the most common concerns for presentation for a geneticist. Presentation prior to the age of one (congenital hearing loss), profound sensorineural hearing loss (SNHL), and bilateral hearing loss are sensitive and should raise concern for genetic causes of hearing loss and prompt referral for genetic testing. Genetic testing particularly in this instance offers the opportunity for anticipatory guidance including possible course of the hearing loss over time and also connection and evaluation for additional congenital anomalies that may be associated with an underlying syndrome vs. isolated genetic hearing loss.

Keywords: genetic, hearing loss, congenital hearing deficit, hearing problems, genetic algorithm

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INTRODUCTION

Every 2–3 children out of 1,000 in the United States are born with hearing loss (HL), making it the most common congenital sensory deficit in humans (1). Sensorineural hearing loss predominates congenital hearing loss, with the causes of HL broadly divided into genetic vs. non-genetic or acquired factors. Over the past 25 years the continual advancement of technology and accuracy of diagnostic testing has revealed genetic etiology for HL occurrences in prelingual children to be as high as 60% (2).

Despite this advancement in knowledge of genetic causes, there is still limited consensus on management of pediatric patients with hearing loss (3). The primary goals of management of pediatric patients with HL are timely and proper diagnosis and determining appropriate aural rehabilitation. The aim of achieving these goals is to optimize communication and language development in the child with hearing loss.

Genetic testing may provide insight into management of hearing loss itself or provide guidance of when to consider additional congenital anomalies in association with a genetic syndrome. For example, individuals with sensorineural hearing loss who have Usher syndrome are at risk for retinopathy and this should be followed closely. In addition, monogenic causes of hearing loss may present with a spectrum within a family. Therefore, diagnosis in a child with profound hearing loss may prompt testing in other family members who have progressive hearing loss or allow for treatment of other affected family members at a younger age.

In this article, we will review an approach to genetic testing for individuals with hearing loss and then discuss the evidence regarding management as a guide to optimizing future practice.

PEDIATRIC HEARING LOSS OVERVIEW

The American Academy of Pediatrics' Joint Committee on Infant Hearing is a leader in endorsing early detection of and intervention for infants with hearing loss. They proposed the 1–3–6 guideline for screening and interventions for pediatric HL. This is designed to maximize the outcome for infants who are deaf or hard of hearing and recommends that all infants should be screened

for hearing loss no later than 1 month of age. Infants that do not pass their screening are recommended to have a comprehensive audiological evaluation [auditory brainstem response (ABR) testing] no later than 3 months of age, and infants that do have confirmed hearing loss should receive appropriate intervention no later than 6 months. Intervention should be from health care and education professionals with expertise in hearing loss and deafness in infants and young children (4).

Infants admitted to the neonatal intensive care unit (NICU) require more in-depth screening based on days of admission. NICU infants admitted to the hospital for more than 5 days should have ABR included as part of their screening (4). Regardless of newborn hearing-screening outcome, all infants should continue to have ongoing monitoring for developmentally appropriate communication skills and auditory behaviors.

NON-GENETIC ETIOLOGY WORK-UP

Clinicians should establish potentially reversible causes of hearing loss as soon as possible. Half of all the non-genetic causes of HL are attributed to infectious disease. TORCHES [toxoplasmosis, rubella, cytomegalovirus (CMV), herpes, and syphilis] infections are known risk factors for SNHL. Out of these infectious causes, congenital CMV is the most common cause of non-hereditary SNHL in childhood. Most estimates from studies conducted in Europe, the United States, and Japan show the prevalence of congenital CMV varies from 0.2 to 2.0%, but much higher in developing countries at 6–14% (5). CMV infection is congenital if diagnosed within 21 days postnatally. CMV infection in infants if acquired postnatally is not associated with SNHL (3). At our institution, we test for congenital CMV using saliva polymerase chain reaction (PCR) and if this is positive confirmation is with urine PCR. Treatment for congenital CMV in children with isolated SNHL is still not fully elucidated. Antiviral therapy, particularly with valganciclovir, is the most commonly used medication for treatment though the benefits in the literature are mixed (3). When a patient is diagnosed with congenital CMV with isolated SNHL, infectious disease consultation is recommended to review the risks and benefits of antiviral therapy and consider treatment.

