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Clinical and molecular analysis of Guangxi patients with Kabuki syndrome and *KMT2D* mutations

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ABSTRACT

Kabuki syndrome (KS) is a multiple congenital anomaly syndrome that is characterized by postnatal growth deficiency, hypotonia, short stature, mild-to-moderate intellectual disability, skeletal abnormalities, persistence of fetal fingertip pads, and distinct facial appearance. It is mainly caused by pathogenic/likely pathogenic variants in the *KMT2D* or *KDM6A* genes. Here, we described the clinical features of nine sporadic KS patients with considerable phenotypic heterogeneity. In addition to intellectual disability and short stature, our patients presented with a high prevalence of motor retardation and recurrent otitis media. We recommended that KS should be strongly considered in patients with motor delay, short stature, intellectual disability, language disorder and facial deformities. Nine *KMT2D* variants, four of which were novel, were identified by whole-exome sequencing. The variants included five nonsense variants, two frameshift variants, one missense variant, and one non-canonical splice site variant. In addition, we reviewed the mutation types of the pathogenic *KMT2D* variants in the ClinVar database. We also indicated that effective mRNA analysis, using biological materials from patients, is helpful in classifying the pathogenicity of atypical splice site variants. Pedigree segregation analysis may also provide valuable information for pathogenicity classification of novel missense variants. These findings extended the mutation spectrum of *KMT2D* and provided new insights into the understanding of genotype-phenotype correlations, which are helpful for accurate genetic counseling and treatment optimization.

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1. Introduction

Kabuki syndrome (KS, MIM# 147920) is a multiple congenital anomaly syndrome which was first described in 1981 [1,2]. It is characterized by postnatal growth deficiency, hypotonia, short stature, mild-to-moderate intellectual disability, skeletal abnormalities, persistence of fetal fingertip pads, short fifth finger, and distinct facial appearance (long palpebral fissures, arched eyebrows and lateral eyebrows that are sparse or notched, broad and depressed nasal tip, large or dysplastic ears, and a cleft or high-arched palate). Other recurrent findings may include: congenital cardiac anomalies, genitourinary anomalies, gastrointestinal anomalies, cleft lip and/or palate and abnormal dentition [3–5]. The clinical diagnosis of KS is often challenging and usually requires long-term monitoring, because the phenotype tends to evolve over time [6]. The prevalence of KS is estimated to be 1 in 32,000 [7].

Pathogenic variants in the *KMT2D* gene (MIM#602113) have been identified as the most common cause (approximately 75%) of KS [8,9]. Mutations in the *KDM6A* gene (MIM#300128) account for less than 5% of cases, whilst the genetic basis for over 20% of KS patients remains unknown [10,11]. Both the *KMT2D* gene and the *KDM6A* gene participate in chromatin remodeling and regulate gene expression [12]. Loss-of-function variants in these two genes cause abnormal cell differentiation, ultimately leading to a characteristic dysmorphism, growth retardation and developmental delay [13]. A high degree of clinical and genetic heterogeneity exists within KS and the dosage sensitivity of causative genes may be related to different clinical manifestations, no specific genotype-phenotype correlations can be delineated [12,14].

So far, hundreds of *KMT2D* mutations have been identified to date. These include missense variants, nonsense variants, indels, frameshifts, splice site variants, multiple exon deletions and structural variants encompassing *KMT2D* gene. In addition, mosaic *KMT2D* mutations, which included point mutations, indels, whole gene deletions, intragenic deletion and duplications of the *KMT2D* gene, have also been reported in KS patients with milder clinical presentation [14,15]. Here, we reported nine KS patients and identified nine *KMT2D* variants, which included five nonsense variants, two frameshift variants, one missense variant and one splice site variant. The splice site variant was non-canonical and abnormal splicing was confirmed by reverse transcription PCR analysis using total RNA that was extracted from peripheral blood of the affected patient. The patients exhibited substantial clinical features of KS and showed broad phenotypic variability. All patients manifested delayed motor developmental milestones. A high prevalence of recurrent otitis media was also noted in our cohort. Our study extended the mutation spectrum of *KMT2D* and provided new insights into the understanding of genotype-phenotype correlations, which are helpful for accurate genetic counseling and treatment optimization.

2. Materials and methods

2.1. Patients and ethics approval

All patients were recruited from Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region. Written informed consent was collected from the parents or legal guardians of all study participants. The study was reviewed and approved by the ethics committee of our hospital.

2.2. DNA extraction

Peripheral blood of patients and their family was collected in vacutainer tubes with ethylenediaminetetraacetic acid by venipuncture. Abdominal amniocentesis was performed under ultrasound guidance. Genomic DNA was isolated using QIAamp DNA Blood Mini Kit (Qiagen, Germany) or Lab-Aid DNA kit (Zeesan, Xiamen, China) by standard procedures according to the manufacturer's protocol.

