Whole Exome Sequencing Aids The Diagnosis of Fetal Skeletal Dysplasia

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Abstract

Background: Skeletal dysplasia is a complex group of bone and cartilage disorders with strong clinical and genetical heterogeneity. Several types have prenatal phenotypes. And it is difficult to make a molecular diagnosis rapidly due to lacking family history and non-specific and limited clinical symptoms in utero. This study aims to diagnose 16 Chinese fetuses with skeletal dysplasia.

Methods: Single nucleotide polymorphism-array (SNP-array) was performed in 12 of 16 samples. If no microdeletions or microreplications related to skeletal dysplasia were detected, whole-exome sequencing (WES) was adopted. And the last four cases only got whole-exome sequencing for analyzing copy number variants and single nucleotide variations at the same time.

Results: Among the 16 cases, 12 patients received definitive diagnosis and we detected one deletion in DMD gene by SNP-array and 15 variants of 6 genes including FGFR3, COL1A1, COL1A2, ALPL, HSPG2 and DYNC2H1. 8 variants of COL1A1, COL1A2, ALPL and HSPG2 are novel. And somatic mosaicism in asymptomatic parent with mutations in COL1A1 or COL1A2 was observed.

Conclusions: In general, our study expanded the prenatal phenotypes in Duchenne muscular dystrophy (DMD)/Becker muscular dystrophy (BMD), found 8 novel variants and elucidated that the utilization of whole-exome sequencing improved the diagnosis yield of skeletal dysplasia and provided useful genetic counseling guidance for parents.

Background

Unexpected skeletal dysplasia affects approximately 1 per 5,000 and is a complex group of bone and cartilage disorders with strong clinical and genetical heterogeneity [1]. In the 2015 revision of the Nosology and Classification of Genetic Skeletal Disorders, 436 disorders are classified into 42 groups according to syndromes, publication, genetic information and nosologic autonomy and 364 different genes are associated with genetic skeletal disorders [2].

Many of these disorders can give rise to prenatal phenotypes. In the past, ultrasound evaluation is a widely used method for detection of congenital anomalies [3]. However, a lack of family history and non-specific and **limited** clinical symptoms in **utero** introduce difficulties in prenatal diagnosis. Recently, with the advances in molecular technology especially next-generation sequencing, high-throughput sequencing has been utilized in prenatal genetic diagnosis. Thirty-one studies published prenatal analysis by WES with the diagnostic rates between 6.2% and 80% [4]. **Notably**, the **application** of targeted exome sequencing in prenatal diagnosis of skeletal dysplasia is **outstanding** as several researches have reported high detection rates from 75% to 83.3% [5-8]. Definitive molecular diagnosis can provide accurate results instead of a suspected clinical impression and information about **subsequent** development of the disease and **treatmentregimens**, thus parents get **consultation** and birth defect intervention could be **implemented**.

In this study, we analyzed 16 cases of fetuses with suspected skeletal dysplasia by WES and aimed to elucidate next-generation sequencing as a useful and efficient aid to precise diagnosis that help **geneticcounseling** and risk assessment for future pregnancies.

Methods

Patients

16 affected patients and available family members were recruited by the Affiliated Suzhou Hospital of Nanjing Medical University with informed consent. The study was approved by the institutional ethics committee. All fetuses were diagnosed with suspected skeletal abnormalities by prenatal ultrasound. Their clinical symptoms were summarized in Table 1. We obtained fetal muscle tissue or cord blood and parents' peripheral blood with the exception of case 9 and 10 that we only got the samples from patients. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to standard extraction methods.

SNP-array

SNP-array analysis was performed on the Affymetrix CytoScan platform (Affymetrix, Santa Clara, CA, USA) following the protocol. After amplified, DNA was hybridized to the Affymetrix 750K array containing 550,000 copy number variation (CNV) markers and 200,000 SNP markers. Data were analyzed by Chromosome Analysis Suite 3.2 (Affymetrix, Santa Clara, CA, USA). CNVs were analyzed and classified by American College of Medical Genetics and Genomics guidelines [9].