Overall in the literature, use of CT in children with hearing loss ranges from 20 to 70% of the time (6). It is superior to MRI in delineating bony anatomy and, therefore, useful in understanding conductive or mixed hearing loss, and CT has also been shown to have a higher yield in identifying enlarged vestibular aqueducts and cochlear anomalies (7). Though it is less expensive and faster than MRI, it does expose children to ionizing radiation, so this risk should be considered when deciding on diagnostic imaging. MRI is preferred for evaluating the cochlear nerve, and the brain and nerve tumors (such as vestibular schwannoma). MRI frequently requires sedation or general anesthesia to perform and the risks associated with this also need to be considered when choosing an imaging modality. When it comes to surgical planning, particularly for possible cochlear implantation, CT or MRI can be used and usually up to the surgeon's discretion. The IPOG study group had 100% agree or partially agree that children who are cochlear implant candidates with profound hearing loss may benefit from CT or MRI to assess for cochlear dysplasia and cochlear nerve hypoplasia/aplasia (8).

If the pediatric HL is considered to be non-genetic and non-infectious in etiology, additional testing should be based on clinical exam findings, imaging, and medical and family history. A "shotgun" approach to obtain an etiologic diagnosis for pediatric HL has been shown to have very low diagnostic yield and to unnecessarily increase cost (9). However, certain investigations are value-additive.

GENETIC WORK-UP

Any infant or child diagnosed with bilateral congenital HL without a known etiology (e.g., infectious) requires a genetics consultation. Comprehensive genetic testing has the highest diagnostic yield of any single test for bilateral SNHL and up to 60% of cases of congenital HL are due to a genetic etiology (8, 10). Originally the only available genetic testing for hearing loss was single-gene testing, most commonly for *GJB2* and/or *GJB6* genes (10). Comprehensive genetic testing (CGT), by using massively parallel sequencing or next generation sequencing (NGS), now improves the genetic diagnostic yield by multiple orders of magnitude over single-gene testing and is becoming the new standard of care due to decreased cost of sequencing and avoidance of multiple tests and appointments (10, 11). IPOG consensus recommendations also state this with 84% agreement or in partial agreement that in the setting of CPG, single-gene testing is of low diagnostic yield and should only be offered as part of an initial workup if a known family history exists. All surveyed agree or partially agree that single-gene testing should be considered if CGT is not available (8). It is important to note that a negative genetic test does not rule out a genetic etiology, as the gene of interest may not have been included due to lack of known role in HL to date or the sensitivity of the test may not have picked up the causative mutation (such as intronic mutations). Whole genome sequencing can be used as well to detect lesser known hearing loss mutations not covered in the NGS testing. **Table 1** lists some of the more commonly available commercial NGS tests in the United States market, which all evaluate for syndromic and non-syndromic causes for congenital hearing loss.

Genetic etiologies for congenital HL can be divided into syndromic or non-syndromic SNHL (NSSNHL). Approximately 70% of cases of genetic-related hearing loss are NSSNHL, which are often contributed to a single gene mutation. The remaining 30% syndromic SNHL cases are often accompanied by other physical abnormalities or systemic clinical manifestations. **Table 2** provides a list of the most common syndromic causes of hearing loss. The number of known NSSNHL related to disease-causing genetic variants continues to grow as the technology and capability of genetic testing has expanded. However, the number of known syndromic SNHL genetic causes has remained

TABLE 1 | NGS tests in US.

	Laboratory	Turn Around Time	# of Genes Analyzed
OtoSeq	Cincinnati children's molecular genetics laboratory	8 weeks	23
Fulgent comprehensive hearing loss NGS panel	Fulgent genetics	3–5 weeks	179
OtoGenetics comprehensive deafness gene testing	OtoGenetics corporation	4 weeks	129
OtoSCOPE	Iowa molecular otolaryngology and renal research laboratories	6 weeks	152
Emory hearing loss expanded panel	Emory genetics laboratory	12 weeks	92
OtoGenome	Laboratory for molecular medicine, partners healthcare personalized medicine	8–12 weeks	110

TABLE 2 | List of syndromic HL.

Autosomal Dominant

- Branchio-oto-renal syndrome (EYA1)
- Treacher-Collins/mandibulofacial dysostosis (TCOF1)
- Stickler syndrome (COL2A1)
- Waardenburg syndrome (PAX3, EDN3)
- Apert syndrome (FGFR2)
- Crouzon syndrome (FGFR2)

Autosomal Recessive

- Pendred syndrome (SLC26A4)
- Jervell and Lange-Nielsen syndrome (KCNQ1)
- Usher syndromes (MYO7A)
- Goldenhar syndrome/oculoauriculovertebral spectrum

X-linked

- Alport syndrome (COL4A5)

Other

- Down syndrome
- CHARGE syndrome
- Turner syndrome
- Kabuki syndrome (KMT2D, KDM6A)
- Nager syndrome (SF3B4)
- Velocardiofacial syndrome/22q11.2 deletion syndrome (TBX1)
- Osteogenesis imperfecta (COL1A1, COL1A2)

relatively stable over the past 10 years with ∼500 known syndromes associated with HL (12, 13).

