

Research article

Application of whole exome sequencing in fetal cases with skeletal abnormalities

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ABSTRACT

Objectives: To investigate the role of whole exome sequencing (WES) technology in fetuses with skeletal abnormalities (SKA) for establishing an appropriate clinical diagnosis and treatment path.

Methods: From April 2019 to August 2020, eight special families were enrolled into the study. Their fetuses showed abnormal SKA by ultrasonic testing during pregnancy, but it is inconsistent with the normal results identified by chromosomal microarray analysis (CMA) of amniotic fluid or abortion. For further diagnosis, WES was performed to detect the causative genes mutations followed by Sanger sequencing.

Results: Among of these eight fetuses with SKA, we found more than half of pathogenic mutations were in COL1A1/2 gene, except for a known hotspot mutation in FGFR3 gene (c.1138G>A). Three heterozygous mutations of COL1A1 gene, c.2885G>A p (Gly962Asp), c.994G>A p (Gly332Arg) and c.1002 + 5G>T, were *de novo* mutations. The c.1002 + 5G>T mutation in COL1A1 was firstly reported. In addition, one fetus carried a novel heterozygous mutation of COL1A1 c.644G>A p (Gly215Asp), which was inherited from the mother. Another novel heterozygous mutation c.2482G>T p (Val828Phe) in the COL1A2 gene was identified in another fetus and was inherited from the father. Among of these COL1A1 mutations, these results might involve in two novel splicing mutations.

Conclusion: Our study reported several novel heterozygous mutations which expands the COL1A1/2 mutation spectrum for prenatal diagnosis of SKA. Most importantly, WES technology is necessary as a routine step of the SKA diagnosis before or during pregnancy, combining with the detection of chromosome level.

1. Introduction

Skeletal abnormalities are common birth defects, with an incidence of 0.023–0.076% (Barbosa-Buck et al., 2012; Duarte et al., 2019; Li et al., 2020; Rasmussen et al., 1996; Schramm and Mommsen, 2018; Stoll et al., 1989). The clinical manifestations of SKA showed variable phenotypes, ranging from mild types to severe types, which resulted from strong genetic heterogeneity. Osteogenesis imperfecta and achondroplasia caused by mutations in COL1A1 or COL1A2 and FGFR3 gene, respectively, are major types of skeletal abnormalities. These causative mutations mostly are inherited in an autosomal dominant pattern. Up to now, many mutations in the COL1A1 and COL1A2 genes have been identified in different populations, but that is dependent on peripheral blood extracted from patients (Ho Duy et al., 2016; Zhang et al., 2012). These collected mutations cannot be completely applied in genetic counseling and prenatal diagnosis, which based on maternal peripheral blood.

Ultrasound examination could be able to assess whether the fetus at risk had karyotype changes or large genomic structural variants, but it still faced challenges and difficulties in better understanding of pathogenesis and the phenotype-genotype correlation. Some point mutations and small deletions or duplications within specific genes were increasingly reported and also caused severe phenotype, even lethality than had been previously thought. However, the rapidly development of next generation sequencing (NGS) technologies gave us an opportunity to provide the early genetic screening and genetic counseling during pregnancy or pre-pregnancy. And many researches showed disorders associated with specific mutations identified by whole exome sequencing could explain the results of ultrasound evaluations, including skeletal disorders. Therefore, we applied WES in eight amniotic fluid or abortion samples with normal results by CMA, but showed skeletal abnormalities by ultrasonic testing.

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2. Material and methods

2.1. Sample collection

The study was approved by the institutional ethics committee of Ningbo Women & Children's Hospital. In this study, we recruited eight pregnant mothers whose ultrasound showed obvious fetal skeletal abnormalities at our fetal medicine center from April 2019 to August 2020. All families hoped to clarify the genetic factors causing fetal skeletal abnormalities and required birth-defect intervention. Parental consent was obtained for collecting aborted fetuses and amniotic fluid samples. Peripheral bloods also were collected from proband's parents.

