Clinical Application of Antenatal Genetic Diagnosis of Osteogenesis Imperfecta Type IV

Authors' Contribution:
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Background: Clinical analysis and genetic testing of a family with osteogenesis imperfecta type IV were conducted, aiming to discuss antenatal genetic diagnosis of osteogenesis imperfecta type IV.

Material/Methods: Preliminary genotyping was performed based on clinical characteristics of the family members and then high-throughput sequencing was applied to rapidly and accurately detect the changes in candidate genes.

Results: Genetic testing of the III5 fetus and other family members revealed missense mutation in c.2746G>A, pGly916Arg in COL1A2 gene coding region and missense and synonymous mutation in COL1A1 gene coding region.

Conclusions: Application of antenatal genetic diagnosis provides fast and accurate genetic counseling and eugenics suggestions for patients with osteogenesis imperfecta type IV and their families.

MeSH Keywords: Genotyping Techniques • Osteogenesis Imperfecta • Prenatal Diagnosis

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Background

Osteogenesis imperfecta (OI), also known as brittle bone disease, is a rare connective tissue disease characterized as increased bone brittleness and collagen metabolic disorder. OI is mainly caused by the gene mutation in COL1A1or COL1A2 of coding type I collagen a chain [1]. Typical clinical manifestations of OI include multiple fracture, short stature, blue sclera, loss of hearing, and dentinogenesis imperfecta (DI) with hereditary and familial features. The incidence of DI ranges from 1/100 000 to 1/25 000 [2,3]. Most OI cases have autosomal dominant inheritance (AD) and others result from autosomal recessive inheritance (AR) [4].

The severity of OI varies greatly, even within families who share a common genetic mutation. Optimal management of OI requires a multidisciplinary approach involving pediatrician, rehabilitation specialist, orthopedic surgeon, dentist, geneticist, social worker/psychologist, and occupational therapist. Bisphosphonate therapy remains the mainstay of medical treatment in OI and has been shown to decrease bone pain, improve muscle strength and mobility, and decrease fracture incidence. Novel therapies are gradually emerging as more evidence reported about the signaling pathways involved in bone formation [5]. This study was designed to advance the antenatal genetic diagnosis of osteogenesis imperfecta type IV within a family.

Material and Methods

Clinical data

The patient (III3) was admitted to our hospital due to complaints about 2 abnormal pregnancies. She received antenatal ultrasonic diagnosis at 30-week pregnancy in 2010. Ultrasonic examination revealed bilateral femur shortness of the fetus (left 40 mm and right 46 mm). Left femur deformity was observed. In 2012, ultrasonic examination at 24-week pregnancy showed that the femur length was 36 mm and 37 mm for humerus, with slight curving, considered as achondroplasia. Karyotype analysis of the amniotic fluid revealed no significant abnormality. DNA detection demonstrated no G→A heterozygous mutation at nucleotide 1138 in exon 10 of FGFR3, a candidate gene of congenital achondroplasia. She chose to terminate the pregnancy during twice visits in the years of 2010 and 2012. The husband of the patient (III4) (body height 175 cm) had a suspected bone fracture before age 1 year. The father-in-law of the patient (II8) had spinal deformity, repeated femur fractures, femur shortness, normal lower extremity, and was unable to walk. He suffered from blue sclera at a young age and was fully recovered now. The sister-in-law of the patient (III5) was of small stature and had a 3-cm difference in

leg lengths. No intermarriage was reported in this family. Other family members presented with no skeletal deformity, blue sclera, DI, or hearing loss.

Methods

Sample collection

The peripheral blood (5 ml) was sampled from the subjects I2, II2, II5, II8, III3, III4, and III5 and transferred to the laboratory under freezing condition.

High-throughput sequencing and library construction

(1) Standard procedures of extraction of DNA of the whole blood

(1.1) The experimental devices, such as DNA extract column, sample container, and centrifuge tube were prepared and labeled in sequence number.

(1.2) A portion of 20 μL QIAGEN proteinase (or proteinase K) was supplemented into the 1.5-mL centrifuge tubes.

(1.3) A portion of 200 μL sample and 200 μL AL buffer solution were supplemented into the centrifuge tubes, shaken for 15 s, centrifuged for a short time, and incubated at 56°C for 15 min.

(1.4) A portion of 200 μL absolute ethyl alcohol (frozen or room temperature) was added into the centrifuge tubes, shaken evenly, and centrifuged briefly to remove the droplets attached to the tube wall.

(1.5) The mixture was transferred to the QIAGEN nucleic acid extraction column carefully to avoid polluting the column opening and centrifuged at 8000 rpm for 1 min. The extraction column was put into a new tube. The original tube containing the filtration was discarded.