The Clinical Genome Resource (ClinGen), a National Institutes of Health (NIH)-funded initiative has built a central resource to provide a semiquantitative framework to assign clinical validity to the gene-disease relationships in the hundreds of genes reported in the literature associated with hearing loss. The gene-disease pairs are classified by either being definitive, strong, moderate, limited, disputed, or refuted. For the hearing loss associated genes ClinGen applied the clinical validity framework on 142 genes associated with non-syndromic and syndromic hearing loss that are included on panels from 17 diagnostic testing laboratories from around the world (14). The clinical validity classifications for these genes are publicly available at: https://search.clinicalgenome.org/kb/gene-validity.

Genetic diagnosis may help practitioners provide anticipatory guidance to families regarding other involved organ systems. This can provide significant insight into the patient's care. All children confirmed to have SNHL should have a vision screen either by their pediatrician, optometrist, or ophthalmologist as part of their work-up, given the 2–3-fold increased risk of ocular abnormalities in children with non-syndromic SNHL (8).

Vanderbilt Children's Hospital Hearing Loss Workup in Pediatric Patients

Bilateral SNHL

1. Mild to Moderate (21-54 dB)
    a. Imaging: Only if change in hearing
    b. Labs: None unless clinical concern
    c. Consultations
        i. Ophthalmology or PCP vision screen
        ii. Genetics: Higher likelihood of determining cause
    d. Other
        i. Hearing aid evaluation
2. Moderate to Severe (55-74dB)
    a. Imaging: CT Temporal Bone
    b. Labs: None unless clinical concern
    c. Consultations
        i. Ophthalmology or PCP vision screen
        ii. Genetics
    d. Other
        i. Hearing aid or cochlear implant evaluation
3. Severe to Profound (>75 dB)
    a. Imaging: CT (MRI if concern for absent/dysplastic nerve or if child will require sedation for CT)
    b. Labs: None unless clinical concern
    c. Consultations
        i. Ophthalmology
        ii. Genetics
    d. Other
        i. EKG (especially if family history of arrhthymia)
        ii. Cochlear implant evaluation

CMV: If failed hearing screen in NICU, newborn nursery, or seen in clinic with new identified hearing loss get saliva PCR within first 21 days of life. If saliva PCR is positive then urine PCR testing for conformation. If congenital CMV is confirmed then consider for ValEar trail and refer to ID.

Unilateral SNHL

1. Mild to Moderate
    a. Imaging: CT Temporal Bone per physician discretion
    b. Labs: None unless clinical concern
    c. Consultations: Hearing aid evaluation, Ophthalmology or PCP vision screen, +/- Genetics
    d. Other
2. Moderate to Severe
    a. Imaging: CT Temporal Bone
    b. Labs: None unless clinical concern

FIGURE 1 | Vanderbilt Children's Hospital hearing loss protocol.

A recent study demonstrated that the majority of ocular abnormalities in children with hearing loss can be identified with today's standard screening methods (15). If a child has profound bilateral SNHL and there is a family history of cardiac arrhythmia or sudden death of unknown etiology, then it is also recommended to obtain an electrocardiogram to rule out Jervell and Lange-Nielsen syndrome.

In the NSSNHL group, 75–80% are inherited in an autosomal recessive pattern, with 15–24% autosomal dominant, 1–2% X-linked, and <1% mitochondrial (2, 13, 16). Abnormalities of *GJB2* are the most common cause of HL in infants, and account for ∼50% of the autosomal recessive HL patients. Since only half of the responsible mutations of *GJB2* are biallelic, there are a significant number with only one mutation therefore screening for only this gene is insufficient (2). *GJB2* is located on chromosome 13q and encodes for the protein connexin 26, while *GJB6* is also located on the same chromosome and lies adjacent to it at the DFNB1 locus. Connexin 30 which forms gap junctions with Connexin 26 is encoded by *GJB6*. Thus, NSSNHL loss can also occur with a mutation in *GJB2* on one allele coupled with a deletion in *GJB6*, and explains the HL in patients when *GJB2* is not inherited in a biallelic fashion (2). Over 110 disease-causing variants in *GJB2* and at least 2 deletions in *GJB6* have been reported (2, 17, 18). It should be noted that the most common *GJB2* disease-causing variants that are found in the Caucasian populations, do not play a large role in other races, particularly those of African descent (10, 19–21). The second most common genetic cause of bilateral SNHL is a mutation in the *STRC* gene on 15q15.3, which can be from partial or complete gene deletions, copy number variants (CNV), or point mutations of the gene (2, 22).