Whole-exome sequencing and mutation analysis

If microdeletions or microduplications related to skeletal dysplasia were not detected, whole-exome sequencing was adopted and DNA of the fetus and parents were used to create the DNA libraries and enriched by xGen Exome research panel v1.0 (Integrated DNA Technologies, Coralville, IA, United States) and were sequenced on the Illumina HiSeq 4000 (Illumina, San Diego, CA, United States). After mapped to the human genome (NCBI37/hg19), the sequencing data were filtrated, analyzed and compared to databases including Exome Aggregation Consortium (ExAC), dbSNP, 1000 Genome Project, Human Gene Mutation Database (HGMD), ClinVar and Leiden Open Variation Database (LOVD). Variants were classified according to American College of Medical Genetics and Genomics recommended guidelines [10].

Verification of gene mutations

Candidate variants would be reviewed by clinicians, genetic technicians and ultrasound experts. If microdeletions or microduplications were detected, these would be verified by multiplex ligation-dependent probe amplification (MLPA) in patients and parents. And if mutations were detected for a single gene, Sanger sequencing would be conducted to validate the mutations.

Table 1

Summary of clinical findings and molecular diagnose. It should be placed in the 76th line.

case	Family history	gestation	Ultrasound findings		Molecular result	
			Skeletal anomalies	Other anomalies	Variant	Inheritance
1	One previous pregnancies with the same skeletal anomalies	24	Feet were ballet-shaped, performed excessive plantarflexion and the angle of tibiofibula and dorsum of foot was near 180°	Polyhydramnios	arr[hg19] Xp21.1(31,690,978 – 31,875,673)x0	maternal
2	Previous pregnancy with short long bones and narrow thorax	25	Narrow thorax TH = 118 mm, short long bones FL = 23 mm HL = 19 mm, polydactylia	Renal sinus separation	<i>DYNC2H1</i> NM_001080463 c.5984C > T and c.10606C > T	AR Biparental inheritance
3		26	Short and curved long bones, FL = 34 mm HL = 36 mm, curved cubitus		<i>ALPL</i> NM_000478.5 c.984_986del and c.1463C > G	AR Biparental inheritance
4		22	Short and curved long bones	Cloverleaf skull, enteric canal echo enhancement	<i>ALPL</i> NM_000478.5 c.978_980del and c.2T > C	AR Biparental inheritance
5		25	Narrow thorax, short long bones, FL = 24 mm HL = 17.6 mm, short rib		<i>FGFR3</i> NM_000142.4 c.742C > T	De novo

TH, Thoracic Circumference; FL, Femur length; HL, humerus length; NT, nuchal translucency; CTR, cardiothoracic ration; AD, autosomal dominant; AR, autosomal recessive; U, unknown

case	Family history	gestation	Ultrasound findings		Molecular result	
			Skeletal anomalies	Other anomalies	Variant	Inheritance
6		24 ⁺ 6W	Short long bones, FL = 29.4 mm HL = 17 mm		<i>COL1A2</i> NM_000089 c.2189G > T	De novo
7		14 ⁺ 3W	X-type lower limbs, upper limbs adductus, nasal bone length 2.2 mm	NT = 4 mm anasarca		
8	Two previous pregnancies with the same skeletal anomalies	19	Short long bones with fracture, short rib, wide orbital septum		<i>COL1A2</i> NM_000089 c.1764 + 3_1764 + 6del	maternal
9		25	Short long bones, lumbosacral portion bent			
10		24	Short limbs, micrognathia, spine misaligned		<i>HSPG2</i> NM_005529.6 c.8553del and c.12532 + 1G > T	U
11		31	Abnormal morphology of ulna			
12	Two previous pregnancies with the same skeletal anomalies	23	Short limbs, narrow thorax, bell-shaped chest	Anasarca CTR > 0.5 pleural effusion	<i>COL1A1</i> NM_000088.3 c.3389G > A	maternal
13		20	Short long bones with abnormal thorax	NT = 4.4 mm	<i>COL1A1</i> NM_000088.3 c.1921G > A	De novo