(1.6) A portion of 500 μL AW1 solution was added into the purifying column, centrifuged at 8000 rpm for 1 min and the waste was discarded. The tube was gently tapped on the tissue paper to remove the residual waste on the edge of the tube opening.

(1.7) A portion of 500 μL AW2 solution was supplemented into the purifying column and centrifuged at 8000 rpm for 1 min.

(1.8) The extraction column was put into a new tube and the original tube containing the filtration was discarded, and then centrifuged at 12 000 rpm for 2 min.

(1.9) The residual liquid in the middle segment of the purifying column was discarded. The purifying column was placed into

a new 1.5-mL centrifuge tube. The covering was opened and dried at room temperature for 3 min. A portion of 50 µL AE buffer solution was supplemented into the purifying column through the column membrane, incubated at room temperature for 5 min, and centrifuged at 8000 rpm for 1 min; nucleic acid sample elution and repeated dissolving by using 50 µL AE.

(1.10) The centrifuge tube was labeled with bar code correspondingly. A portion of 1-2 µL DNA was prepared for NanoDrop detection and the concentration of samples were recorded.

(2) Construction of library

(2.1) Sampling fragmentation and purification: the sample was subject to fragmentation into 100–700 bp. A portion of 4 µL was extracted for subsequent electrophoresis. The fragmented samples were transferred into the plate via 96-well plate for product purification using magnetic beads.

(2.2) Terminal repair: terminal repair reaction solution was prepared and packaged into PCR plates, mixed evenly with DNA template, and then closed for centrifuging. The sample was subject to a warm bath at 20°C for 30 min. DNA recovery was performed from the reaction products by using magnetic beads.

(2.3) Addition of 3'-A overhangs (A-Tailing): reaction solution was prepared and packaged into PCR plate, mixed evenly with DNA template, and closed for centrifuging.

(2.4) A-Tailing reaction system: the sample was subjected to a warm bath at 37°C for 30 min, shaken evenly, and then centrifuged.

(2.5) Adapter ligation: Adapter ligation reaction solution was prepared and packed into PCR plates separately, mixed evenly with DNA template, and closed for subsequent centrifuging. The sample was subject to DNA purification after overnight warm bath at 16°C for 12-16 h.

(2.6) Pre-LM-PCR of non-captured sample: Pre-LM-PCR reaction solution was prepared and packed into PCR plates separately, mixed evenly with DNA template, and closed for subsequent centrifuging. The sample was subject to the operating procedure of the PCR device.

(2.7) DNA recovery was performed from the purified reaction products by using magnetic beads. The obtained DNA was utilized as the DNA library.

(2.8) Chip hybridization and elution: the hybridization solution of the sample and chip was transferred into the hybridization device at 47°C for 64-72 h. The cleaning solution and streptomycin magnetic bead were prepared. DNA elution from the magnetic beads was conducted according to the manufacturers' instructions. The prepared LM-PCR reaction solution was packed into the tubes separately and the sample was put into the PCR analyzer. After the operating procedures, DNA purification was conducted. The construction of the DNA library was completed after hybridization.

(3) Production of DNA cluster: the surface of the patent chip was ligated with 1 layer of mono-chain primer. DNA fragment was "fixed" on the chip via base complementary with the primer on the chip surface after DNA mono-chain. The other end (primer complementation adjacent to 5'or 3') was "fixed" and formed a "bridge", 30 cycles of amplification for 1000 times of amplification for each single molecule, forming a monoclonal DNA cluster. Following production of the DNA cluster, amplification was subject to linearization. The sequence primer was subsequently hybridized with the target region of the universal sequence.

(4) DNA sequence: the principle of sequencing by synthesis was utilized. The reconstructed DNA polymerase and dNTP with 4 fluorescent labels were supplemented. These nucleotides are "reversible terminator" because 3' hydroxyl terminal contains chemical segmentation region, which merely allows for a single base mixture for each cycle. The category of nucleotides on each template after the first cycle of reaction was read by using laser scan reaction plate surface. Then, these genes were subject to chemical segmentation, 3' end viscosity was restored, and the polymerization of the second nucleotide was initiated. Step by step, the reaction proceeded until the sequence of each template was subject to polymerization for double strands. The fluorescent signal was collected for each cycle to analyze the sequence of each template DNA segment. Data analysis: automatic read of the base, and the data were transferred to the automated analysis channel for quadratic analysis.