2.3. Whole-exome sequencing and data analysis

The DNA was quantified with a Nanodrop spectrophotometer (Life Corp, MA, USA). Exome sequences was enriched with an Agilent SureSelect Human All Exon v6 kit (Agilent Technologies, CA, USA). And the purified DNA libraries were sequenced using an Illumina HiSeq2500 sequencer (Illumina, CA, USA) with a read depth at least 100× and more than 95% of the targeted regions were covered over 20×. After removing the redundant reads, all SNPs and InDels were identified using the mapped reads and annotated with ANNOVAR software. All the candidate variants were further validated and segregated by Sanger sequencing in all available family members. The detected variants were interpreted and classified according to the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines [16].

2.4. In silico analysis

In silico analyses to predict missense mutation effects were performed using REVEL (https://sites.google.com/site/revelgenomics/), PolyPhen 2.0 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/). For identified intronic variants, the potential splice effects were predicted by the varSEAK online tool (https://varseak.bio).

2.5. RNA extraction and cDNA analysis

Fresh peripheral blood leukocytes were obtained from the participants using EDTA anticoagulation tubes. Total RNA was isolated with TRIzol reagent (Invitrogen, CA, USA). It reverse-transcribed to cDNA using a First-strand cDNA Synthesis Kit (Takara Biotechnology, Dalian, China) and amplified by PCR with Taq polymerase (Takara Biotechnology, Dalian, China). The obtained PCR products were detected by 1.5% agarose gel electrophoresis. The products were purified and sequenced with an ABI 3130 genetic analyzer (Applied Biosystems).

3. Results

Nine unrelated KS cases with *KMT2D* variants (NM_003482.4) were identified by whole-exome sequencing (Table 1, Table S1 andFigure S1-2). These included a fetus, an infant who died at one and a half months of age and seven children (one female and six males). When calculating phenotypic proportion, we excluded the affected fetus and the infant who died early (cases 3 and 5). In addition, we analyzed the mutation types of the pathogenic *KMT2D* variants that are documented in the ClinVar database (Table S2) and compared them with our cohort.

3.1. Patient 1

A 5-year-old female patient was delivered at 35 + 6 weeks of pregnancy, with birth weight of 2630 g. She was hospitalized immediately after delivery for premature birth and neonatal infection. During her hospital stay, neonatal hypoglycemia, hyperbilirubinemia, patent ductus arteriosus and patent foramen ovale were diagnosed. Three years later, she developed a recurrent infection, including respiratory infections, otitis media and rash. And global developmental delay was noted by six months of age, which included motor delay, short stature, mental retardation and intellectual disability. In addition, the clinical characteristics included premature anterior fontanel closure, hypertelorism, exophthalmos, depressed nasal tip, broad nasal root, allergic rhinitis and mild hearing impairment in the left ear.

By WES, a novel heterozygous c.3249C > A(p.Cys1083Ter) in the *KMT2D* gene was identified in this girl. The nonsense variant has been reported by Quinlan-Jones E et al. [17], and it was classified as being pathogenic according to ACMG/AMP variant classification scheme (PVS1 + PS2 + PS4_supporting + PM2_supporting).

3.2. Patient 2

A 2-year-old male patient was delivered at 36 + 5 weeks of pregnancy, with birth weight of 2850 g and birth length of 50 cm. He had a history of phototherapy for jaundice at birth. And bilateral adenitis of unknown origin of the lacrimal glands were diagnosed after birth. Due to motor retardation, he was admitted to our hospital for a further assessment at the age of five months. Upon admission, a clinical examination revealed that microcephaly, premature anterior fontanel closure, micrognathia, hypertelorism, high palate and lower limb asymmetry. Echocardiography revealed a ventricular septal defect and a small atrial septal defect. X-ray showed dislocation of the left hip. Brain MRI showed morphological changes of corpus callosum, considering the possibility of corpus callosum dysplasia. In addition, the child presented with moderate developmental delays and otitis media.

Finally, applying WES, we revealed a heterozygous c.12592C > T (p.Arg4198Ter) in the *KMT2D* gene in the boy. This nonsense variant has been reported in at least six patients with KS [9,18–21]. The variant was absent in both parents, and it was classified as being pathogenic according to ACMG/AMP guidelines (PVS1 + PS2 + PS4_moderate + PM2_supporting).

3.3. Patient 3

A 30-year-old woman was referred for genetic counseling at 23 weeks of gestation because of fetal abnormalities in one twin on prenatal ultrasound. Abnormal ultrasound findings included micrognathia, abnormal position of the right kidney, strephenopodia of the right foot and single umbilical artery.