TH, Thoracic Circumference; FL, Femur length; HL, humerus length; NT, nuchal translucency; CTR, cardiothoracic ration; AD, autosomal dominant; AR, autosomal recessive; U, unknown

case	Family history	gestation	Ultrasound findings		Molecular result	
			Skeletal anomalies	Other anomalies	Variant	Inheritance
14		25	Short and curved long bones		<i>COL1A2</i> NM_000089 c.1010G > A	De novo
15		22	Short left humerus and curved ulna and radius			
16		U	Short long bones		<i>FGFR3</i> NM_000142.4 c.1138G > A	De novo
TH, Thoracic Circumference; FL, Femur length; HL, humerus length; NT, nuchal translucency; CTR, cardiothoracic ration; AD, autosomal dominant; AR, autosomal recessive; U, unknown						

Results

A total of 16 cases were investigated and all of them underwent a detailed ultrasound examination during pregnancy with suspected skeletal dysplasia. Several parameters have been visualized: biparietal diameter, head circumference, abdominal circumference, chest circumference, length of the long bones, shape of long bones, mandibular size and shape, abnormal posturing of the extremities and other congenital anomalies. In 16 cases, we detected one deletion in *DMD* and causative variants in 6 genes including *FGFR3*, *COL1A1*, *COL1A2*, *ALPL*, *HSPG2* and *DYNC2H1* with a detection rate of 75% (Table 1).

Abnormalities detected by SNP-array

12 cases were tested for CNVs by SNP-array. We found a 184kb deletion in chromosome Xp21.1 which spanned exon 49 to exon 53 of the *DMD* gene in case 1. At 24 weeks of gestation, the ultrasound test showed that the fetal feet were ballet-shaped, performed excessive plantar flexion and the angle of tibiofibula and dorsum of foot was near 180°. However, the fetus didn't accept prenatal genetic test. When he was born, we received cord blood to conduct SNP-array. Further analysis by MLPA verified that both he and his mother harbored this deletion (Figure 1a).

Abnormalities detected by WES

For other 15 cases, WES was used to find genetic etiology. Fortunately, 15 variants in 6 genes associated with skeletal dysplasia were detected in 10 cases confirmed by Sanger (Figure 1b). Patient 2 had compound heterozygous mutations in *DYNC2H1*: c.5984C>T (p.A1995V) and c.10606C>T (p.R3536X) which had been reported in 2018 [11]. Hypophosphatasia resulting from mutations in *ALPL* were found in case 3 (c.984_986del, c.1463C>G) and case 4 (c.2T>C, c.978_980del). *FGFR3* mutation c.742C>T was detected in case 5 and c.1138G>A was detected in case 16. Mutations in collagen genes were identified in five cases, of which case 6' c.2189G>T (p.G730V) in *COL1A2*, case 13' c.1921G>A (p.G641R) in *COL1A1* and case 14' c.1010G>A

(p.G337D) in *COL1A2* were de novo while c.1764+3_1764+6delAAGT in *COL1A2* in case 8 and c.3389G>A in *COL1A1* in case 12 were maternally inherited. In case 10, clinically significant mutations in *HSPG2* were found (c.8553del and c.12532+1G>T). However, no pathogenic variants were identified in case 7, 9, 11 and 15.

Discussion

Among the 16 cases in the present study, 12 cases received a definitive molecular diagnosis, including a microdeletion and 8 novel variants.