2.2. Whole exome sequencing

Genomic DNA was extracted from the peripheral blood and samples from abortion or amniotic fluid using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's procedures. First, we analyzed fetal chromosomal abnormalities in recruited eight families by CMA, but there were no variations in them were identified, unfortunately. Then, we applied whole exome sequencing to elucidate the causations of fetal skeletal abnormalities. Depending on the cost and family selection, trio-WES (proband and parents) was applied for case 1, 2, 3 and 5 and proband-only WES was performed in case 4, 6, 7 and 8 to detect the causative genes mutations. WES was applied using the Agilent SureSelect XT Human All Exon V6 kit (Agilent Technologies, USA) and Illumina HiSeq 2500 System (Illumina, USA). Data analysis was performed using NextGENe (SoftGenetics LLC, USA) and candidate variants were screened by Ingenuity Variant Analysis (Ingenuity Systems, USA) (<https://variants.ingenuity.com>).

2.3. Sanger sequencing

The candidate causative mutations discovered via whole exome sequencing of probands were confirmed by Sanger sequencing. The sequence covered identified mutations of *COL1A1* gene, including the exon-intron boundaries, were amplified by polymerase chain reaction (PCR) using Faststart Taq DNA polymerase (Roche, Switzerland). The amplified DNA fragments were purified and sequenced in both directions using ABI 3130 Genetic Analyzer. The sequencing data were compared with the reference sequence of *COL1A1* (NM_000088.3) in the NCBI database.

3. Results

3.1. Clinical features

Eight families were referred to the Ningbo Women & Children's Hospital due to fetuses with skeletal abnormalities by ultrasonic testing (Figure 1, Table 1). Most of probands' parents were in normal physical condition, except for case 1. The mother in this family was found to be blue sclerae, short stature (132 cm), abnormal joint development in hands and fractures were happened more common after birth. In this pregnancy, a fetal skeletal abnormality was suspected because the femur of the fetus was slightly angled on both sides, while she had a history of healthy pregnancy and gave birth to a normal male child. Three other families, case 2–3 and 5, also had a normal child birth, as in case 1. Case 2, ultrasonic scan found the long bones of fetal four limbs are curved into angles and the fetus presented poor ossification of the skull and vertebrae and hypertelorism at 24 weeks and 5 days' gestation. Case 3, at 20 weeks and 6 days' gestation, the fetus presented abnormality of calvarial morphology, a strawberry-shaped skull, limb undergrowth, short long