Trio-based WES revealed a novel heterozygous variant, c.9566delG (p. Gly3189fs*7), in the *KMT2D* gene of the sick fetus. The frameshift variant was not detected in pregnant woman and her husband, and it was classified as being pathogenic according to ACMG/AMP variant classification guidelines (PVS1 + PS2 + PM2_supporting).

3.4. Patient 4

A 15-year-old boy was the fourth child born to a healthy and non-consanguineous couple. He was born at term after an uneventful pregnancy. At 13 years of age, he was admitted to hospital due to learning disability and muscle weakness. According to his family, he began to speak his first words when he was 18 months old. He started to walk at age of three years. Despite developmental retardation, he could recite some ancient Tang poetries and talk to other people when he was five years old. Since then, however, he presented with developmental regression, arm weakness and autism behavior. For now, he can only write his name and numbers. Medical

Table 1
Summary of clinical and molecular features of the patients with Kabuki syndrome in our cohort.

Patient #	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	Affected children
Gender	Female	Male	NA (Fetus)	Male	Male	Male	Male	Male	Male	
Age at diagnosis	1 year	5 months	23 gestational weeks	14 years	1 month	7 years	8 years	8 years	7 years	
Nucleotide change	**c.3249C > A**	c.12592C > T	**c.9566delG**	**c.10507 + 6T > C**	c.13606C > T	**c.8077C > T**	**c.7744delG**	c.16273G > A	c.12688C > T	
Amino acid change	p.[Cys1083Ter]	p.[Arg4198Ter]	p.[Gly3189fs*7]	–	p.[Arg4536Ter]	p.[Gln2693Ter]	p.[Val2582fs*1]	p.[Glu5425Lys]	p.[Gln4230Ter]	
Exon/Intron	E12	E40	E35	Intron38-donor	E41	E33	E32	E52	E40	
Inheritance	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	NA	*De novo*	
Reference	[17]	[9,18–21]	Present study	Present study	[8,9,21]	Present study	Present study	[21–26]	[13]	
Short stature	+	+		+		+	+	+	+	7/7
Failure to thrive				+	+			+		2/7
Developmental delay (DD)/ Intellectual disability (ID)	DD/ID	DD/ID		Moderate ID		Mild ID	IQ Normal	IQ Normal	DD/ID	5/7
Behavioral abnormality								+		1/7
Motor delay	+	+		+		+	+	+	+	7/7
Speech impairment		+		+		+			+	4/7
Hypotonia				+					+	2/7
Seizures						+				1/7
Persistent fetal finger fat pads				+			+			2/7
Brachydactyly				+	+		+	+		3/7
Clinodactyly				+				+		2/7
Hypoplastic fingernail/toenail				+				+		2/7
Craniofacial	+	+								2/7
Abnormality of the cerebrum	+	+								2/7
Skeletal anomalies			+			+				1/7
Upper limb phocomelia					+					–
Small hand						+				1/7
Non-traumatic joint dislocation		+		+				+		3/7
Lax joints		+								1/7
Visceral malformation	+	+	+		+					2/7
Congenital heart defect					+					–
Abnormal respiratory tract	+									1/7
Gastrointestinal anomaly							+			1/7
Genitourinary anomaly			+	+		+	+			3/7
Endocrine problem	+				+					1/7
Obesity				+			+			2/7
Immunological	+									1/7
Frequent infection	+			+			+			3/7
Skin problem	+						+	+		3/7
Recurrent otitis media	+	+		+			+		+	5/7
Microcephaly		+								1/7
Prominent forehead							+			1/7
Arched eyebrows				+			+		+	3/7
Lateral eyebrows sparse or notched							+		+	2/7

(*continued on next page*)

Table 1 (*continued*)

Patient #	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	Affected children
Long palpebral fissures				+				+	+	3/7
Long eyelashes								+		1/7
Everted lower eyelids								+		1/7
Hypertelorism	+	+			+		+			3/7
Exophthalmos	+									1/7
Epicanthus						+	+			2/7
Depressed nasal tip	+				+		+		+	3/7
Broad nasal root	+				+		+		+	3/7
Short philtrum				+					+	2/7
Large ears				+			+	+	+	4/7
Low-set ears						+				1/7
Hearing loss	+			+					+	3/7
Morning glory anomaly									+	1/7
Micrognathia		+	+							1/7
Thin upper and full lower lip									+	1/7
High/cleft palate		+				+				1/7
Cleft palate									+	1/7
Lip pits								+		1/7
Oligodontia/abnormal incisors				+			+	+		3/7
Webbed neck					+					–
Lacrimal adenitis	+									1/7
Score#	3	5		4		3	4	4	2	

Nucleotide numbering is based on NM_003482.4. Abbreviations: +, present; -, absent. # The results were based on the scoring system reported by Makrythanasis et al.