Results

No loss or repetition of large-size fragment was observed in the coding region of genes (COL1A1, COL1A2 and CRTAP) related to osteogenesis imperfecta types I, II, III, IV, and VII in this studied family. No gene mutation was found in the coding region of *COL1A1*, *COL1A2* and *CRTAP* genes in subjects I2, II2, II5, and III3.

Genetic testing of the fetus of III5, III4, and II8 revealed a missense mutation of c.2746G>A, pGly916Arg in *COL1A2* gene coding region (Figures 1 and 2).

One missense mutation (p.Pro549Ala) and 4 samesense mutations (p.Thr29Thr, p.Asp82Asp, p.Pro482Pro, and p.Val626Val)
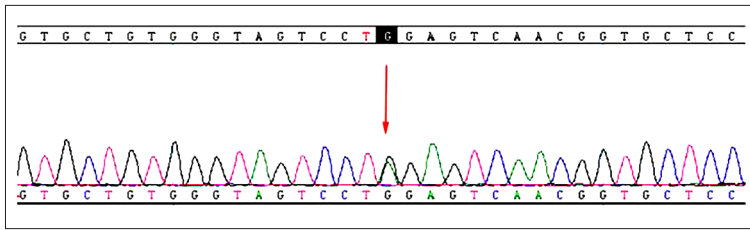
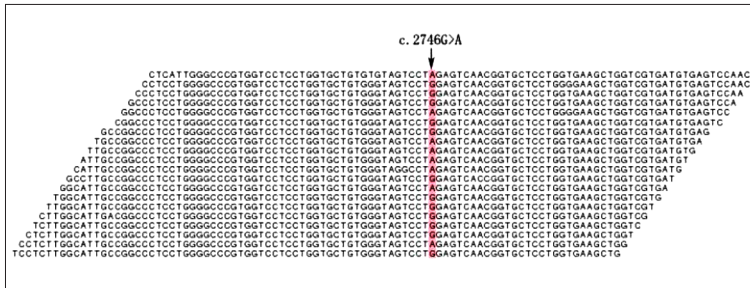**Figure 1.** Peak chart of COL1A2 gene: c.2746G>A(p.Gly916Arg).

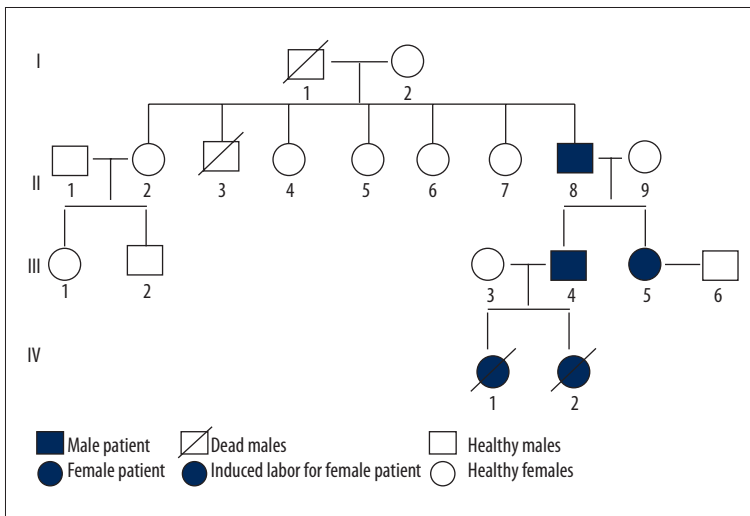**Figure 2.** Comparison chart of sequence.

Figure 3. Flowchart of family tree.

were observed in the coding region of *COL1A2* gene in the known polymorphism mutational sites of subject II8. One missense mutation (p.Thr1075Ala) and 1 samesense mutation (p.Thr766Thr) were found in the coding region of *COL1A1* gene. No frameshift, missense, samesense, or splice mutation was found in the coding region of *CRTAP*. One missense (p.Pro549Ala) and 4 samesense mutations (p.Thr29Thr, p.Asp82Asp, p.Pro482Pro, and p.Val626Val) were observed in the coding region of *COL1A2* gene. One missense mutation (p.Thr1075Ala) and 1 samesense mutation (p.Thr766Thr) were found in the coding region of *COL1A1* gene. Two samesense mutations (p.Leu71Leu and p.Asp178Asp) were found in the coding region of *CRTAP*.