The diagnostic yield of genetic testing in children with unilateral SNHL ranges from 1 to 5% in the literature (23, 24). However, it can have huge implications on the patient's care including screening and should be considered early on in the work up. In particular, all individuals with bilateral congenital sensorineural hearing loss should be sent for genetics work up. In addition, a family history of hearing loss should prompt referral.

IMAGING IN HEARING LOSS

Radiographic imaging can play an important role in diagnosis in genetic or non-genetic etiologies for pediatric HL. See **Figure 1** for our institution's protocol for imaging depending on the degree of hearing loss, unilateral vs. bilateral, or if post-meningitis. Whether a computed tomography (CT) of the temporal bones or magnetic resonance imaging (MRI) is ordered, children with more severe SNHL are more likely to have an abnormality on imaging (3). Currently, there is neither consensus on the best temporal bone imaging modality nor on the timing of obtaining a scan. However, an international working group of pediatric otolaryngologists—IPOG—expressed a majority opinion that temporal bone imaging should not be performed during the neonatal period unless indicated for other reasons (i.e., brain MRI) (8). The same IPOG publication sought to obtain consensus surrounding pediatric hearing loss. In a survey, 76% IPOG members agree or partially agree that temporal bone imaging is of low diagnostic yield when routinely employed in the setting of symmetric bilateral hearing loss. Note they did not detail this or break it down into severity of the hearing loss. All agree or partially agree that for unilateral, asymmetric or mixed loss, the diagnostic yield of imaging is higher and should be considered. All surveyed also agree or partially agree that children that are cochlear implant candidates with profound hearing loss may benefit from CT or MRI.

CONCLUSION

Identifying a genetic etiology has been shown to have many clinical benefits as well as psychosocial and emotional benefits for patient families. Obtaining a genetic diagnosis has been shown to improve parental psychological well-being, mostly by alleviating guilt and accelerating involvement in rehabilitation (10, 25–27). It also allows for parental education of future medical or educational needs and potential risk of hearing loss in future offspring (10). If definitive results are found on genetic testing, this may also preclude the need for imaging and further costs (24). Finally, for children requiring rehabilitation through cochlear implantation, genetic testing may help benchmark expected outcomes against specific lesions that have been characterized by busy sequencing centers that aggregate both genotypic and phenotypic (audiometric and speech-related) information.

AUTHOR CONTRIBUTIONS

RB responsible for writing manuscript draft, editing, and figure/table creation. JD, CW, and FV responsible for writing and editing manuscript and creation of figure protocol. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.536730/full#supplementary-material

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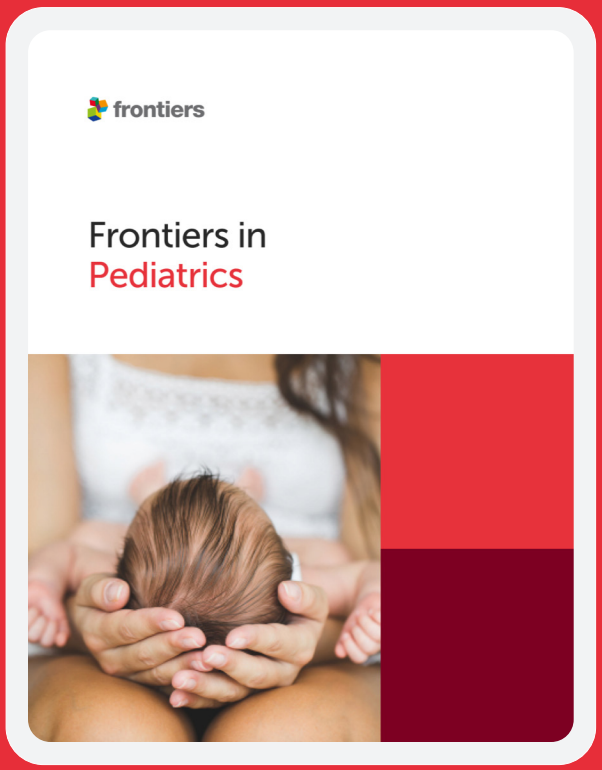
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