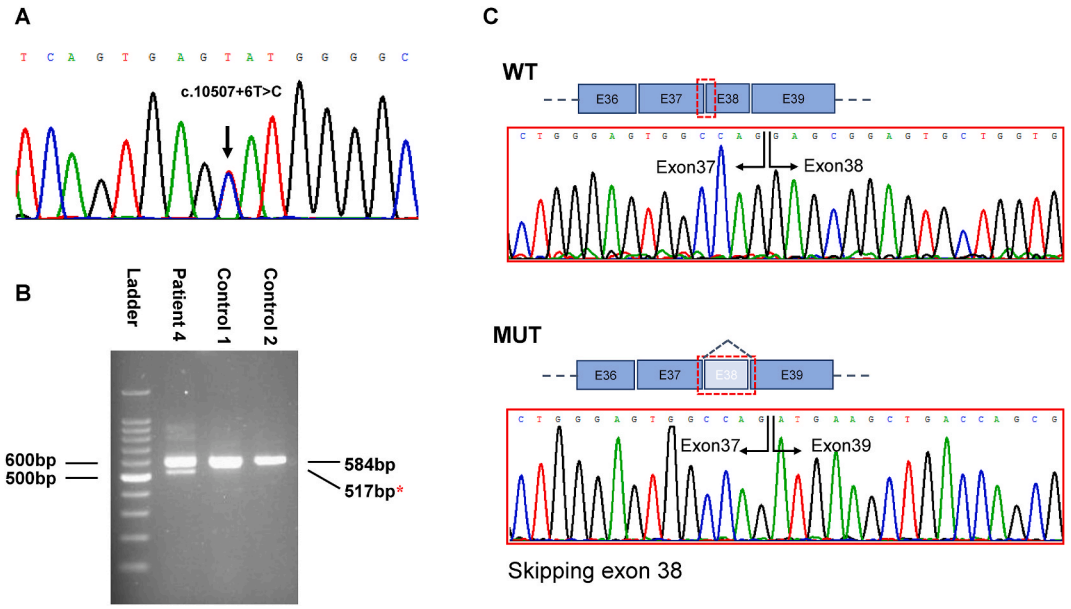

Fig. 1. Results of Genomic DNA and cDNA Sequencing in Patient 4 (A) DNA sequencing results in the *KMT2D* gene. The mutation site is indicated by an arrow. (B) Reverse transcription PCR products for *KMT2D* mRNA transcripts from healthy and patient individuals were separated through 1.5% agarose gel electrophoresis, the aberrant mRNA fragment is indicated with a red asterisk. (C) The results of cDNA sequencing. The corresponding physical maps are annotated above the sequence diagrams. The positions of base sequences are marked with red dotted boxes. Skipped exon is indicated by lighter colored box. WT = wild-type sequence (584 bp); MUT = aberrant transcript (517 bp).

examination showed that short stature (−3.8 SD), large ears, facial freckles, thick and arched eyebrows, lateral eyebrows sparse, long palpebral, short philtrum, depressed nasal tip, high palate, oligodontia and abnormal incisors, wide gaps between incisors, restricted elbow valgus, brachydactyly and clinodactyly of fifth fingers, persistent fetal finger fat pads, narrow feet, brachydactyly of fifth toes, nail dysplasia, microphallus and microrchidia.

Via WES of the proband, we identified a novel heterozygous *KMT2D* variant, c.10507+6T > C, in the splice site of the intron 38, which was not detected in neither of his parents by Sanger sequencing (Fig. 1A). This splice site variant is absent in the general population, including dbSNP, the Genome Aggregation Database (gnomAD), and the 1000G database. According to the ACMG/AMP guidelines for the interpretation of sequence variants, the PVS1 evidence code cannot be applied for classification of the non-canonical splice site variant. However, the substitution would reduce the score of the authentic donor site (−48.19%) and activate a cryptic site 24 nt downstream of 5′ splice site, which was predicted by the varSEAK online tool. To analyze the effect of the variant on pre-mRNA splicing, extraction of mRNA from peripheral blood leukocytes of the proband was performed. Sanger sequencing of the patient's cDNA sample demonstrated the presence of a truncated messenger RNA transcript that skipped exon 38 (67bp), which causes a frameshift effect and triggers a nonsense-mediated mRNA decay (Fig. 1B–C). Based on the analysis of cDNA, the substitution (c.10507+6T > C) can be classified as pathogenic in accordance with the ACMG/AMP variant classification criteria guidelines (PVS1 + PS2 + PM2_supporting).