The family tree was illustrated based on the clinical analysis and genetic testing outcomes, as illustrated in Figure 3

Discussion

Patients diagnosed with OI present with significant genetic heterogeneity. Different gene mutations-induced OI patients have continuous varying interval in phenotype. OI patients are characterized as having normal features, short stature, severe deformity, multiple fracture or progressiveness fracture, and even fetus death during the perinatal period [6]. Therefore, it is a challenging task to classify OI based on symptom and signs. Analysis of OI-related gene mutations plays a pivotal role in the classification and diagnosis of OI. In 1979, Sillence classified OI into 4 types according to the severity of phenotype: type I (slight OI accompanied with blue sclera) <type IV (normal sclera and mild deformity) <type III (progressive fracture) <(perinatal death). Type I OI results from COL1A1 mutation and types II-IV from COL1A1 or COL1A2 mutations [4,7,8]. Recently, mutant genes of types V-XIV have been described, including *IFITM5, SERPINF1, CRTAP, LEPRE1, PPIB, SERPINH1,*

FKBP10, *SP7*, *BMP1,* and *WNT1*. Slight mutation of CRTAP phenotype is regarded as type VII and similar to type II when the mutation is serious [9–16]. Consequently, the diagnosis of achondroplasia of the second fetus of patient III3 was excluded from this study. Then, the preliminary diagnosis of familial OI was made. OI was classified into types I, II, III, IV, and VII.

In this study, the subjects III5, III4, and II8 carried c.2746G>A (p.Gly916Arg) mutation. The mutational site has not been reported to be pathogenic and the incidence of this mutation is extremely low. SIFT (sorting intolerant from tolerant) and Polyphen (polymorphism phenotyping) calculation methods were conducted to predict protein function and revealed the harmful outcomes. It is speculated that c.2746G>A (p.Gly916Arg) mutation probably acts as the pathogenic mutation within this family.

Over 1000 types of gene mutations have been reported to result in OI. The type and site of gene mutations could affect phenotype and even genotype and phenotypic relation to a certain extent [17]. Especially for familial OI, OI induced by the same type and even same mutation may present with phenotypes of different severities [18]. The family members received clinical tests, as well as genetics and molecular analysis aiming to elucidate the underlying mechanism: subject II8 had severe phenotype and was unable to walk; subject III3 twice experienced induced labor due to skeletal deformity of the fetus; and subjects III4 and III5 presented with relatively mild phenotype. This evidence shows that c.2746G> A mutation in the coding region of *COL1A2* may be the molecular basis. This mutation leads to the substitution of glycine by arginine and structural changes in type I collagen. Previous studies demonstrated that missense mutation-induced structural changes in type I collagen are categorized into types II–IV with severe phenotype [19]. The type of amino acid mutation equally influences the phenotypic severity. Meini et al. [20] demonstrated that 18.9% (55/290) of 290 mutational sites of COL1A2 were replaced by glycine, manifested as lethal type II OI. In this study, except for c.2746G> A mutation, other mutations were found at polymorphism mutational site, suggesting that these mutational sites probably changed the secretory production of type I collagen, thereby leading to significant heterogeneity between genotype and phenotype in this family.

The members of this family show the clinical characteristics of *COL1A1/COL1A2*-related OI and they were eventually diagnosed with familial type IV OI. At present, antenatal ultrasound and gene detection are available for antenatal diagnosis of OI. Throughput sequencing technique could be applied in the detection of OI during early gestational period because it shortens the diagnosis time of OI, enhances the diagnostic reliability, and reduces the cost; it is becoming a pivotal tool in laboratory diagnosis of OI [21]. Antenatal ultrasound could reveal obvious femur and short limb deformity during 20-week pregnancy when making a diagnosis of type IV OI [22], which plays a vital role in guiding the pregnancy outcomes of patients.

As this study proceeded, subject III5 had been pregnant. Genetic testing showed c.2746G>A (p.Gly916Arg) mutation in the coding region of *COL1A2* gene in the fetus of subject III5. At 20-week pregnancy, the karyotype of amniotic fluid was 46, XX. Multiple antenatal ultrasonic examinations revealed no skeletal deformity. We recommended subject III5 to receive ultrasonic examination from antenatal to the third trimester of pregnancy. If no skeletal abnormality of the fetus was observed, she may be suspected with slight OI and suitable for pregnancy. For those subjects with skeletal abnormality, the feasibility of normal labor should be carefully evaluated.

Conclusions

At present, antenatal diagnostic procedures of OI mainly proceed during the secondary prevention stage of birth defects, which cannot fundamentally solve the problems of patients and their family members. PGD, as the primary preventive means, could be applied in termination of inheritance of pathogenic genes or selection of embryonic implantation without OI-related gene mutations. In this study, subject III3 twice experienced adverse pregnancy. Therefore, PGD was considered to provide more evidence to another pregnancy.

Conflict of interest disclosures

None.

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