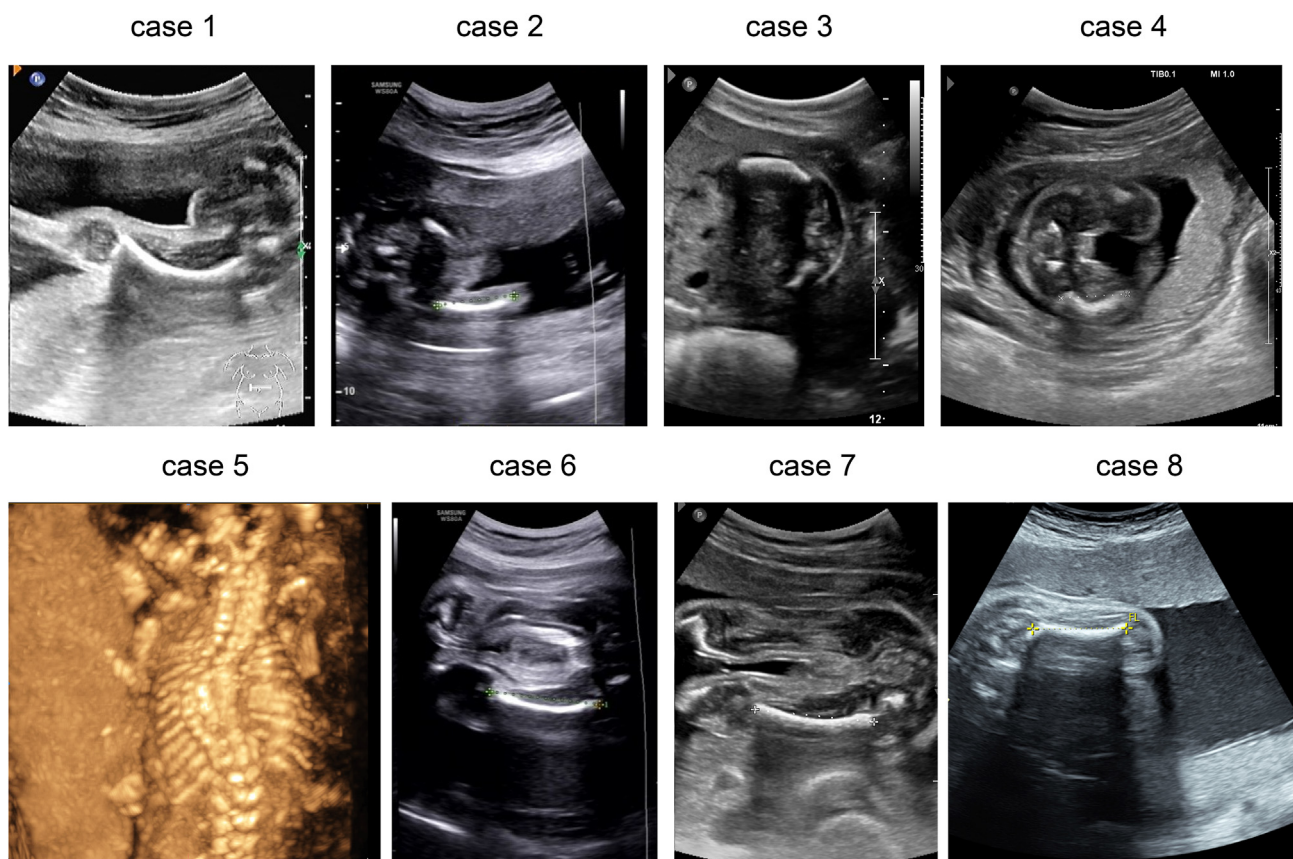


Figure 1. Fetuses with skeletal abnormalities. Ultrasound scans of eight fetuses showed abnormal skeletal phenotypes, such as short long bones or bowing of the long bones.

Table 1. Summary of ultrasound and WES results in eight fetuses.

Case No.	Weeks of pregnancy	Ultrasound information	WES results		Pathogenicity	Sample	The age/phenotype of the parents		Inheritance
			Chromosome position	Genetic mutation			Mother	Father	
1	22	Light spot in heart of fetus, mild bilateral femoral bowing	chr17:48275145	<i>COL1A1</i> c.644G>A, p (Gly215Asp)	Pathogenic (PVS1+PM2+PM5+PP2+PP3)	Amniotic fluid	33/Blue sclerae, short stature, recurrent fractures, genu valgum, skeletal dysplasia	41/Normal	Mother
2	24 ⁺⁵	Bowing of the long bones, poor ossification of the skull and vertebrae, hypertelorism	chr17:48266581	<i>COL1A1</i> c.2885G>A, p (Gly962Asp)	Likely pathogenic (PS2+PM2+PP3)	Amniotic fluid	36/Normal	36/Normal	de novo
3	20 ⁺⁶	Abnormality of calvarial morphology, short long bone, limb undergrowth, bowing of the long bones, fractures of the long bones	chr17:48273524	<i>COL1A1</i> c.994G>A, p (Gly332Arg)	Pathogenic (PS2+PM1+PM2+PP3+PP5)	Aborted fetus	36/Normal	38/Normal	de novo
			chr1:22181841	<i>HSPG2</i> c.5953G>A, p (Ala1985Thr)	VUS (PM1+PM2)				
4	20	Short long bones, bowing of the long bones	chr17:48273511	<i>COL1A1</i> c.1002+5G>T	Likely pathogenic (PS2+PM2+PP3)	Aborted fetus	22/Normal	24/Normal	de novo
			chr1:22204738	<i>HSPG2</i> c.2626A>G, p (Ile876Val)	VUS (PM1+PM2+BP4)				
5	22 ⁺⁴	Abnormality of the thoracic spine, intervertebral space narrowing, Abnormality of the ribs	chr7:94052347	<i>COL1A2</i> c.2482G>T, p (Val828Phe)	VUS (PM2+ PP3)	Aborted fetus	36/Normal	39/Normal	Father
6	31 ⁺⁶	Proximal limb shortening	chr4: 1806119	<i>FGFR3</i> c.1138G>A, p (Gly380Arg)	Pathogenic (PS2+PS4+PM2)	Aborted fetus	40/Normal	42/Normal	de novo
7	25	Short femur, polyhydramnios	chr4: 1806119	<i>FGFR3</i> c.1138G>A, p (Gly380Arg)	Pathogenic (PS2+PS4+PM2)	Amniotic fluid	37/Normal	37/Normal	de novo
8	25 ⁺⁴	Short long bones	chr4: 1806119	<i>FGFR3</i> c.1138G>A, p (Gly380Arg)	Pathogenic (PS2+PS4+PM2)	Amniotic fluid	28/Normal	39/Normal	de novo