3.5. Patient 5

The patient was a one-month-old male infant, born at 38 + 4 weeks of pregnancy by caesarean section due to fetal distress, with birth weight of 4070 g and birth length of 50 cm. Apgar scores at 1, 5 and 10 min were all 10. The patient was hospitalized immediately after delivery due to apnea and neonatal pneumonia. The physical examination show that webbed neck, hypertelorism, depressed nasal tip, short and stubby fingers, short upper limb and hypotonia. During his hospital stay, neonatal hypoglycemia and hyperbilirubinemia were noted. Echocardiography showed interrupted aortic arch with ventricular septal defect, patent ductus arteriosus, tricuspid insufficiency, and patent foramen ovale. Holter monitoring showed ventricular arrhythmias. Magnetic resonance imaging of the brain showed bilateral ventricular enlargement and white matter signal abnormality. The child died one and a half months due to respiratory failure.

Through WES, we identified a heterozygous *KMT2D* variant, c.13606C > T (p.Arg4536Ter), in the neonatal patient. This nonsense variant has been reported in at least three patients with KS [8,9,21]. The identified variant was absent in both parents, and it was classified as being pathogenic according to ACMG/AMP guideline (PVS1 + PS2 + PS4_ supporting + PM2_supporting).

3.6. Patient 6

A 7-year-old boy, born to non-consanguineous healthy couple after 37 weeks of gestation. At birth, difficult breathing was noted and he was admitted to the Neonatal Intensive Care Unit. The boy suffered from language and motor retardation. He started walking around two years but he still manifested as easy falling when walking. He started to speak his first words at the age of two, and now he was able to communicate basic needs in short sentences. The boy also had a history of three febrile seizures. Medical examination showed that short stature, facial freckles, high palate, epicanthus, low-set ears, abnormality of the thorax, small hand. An abdominal ultrasound revealed unilateral renal duplication.

Using WES, a novel heterozygous *KMT2D* variant, c.8077C > T (p.Gln2693Ter), was identified in the male patient. The variant was absent in both parents, and it was classified as pathogenic according to ACMG/AMP guideline (PVS1 + PS2 + PM2_supporting).

3.7. Patient 7

A 9-year-old boy was born as second child of non-consanguineous healthy parents after an uneventful full-term pregnancy. He was admitted because of short stature. The boy was diagnosed with gastric volvulus in infancy and suffered from feeding problem. He started walking at 17 months of age. Physical examination revealed short stature, prominent forehead, arched eyebrows with lateral eyebrows sparse or notched, hypertelorism, epicanthus, depressed nasal tip, broad nasal root, large ears, oligodontia, hypoplasia of penis, persistent fetal finger fat pads and short fifth toe.

WES performed at 8 years of age identified a de novo heterozygous *KMT2D* variant, c.7744delG(p.Val2582fs*1). This frameshift variant can be classified as pathogenic in accordance with the recommendation of the ACMG/AMP guideline (PVS1+PS2+PM2_supporting).

3.8. Patient 8

An 8-year-old boy, born to a healthy and non-consanguineous couple following an uneventful full-term pregnancy. He had a 17-year-old healthy sister. The boy was admitted to our hospital with a complaint of short stature. According to his mother, except for raising his head, his developmental milestones of turning over, sitting up, crawling, and walking were achieved at roughly normal times. He had trouble sleeping and poor appetite. His academic performance was below average. Physical examination revealed short stature (118.5 cm, -3SD), poor eye contact, facial nevus, long palpebral fissures, long eyelashes, euryblepharon, large ears, cleft lower lip, agenesis of central incisor, short small finger, curved phalanges of the 5th finger, abnormal dermatoglyphics, finger joint laxity and fifth toenail dysplasia.

Via WES of the proband, we identified a heterozygous *KMT2D* variant, c.16273G > A (p.Glu5425Lys), which was not detected in her mother, but genetic testing was not completed on the father since his father had expired several years. The substitution has been reported in at least seven patients with KS [21-26-f] and it was classified as likely pathogenic, according to the ACMG/AMP guidelines (PS4_moderate + PM2_supporting + PP2+PP3_moderate).

3.9. Patient 9

A 7-year-old boy was the second child born to a healthy and non-consanguineous couple. He was born with a soft cleft palate. He was diagnosed with morning glory optic disc and left side hearing loss at five months. The boy was admitted to hospital for developmental delay. According to his parents, he exhibited severe motor retardation and walked unaided at the age of 4 years. He also suffered from recurring otitis media. Physical examination showed that short stature, hypotonia, arched eyebrows with sparseness of the lateral third of the brow, long eyelashes, broad nasal root, depressed nasal tip, large cup-shaped ears, short philtrum. The craniocerebral MRI showed no obvious abnormality.