In case 1, a deletion of 184 kb fragment in the Xp21.1 segment of chromosome X was found in the infant and inherited from his mother. He exhibited abnormal posturing of the lower extremities which were not reported previously. To our knowledge, only one fetus was reported and exhibited fetal growth restriction and oligohydramnios [12]. In 2010, Murugan et al found an in-frame deletion of exon 49 to 53 detected by MLPA and mPCR [13]. According to frame shift hypothesis [14], it usually caused BMD. Unfortunately, our patient was dead and we could not get more information. Therefore, our patient suffered several new symptoms in intrauterine period and expanded the phenotype spectrum of DMD/BMD. In case 2, two compound heterozygous causative mutations in *DYNC2H1* gene were detected which was associated with short-rib thoracic dysplasia 3 [15]. Short rib-polydactyly syndrome 3 (SRPS 3) was an autosomal recessive disease overlapping with Jeune asphyxiating thoracic dystrophy belonging to the ciliopathy but it was more severe and characterized by early prenatal expression, lethality and variable malformations [16].

Moreover, we found mutations in *ALPL* in case 3 and 4. Pathogenic variants in *ALPL* cause hypophosphatasia characterized by defective mineralization of bone and/or teeth in the presence of low activity of serum and bone alkaline phosphatase (ALP) [17]. c.984_986del in case 3 was previously reported in 2012 and 2015 [18, 19], which result in the deletion of Leucine at the β -sheet and decreased activity of its coding protein the tissue-nonspecific isoenzymes of alkaline phosphatase (TNSALP) [20]. Thus, c.984_986del may reduce the enzymatic activity, too. c.1460C > T (p.A487V) and c.1466G > C (p.C489S) were reported early, of which the latter one exhibited a diminished ALP activity, less located on the cell surface and failed to become the mature form [21, 22]. This suggested that p. 487–489 may play an important role in enzymatic activity. c.2T > C caused the absence of the start codon and generated a transcript starting at Met56 as c.3G > A did which does not exhibit enzymatic activity, has no significant effect on the wild type ALPL protein and cannot be attached to the cell membrane [23].

Patient 5 harbored c.742C > T in *FGFR3* gene which has been detected in different racial types [24–27]. And patient 16 had a hot mutation c.1138G > A in *FGFR3*. These fetuses' ultrasound scan all revealed a narrow chest with shortening of the long bones. Besides, c.8553del and c.12532 + 1G > T in *HSPG2* was identified in case 10. *HSPG2* is an essential gene and its mutations could lead to Schwartz-Jampel syndrome, type 1 (SJS) and severe neonatal lethal Dyssegmental dysplasia, Silverman-Handmaker type (DDSH) [28, 29]. c.8553del produced a truncated protein that terminated at 2878 sites lacking part of domain 1 and the whole domain 2 and c.12532 + 1G > T at the C-terminal region may yield abnormal transcript as predicted by Human Splicing Finder. Unfortunately, we didn't get the parents' DNA so that the compound heterozygous condition couldn't be confirmed. Trio sequencing of patient 7 and the parents detected a heterozygous missense variation c.4813C > T in *FLNB* which was inherited from the normal father with 62 of 120 (51.7%) reads. Though several cases with

family history presenting an autosomal dominant trait has been reported [30, 31], the father in case 7 didn't have malformations associated with FLNB-Related Disorders like short stature, club feet and facial dysmorphisms [32].