bone, bowing of long bones and fractures of the long bones. Case 5, at 22 weeks and 4 days' gestation, the fetus presented abnormal the thoracic spine and ribs and intervertebral space narrowing. This mother had been pregnant twice and had an abortion in one pregnancy and a healthy child in another. In other cases (cases 4, 6–8), the major characteristic of fetuses was markedly short long bones.

3.2. Genetic analysis

Using whole exome sequencing, we identified two main causative genes, *COL1A1/2* and *FGFR3*, which had been reported were correlated to skeletal abnormalities. These two genes classified our cases into two groups (Table 1). A heterozygous mutation c.1138G>A p (Gly380Arg) in

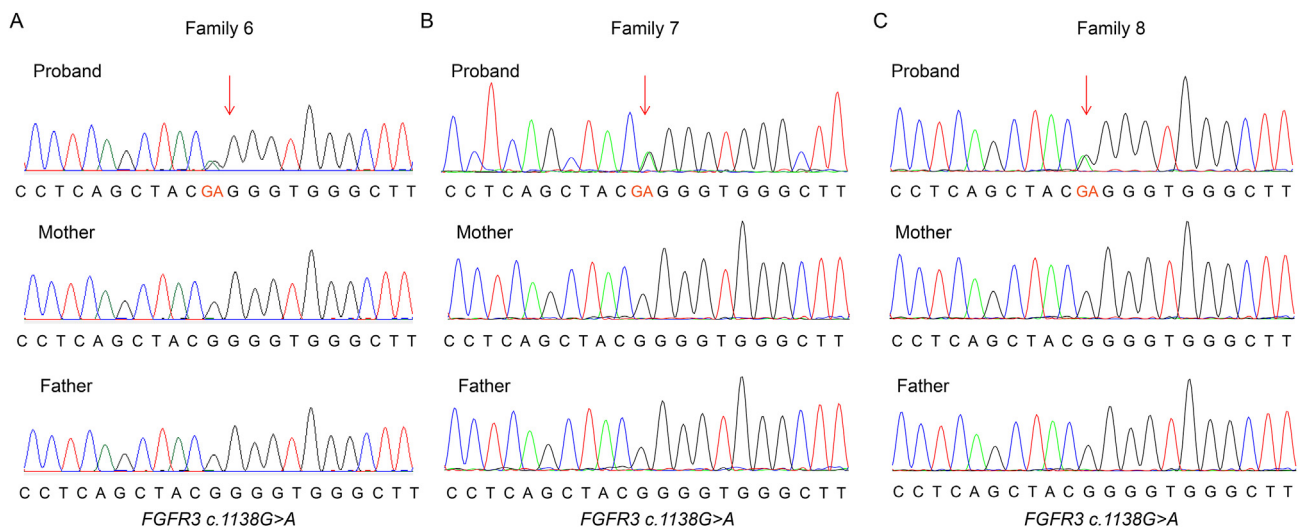


Figure 2. The Sanger verification of the c.1138G>A p (Gly380Arg) mutation in the *FGFR3* gene. The Sanger sequencing revealed that this mutation was de novo, and was not carried by the parents in family 6 (A), family 7 (B), and family 8 (C).