A de novo heterozygous *KMT2D* variant, c.12688C > T(p.Gln4230Ter), was identified in the child by WES. This nonsense variant has been reported [13] and it was classified as pathogenic according to the recommendation of the ACMG/AMP guideline (PVS1 + PS2 + PS4_supporting + PM2_supporting).

4. Discussion

The *KMT2D* gene (NM_003482.4) consists of 54 coding exons (NM_003482.4) that encode a 5537 amino-acid protein, which includes a PHD-zinc finger, PHD-finger, HMG-box, two F/Y-rich regions and a SET domain. The protein is a histone methyltransferase to methylate the Lys-4 position of histone H3, which is an epigenetic marker of transcriptionally active genes [27,28]. The *KMT2D* gene is expressed in most cells and tissues and is critically involved in the regulation of development, differentiation, metabolism and tumor suppression [29]. Somatic mutations in *KMT2D* are associated with a wide variety of cancers [30] and germline *KMT2D* mutations are the major genetic cause of KS.

The *KMT2D* gene is intolerant to variation, with high probability of loss-of-function intolerance (pLI score = 1) and missense variant intolerance (Z score = 3.71) in gnomAD. Loss of *KMT2D* function inhibits the oxygen-responsive gene programs that is essential for neural progenitor maintenance, ultimately causing precocious neuronal differentiation [31]. And Lehman et al. reported that

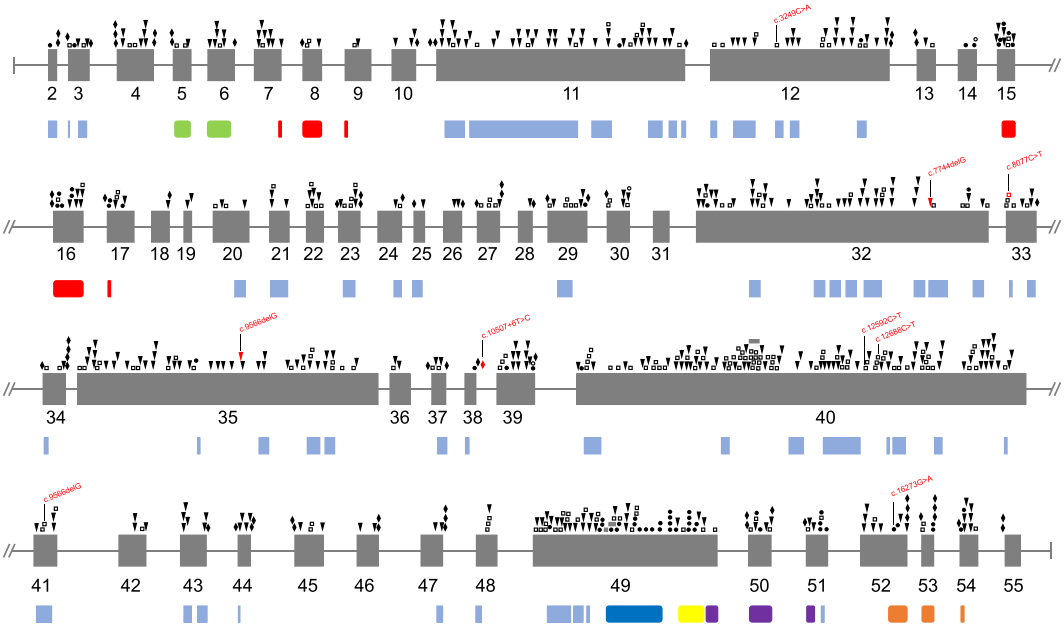

Fig. 2. The spectrum of *KMT2D* mutations. The schematic map (gray boxes denote exons and exon numbers are written under the boxes) of the genomic structure of the *KMT2D* gene are drawn in scale. The corresponding encoded protein domains are indicated below the exons by colored boxes (light blue, compositionally biased region or short sequence motif; light green, PHD-like zinc-binding domain; red, PHD-finger; dark blue, PHD-zinc-finger like domain; yellow, FYRN; purple, FYRC; orange, SET). Pathogenic or likely pathogenic variants are labelled above the schematic map by mutation category (solid dot, missense; diamond, splicing; hollow square, nonsense; triangle, frameshift; gray strip, in-frame deletion/duplication; hollow dot, synonymous). The novel variants in this study are indicated red symbols.

patients with whole-gene deletions of *KMT2D* or pathogenic truncation that occur in the first half of the gene may present with more severe intellectual disability [32]. Currently, in addition to structural variants and multiple exon deletions, 701 pathogenic and likely pathogenic variants of the *KMT2D* gene have been recorded in the ClinVar database (Table S2). These variants are distributed throughout almost all coding exons of the *KMT2D* gene, with exception of exons 31 and 55 (Fig. 2). Most variants are loss-of-function variants (89.87%) and include 346 frameshift variants (49.36%), 211 nonsense variants (30.10%) and 73 splice site variants (10.41%). And nearly a quarter of truncating mutations (24.2%, 136 in 563) occur in exon 40, which codes for 16.8% of KMT2D protein.