Furthermore, variants related to type I collagen genes and caused osteogenesis imperfect (OI) were identified in 5 cases. Type I collagen is a heterotrimer, containing two $\alpha 1(I)$ and one $\alpha 2(I)$ chains assembling by procollagen chains with N-terminal and C-terminal globular propeptides flanking the helical domain [33]. The helical domains contain Gly-Xaa-Yaa triplets where glycine was substituted most frequently causing OI [34]. In our study, we detected 4 missense mutations and one splicing mutation variant: c.1921G > A and c.3389G > A in *COL 1A1* and c.1010G > A, c.2189G > T and c.1764 + 3_1764 + 6del in *COL 1A2* respectively (Fig. 2b). Four missense mutations were glycine substitutions of Gly-X-Y which would delay helical folding, prolonging access time for modifying enzymes. Former researches has described two infants with c.2188G > T and c.2188G > C in *COL 1A2* changing the same amino acid site with c.2189G > T and separately exhibiting the same deformities like blue sclera, wormian bones, shortening and bowing of the upper and lower limbs [35, 36]. The similar situation was occurred in c.1010G > A and c.1921G > A. And c.3389G > A in *COL 1A1* has been listed and defined as likely pathogenic variant in ClinVar. The splicing mutation c.1764 + 3_1764 + 6del in *COL 1A2* in case 8 may yield abnormal transcript as predicted by Human Splicing Finder. Notably, the variants in case 8 and 12 were maternally inherited. Both of the mothers experienced induced abortion in second trimester twice due to the same skeletal dysplasia malformation, suggesting the mosaicism. The mutated allele c.3389G > A was present in 17 of 66 (25.8%) reads in WES and the mutation-related signal was smaller than that of the wild-type allele in sanger sequencing which suggested that it is a mosaic mutation in the mother of case 12. After that, we tried to recall the asymptomatic mother, however, she didn't received radiographical examination and we only knew that her sclera and height were normal and didn't suffer bone fracture before. Similarly, the mutated allele c.1764 + 3_1764 + 6del was present in 22 of 68 (32.3%) reads in the mother of case 8 who presented extremely mild symptoms like short stature compared with the fetus. Nevertheless, the ratio of the fetus was 37.8% (17/45) similar to the mother. We supposed that other factors such as underlying genetic modifiers may affect the phenotypes.

Conclusion

In summary, we detected one copy number variation and 15 single nucleotide variants of 6 genes in 12 families with suspected skeletal abnormalities by prenatal ultrasound. The results of this study elucidated that the utilization of whole-exome sequencing improved the diagnosis yield of skeletal dysplasia and provided useful genetic counseling guidance for parents. At the same time, we expanded the prenatal phenotypes of DMD/BMD and also found two cases with type I collagen variants from asymptomatic parent. It disclosed the advantage of next generation sequencing in the detection of somatic mosaicism. Further studies will be performed to evaluate the application of prenatal whole-exome sequencing for skeletal dysplasia.

Abbreviations

SNP-array: Single nucleotide polymorphism-array

WES: whole-exome sequencing

DMD: Duchenne muscular dystrophy

BMD: Becker muscular dystrophy

CNV: copy number variation

ExAC: Exome Aggregation Consortium

HGMD: Human Gene Mutation Database

LOVD: Leiden Open Variation Database

MLPA: multiplex ligation-dependent probe amplification

SRPS 3: Short rib-polydactyly syndrome 3

ALP: alkaline phosphatase

TNSALP: tissue-nonspecific isoenzymes of alkaline phosphatase

SJS: Schwartz-Jampel syndrome, type 1

OI: osteogenesis imperfect

DDSH: Dyssegmental dysplasia, Silverman-Handmaker type

Declarations

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TH collected and analyzed the data and drafted the manuscript; YLL provided clinical information; TH, XJJ carried out the molecular analyses. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was in accordance with the tenets of the Declaration of Helsinki and was approved by the Affiliated Suzhou Hospital of Nanjing Medical University ethics committee.

Consent for publication

Patient's parents have written informed consent.

Competing interests

There is no conflict of interest.

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Figures

Figure 1

Genetic detection of some cases: a The MLPA results of case 1 indicated the deletion of exon 49 to exon 53 in DMD gene. b Sanger sequencing testified mutations of some cases with OI. Variants were indicated by black arrows.

Figure 2

Ultrasound pictures of case 4, 6, 8 and 10: a Short femur and cloverleaf skull of P4. b Short femur of P6. c Short long bones and wide orbital septum of P8. d Short limbs, micrognathia and spine misaligned of P10.