the exon 8 of *FGFR3* gene was found in three fetuses, case 6–8, that is the most common alternation identified in individuals with achondroplasia (Accogli et al., 2015; Bessenyi et al., 2013; Georgoulis et al., 2011; Rousseau et al., 1994; Xue et al., 2014). The mutation in all of three fetuses was *de novo* by Sanger sequencing (Figure 2) and was predicted to be pathogenic according to the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015) based on the evidence chain (PS2+PS4+PM2).

We also identified novel heterogeneous mutations in *COL1A1* and *COL1A2* genes. Osteogenesis imperfecta caused by the *COL1A1/2* is an autosomal dominant disorder. Among causative *COL1A1* mutations, we not only identified two pathogenic substitution mutations of glycine within the Gly-X-Y triplet domain of the triple helix which were reported before, c.2885G>A p (Gly962Asp) and c.994G>A p (Gly332Arg), in case 2 and case 3, respectively. We also found a novel heterogeneous mutation in the *COL1A1* gene in case 4, c.1002 + 5G>T, which might involve in splicing regulation. These cases were sporadic. In case 5, a novel heterozygous mutation of c.2482G>T p (Val828Phe) in the *COL1A2* gene was identified in both the fetus and fetus's father by trio-based WES (Figure 3A). The result suggested the mutation of *COL1A2* gene was inherited from the proband's father, while it classified as a variant of uncertain significance, according to the ACMG guidelines based on the evidence of PM2 and PP3 (Richards et al., 2015).

c.644G>A p (Gly215Asp) in the *COL1A1* gene was the first reported in case 1. This mutation inherited from proband's mother was confirmed by trio-based WES (Figure 3B). The mutation site of the G nucleotide substituted by the T nucleotide is located 2 bases downstream of the splice site. Thus, *COL1A1* c.664G>T mutation was predicted to be pathogenic according to the ACMG guidelines (Richards et al., 2015) because of the evidence chain (PVS1+PM2+PM5+PP2+PP3). Another novel heterogeneous mutation in case 4, c.1002 + 5G>T, was identified in the intron 15 of *COL1A1* gene and also located at an exon-intron boundary region that might contribute to aberrant splicing. Subsequently, Sanger sequencing was used to verify the *COL1A1* c.1002 + 5G>T mutation in all family members (Figure 3C). The results showed the mutation in *COL1A1* gene was a *de novo* mutation due to confirmed in the fetus, but not detected in parents. According to the ACMG guidelines (Richards et al., 2015), the c.1002 + 5G>T mutation in *COL1A1* gene was classified as likely pathogenic based on the evidence chain (PS2+PM2+PP3).

4. Discussion

Markedly variable phenotypes of fetuses are associated with genomic imbalances during early pregnancy. CMA is a useful primary technique used to identify larger chromosomal abnormalities in the initial genetic