In general, missense variants perturb the secondary structure of the KMT2D protein and cause reduced histone methylation levels [24]. They may lead to a novel multiple-malformation syndrome which is noticeably distinct from KS [33]. Furthermore, a potential gain-of-function mechanism (56-amino-acid region flanked by Leu3525 and Lys3583) has been hypothesized for this differential phenotype [33,34]. In the ClinVar database, more than one third of the missense variants (25/65) and two in-frame deletion/duplication variants (2/3) are found in exon 49. This may be because exon 49 encodes multiple functional domains and conserved motifs/residues (Fig. 2). All three synonymous substitutions are located in the last nucleotide of exons 14, 30 and 39 and the pathogenicity of these variants may be related to aberrant splicing. In this study, nine *KMT2D* variants were identified, of which four were novel, and eight arose de novo (Table 1). The loss-of-function variants accounted for 88.89% of variants in our cohort, which was consistent with the ratio in the ClinVar database.

To date, 322 splice -site variants of the *KMT2D* gene have been documented in the ClinVar database (Table S3). Of the 69 canonical $\pm$ 2 splice -site variants, 63 (91.30%) have been recorded as pathogenic or likely pathogenic, whilst six have been recorded as variants of uncertain significance (VUS). Of the 253 non-canonical splice-site variants, only 10 (3.95%) have been recorded as pathogenic or likely pathogenic. After filtering out the intronic variants that are more than 10 bp away from exon-intron boundaries, 97 variants remain. These include eight pathogenic or likely pathogenic variants (8.25%) and 39 VUS (40.21%). For non-canonical splice -site variants that lack *in vitro* or *in vivo* experimental data, it is difficult to conclusively determine whether these alterations are benign or disease-causing. In this study, the splice -site transition was considered to be a pathogenic variant, by RNA analysis. The results showed that efficient mRNA analysis, using biological material from patients, is helpful for pathogenicity classification of the non -canonical splice-site variants.

The *KMT2D* gene plays a critical role in early vertebrate development and its decreased activity leads to craniofacial, cardiac and brain abnormalities, which are associated with the KS phenotype [13]. Margaret et al. analyzed the clinical information from 399 patients who harbored pathogenic *KMT2D* variants. They found that the most common manifestations were intellectual disability, fetal

fingertip pads and congenital heart defects, which occurred in more than half of the patients [5]. Intellectual disability, as one of cardinal features of KS, is associated with a deficiency of the dentate gyrus granule cell layer, and deficits in neurogenesis and hippocampal memory. These features are caused by impaired histone acetylation and methylation of *KMT2D* [12]. In our cohort, five of seven patients presented with intellectual disability but only two of seven patients had fetal fingertip pads, and one presented with ventricular septal defect. In addition, the decease infant (case 5) presented with a ventricular septal defect, interrupted aortic arch, patent ductus arteriosus, tricuspid valve closure, and arrhythmia. The fetus (case 3) presented with a permanent superior vena cava and single umbilical artery. It has been reported that up to 80% of KS patients with *KMT2D* variants have congenital heart disease. We suspect that some KS children with congenital heart disease remain undiagnosed in our region. Thus, for KS children, clinical assessment, electrocardiogram and echocardiography should be performed at the time of diagnosis. Appropriate treatment and recovery from cardiac disease would improve their quality of life [35].