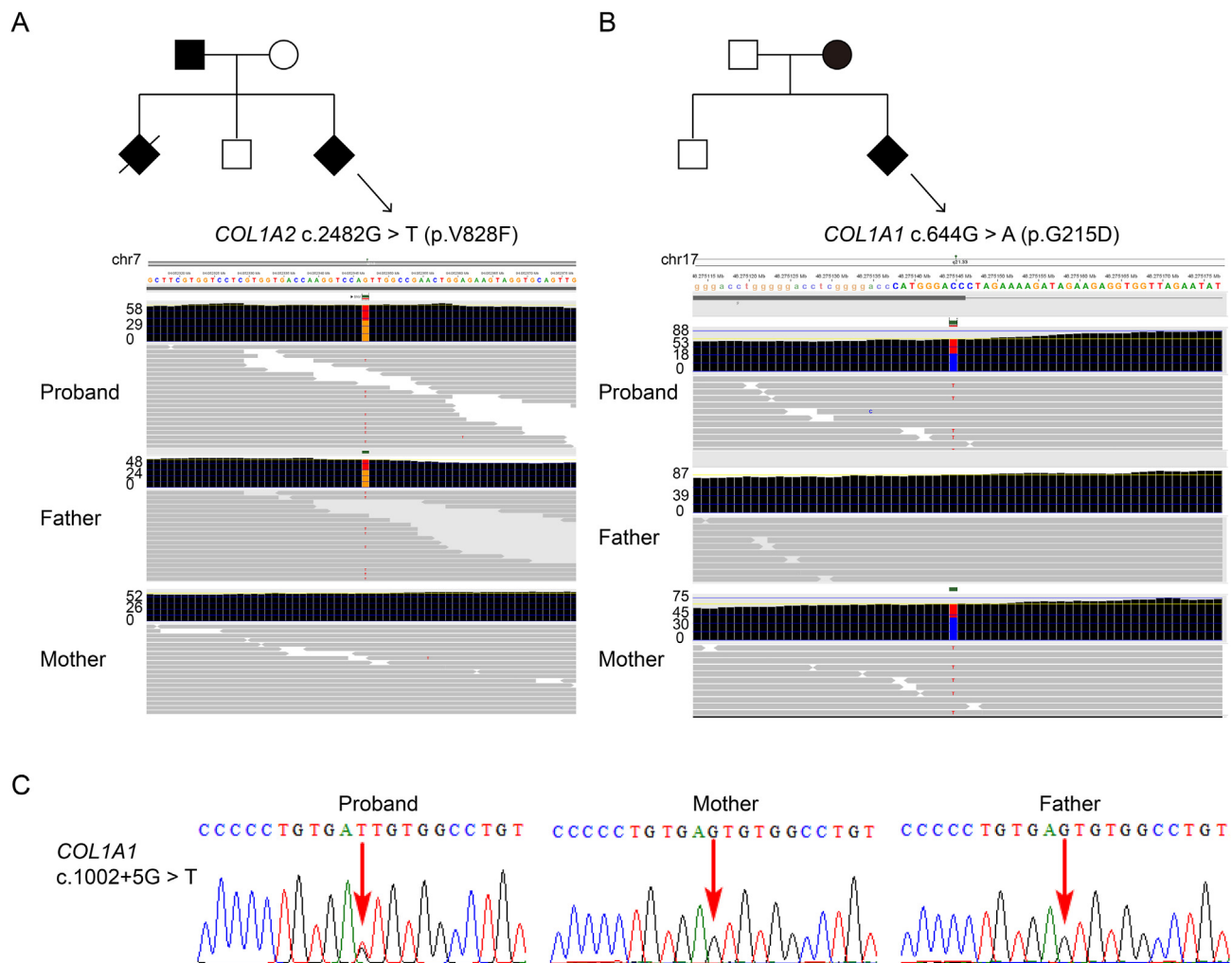


Figure 3. WES or sanger sequencing results of fetuses and their parents (A–B) showed heredity maps and results of trio-based WES in case 5 (A) and case 1 (B), respectively. Trio-based WES indicated one fetus carried a c.2482G>T p (Val828Phe) mutation in *COL1A2* inherited from the father (A) and the other carried a c.644G>A p (Gly215Asp) mutation in *COL1A1*, which was inherited from the mother (B). The numbers in the left represented the read depth of WES. (C) Sanger sequencing result verified the *COL1A1* c.1002 + 5G>T mutation was carried by a proband in case 4. This mutation was a *de novo* mutation, because the fetus's parents are not carriers.

screening process as fetal karyotype is no longer used (Grati et al., 2015; Jang et al., 2019; South et al., 2013; Zhu et al., 2016). However, pathological chromosomal imbalances can be detected by CMA in less than 30% of fetuses with abnormal ultrasound findings (Fiorentino et al., 2013). Considering the limitations of using CMA to detect some diseases caused by point or novel mutations, WES and WGS can identify these mutations simultaneously. Although WGS has an advantage over WES in identifying mutations within regulatory regions, the cost of WGS is much higher than that of WES. Skeletal abnormalities are associated with disturbances of bone growth, which could be caused by diverse mutations in different genes involved in the development of the skeletal system, such as FGF receptor (*FGFR*) family of genes (Yan et al., 2020). Therefore, to compare the detectability of skeletal abnormalities in prenatal diagnosis, we performed CMA and WES at the same time. In all our eight cases, CMA presented normal results. Several pathogenic or novel heterozygous mutations in *FGFR3*, *COL1A1*, *COL1A2* and *HSPG2* genes were identified by WES. These results suggested WES is a better genetic test than CMA to diagnose skeletal abnormalities.

After sequencing, our research found three cases had the same heterozygous mutation in the *FGFR3* gene c.1138G>A p (Gly380Arg) showed the same clinical features. All of these fetuses presented short long bones. c.1138G>A in the *FGFR3* gene is a hotspot mutation (Tang et al., 2021). A similar result also was described in *COL1A1* c.2461G>A p (Gly821Ser) (Ho Duy et al., 2016). The uniformity of the mutation in a specific gene might give us a direction to answer the reason of this specific point mutation in the future. Whether the mutation pattern is different between human populations.

Previous researches showed 90% of osteogenesis imperfecta cases are caused by a causative variant in the *COL1A1* or *COL1A2* genes (Augusiak-Duma et al., 2018; Zhytnik et al., 2017). However, in addition to *COL1A1/2* gene, rarely patients with homozygous or compound heterozygous mutations in other genes also presenting severe or lethal phenotypes were reported (Baldrige et al., 2008; Liu et al., 2019; Willaert et al., 2009). In our research, two fetuses in case 3 and 4 had multiple mutations in the same genes, a pathogenic or likely pathogenic mutation in *COL1A1* and a mutation of uncertain significance in *HSPG2* (Table 1). Mutations of the *HSPG2* gene are associated with Schwartz-Jampel Syndrome (SJS) and Dyssegmental Dysplasia Silverman-Handmaker Type (DDSH) (Arikawa-Hirasawa et al., 2001; Lin et al., 2021; Nicole et al., 2000). Both disorders have common clinical manifestations that involved in skeletal abnormalities (Martinez et al., 2018; Stum et al., 2006), including bowing of the long bones. The phenotype was also presented in our cases and previous studies (Tang et al., 2021).

There are some limitations in our study. First, we are unable to determine the correlation between combined mutations in multiple genes causing the same phenotype and clinical severity because we do not have a sufficient number of cases for statistical analysis. This is a major limitation in our study. Second, considering the family's financial situation, only half of families (case 1, 2, 3 and 5) performed trio-WES to detect the causative genes mutations. Third, the clinical information is not complete. We only collected ultrasound results and the family history, as all pregnant women chose to terminate their pregnancy after finding an ultrasound abnormality or informing the WES results.

In summary, we identified two novel heterogeneous mutations in the *COL1A1* gene, c.664G>T (p.G215D) and c.1002 + 5G>T, and a novel missense mutation in *COL1A2* gene, c.2482G>T p (Val828Phe). It will broaden the mutation spectrum of skeletal abnormalities in prenatal diagnosis. Whole exome sequencing is useful for skeletal abnormalities diagnosis and could contribute to expanding genotype-phenotype correlations.

Consent to publish

Written informed consent for publication was obtained from all participants.

Declarations

Author contribution statement

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

Lulu Yan: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Haibo Li & Bihua Zhou: Performed the experiments.

An'er Chen: Contributed reagents, materials, analysis tools or data.

Liyun Tian: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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