The most common clinical phenotypes observed in our patients were motor delay (7/7) and short stature (7/7) but the former was not common in the literature [5]. Shangguan et al. evaluated the phenotypic spectra of 47 unrelated Chinese KS patients and found that the vast majority (80.4%) presented with intellectual disability. More than half of the patients (57.4%) had short stature. However, motor delay has not been described as a common clinical feature [25]. Motor delay in KS patients is mainly related to infantile hypotonia and attention deficit [36]. Patients often manifest short stature due to poor feeding and nutrition, growth hormone (GH) deficiency and disrupted endochondral ossification of the long bone growth plates [37,38]. Children with KS can benefit from an endocrinological evaluation and GH treatment [3]. Furthermore, due to the frequent association with congenital heart malformations and gastrointestinal abnormalities, neonatal care and infection control are very important for infants with KS. For such children, infections can potentially be fatal. The feeding problems generally abate with increasing age but a certain percentage of patients experienced excess weight or even obesity. Proper feeding and nutritional status are crucial for improving quality of life, immune reconstitution and reducing the risk of infection [39]. In summary, we recommend that in patients with motor delay, short stature, intellectual disability, language disorder and facial deformities, KS should be strongly considered. The prevalence of recurrent otitis media (5/7) was found to be significantly higher in our cohort than in the previous report (30.0% and 27.6%, respectively) [18] and this feature can also be considered as a distinctive characteristic used to help support the clinical diagnosis of KS. Early reports also described a high prevalence of otitis media in children with KS (55% [Niikawa et al., 1988], 92% [Peterson-Falzone et al., 1997], 72% [Kawame et al., 1999], 86%[Lin et al., 2015]) [7,23,41,42]. But with the earlier diagnosis and subsequent treatment, otitis media have become less common in KS patients. Nevertheless, as a major cause of acquired hearing impairment, otitis media still requires special attention for a KS diagnosis. External ear dysmorphism and inner ear malformation increase the risk of otitis media in KS patients. Recurrent otitis media, caused by *KMT2D* mutations, and antibiotic therapy may also be associated with B-cell developmental defects and impaired lymphopoiesis. Ear abnormalities, combined with hearing loss, orofacial anomalies and altered dentition, may also lead to speech impediment [23,40,41]. In our study, four out of seven patients exhibited speech impediment.

It has previously been reported that KS patients with *KDM6A* variants have an increased risk of hyperinsulinism, when compared to those with *KMT2D* pathogenic variants. Hyperinsulinism is associated with the deregulation of B-cell development due to KDM6A demethylation of the H3K27 protein [43,44]. In our cohort, two patients with *KMT2D* variants presented with hyperinsulinism and neonatal or infantile hypoglycemia. This suggested that hyper-insulinemic hypoglycemia may be a common genetic event in all KS patients, not just individuals with *KDM6A* variants. This needs to be explored further in future research. Although severe visual impairments are rare, individuals with KS tend to present with ocular abnormalities [36]. One of our patients was diagnosed with morning glory optic disc abnormality. A careful ophthalmologic and systemic evaluation is important for KS children, to alleviate treatable visual impairment as early as possible.

There are several syndromes and genes associated with developmental delays and facial deformities. These included Rett syndrome, CHARGE syndrome, Fragile X syndrome, Prader-Willi and Angelman syndrome [45–47]. To propose diagnostic criteria for KS, Makrythanasis et al. developed a phenotypic scoring system [18]. According to their proposed scoring system, our patients had low scores, with an average of 3.6 (range: 2–5, Table 1). The results indicated that the clinical manifestations of KS vary greatly among individuals, which highlights the importance of molecular testing for genetic diagnosis of KS, particularly when clinical diagnosis is equivocal. However, the genetic basis of approximately 20% of KS patients remains unknown and further investigation is needed [10,11]. The molecular diagnosis of patients with suspected KS by Sanger sequencing is also challenging due to the large size of the known causative genes (*KMT2D* and *KDM6A*). Hence, whole -exome sequencing is a cost-effective method for the analysis of a wide range of genetic disorders with clinical variability. For VUS, family verifications and functional tests are very important for evaluation of the pathogenicity. Prenatal whole -exome sequencing is a good option for fetal diagnosis of neurodevelopmental disorders and prenatal genetic testing is of great significance for the prevention of birth defects and developmental disabilities. For infants with neurodevelopmental disorders, the most important finding is that early diagnosis and interventions are especially beneficial for both patients and their family [48].

In conclusion, we described the clinical features of nine sporadic KS patients with considerable phenotypic heterogeneity. We highlighted the importance of motor delay and recurrent otitis media in the clinical diagnosis of KS. We identified nine *KMT2D* variants, which included a non-canonical splice site variant whose aberrant splicing was verified by RNA analysis. Our findings contributed additional molecular evidence and expanded the phenotypic spectrum of KS. This will help us to better understand the genotype-phenotype correlations and provide accurate genetic counseling and optimal treatment.

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Author contribution statement

Sheng Yi：;Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Xiaofei Zhang, Jingjing Huang, Jiale Qian, Pingshan Pan：;Conceived and designed the experiments.

Qi Yang, Shang Yi, Shujie Zhang, Qiang Zhang：;Analyzed and interpreted the data.

Xunzhao Zhou：Performed the experiments; Analyzed and interpreted the data.

Xianglian Tang: Performed the experiments; Wrote the paper.

Limei Huang：;Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Qinle Zhang：;Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Zailong Qin：;Analyzed and interpreted the data; Wrote the paper.

Jingsi Luo：;Conceived and designed the experiments; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20223.

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