Investigation on combined copy number variation sequencing and cytogenetic karyotyping for prenatal diagnosis

Jinman Zhang1,2, Xinhua Tang1,2, Jilin Hu2, Guilin He2, Jian Wang2, Yingting Zhu4* and Baosheng Zhu1,2*

Abstract

Background: We aimed to evaluate the clinical value of copy number variation-sequencing (CNV-Seq) in combination with cytogenetic karyotyping in prenatal diagnosis.

Methods: CNV-Seq and cytogenetic karyotyping were performed in parallel for 9452 prenatal samples for comparison of the diagnostic performance of the two methods, and to evaluate the screening performance of maternal age, maternal serum screening, fetal ultrasound scanning and noninvasive prenatal testing (NIPT) for fetal pathogenic copy number variation (CNV).

Results: Among the 9452 prenatal samples, traditional karyotyping detected 704 cases (7.5%) of abnormal cytogenetic karyotypes, 171 (1.8%) chromosome polymorphism, 20 (0.2%) subtle structural variations, 74 (0.7%) mutual translocation (possibly balanced), 52 (0.6%) without karyotyping results, and 8431 (89.2%) normal cytogenetic karyotypes. Among the 8705 cases with normal karyotype, polymorphism, mutual translocation, or marker chromosome, CNV-Seq detected 63 cases (0.7%) of pathogenic chromosome microdeletion/duplication. Retrospectively, noninvasive prenatal testing (NIPT) had high sensitivity and specificity for the screening of fetal pathogenic CNV, and NIPT combining with maternal age, maternal serum screening or fetal ultrasound scanning, which improved the screening performance.

Conclusion: The combined application of cytogenetic karyotyping and CNV-Seq significantly improved the detection rate of fetal pathogenic chromosome microdeletion/duplication. NIPT was recommended for the screening of pathogenic chromosome microdeletion/duplication, and NIPT combining with other screening methods further improved the screening performance for pathogenic fetal CNV.

Keywords: High-throughput sequencing, copy number variation, Prenatal diagnosis, Noninvasive prenatal testing, Chromosomal diseases
Background
Different levels and types of genetic variation exist in the human genome, ranging from single nucleotide mutations to structural or numerical chromosome abnormalities. One or more genetic variations may exist in an individual, and some genetic variations cause severe congenital malformations or death. In addition to triploid and numerical chromosome abnormalities, pathogenic chromosome microdeletion/duplication also leads to poor fetal prognosis. For example, Wolf-Hirschhorn syndrome mostly results in developmental retardation, unusual faces and structural abnormalities, and Miller-Dieker syndrome can be complicated by pachygyria; 22q11.21 microdeletion syndrome often has various degrees of cardiac malformations [1–6]. If pathogenic chromosome microdeletion/duplication can be diagnosed prenatally, the births of children with such severe congenital defects can be avoided.

However, the target diseases of traditional maternal serum screening are limited to common aneuploidies. Fetal ultrasound scanning is mainly used to monitor fetal growth and development, and to find structural fetal abnormalities and soft markers. Noninvasive prenatal testing (NIPT) using maternal plasma cell-free fetal DNA has made prenatal screening for pathogenic chromosome microdeletion/duplication possible.

Traditional cytogenetic karyotyping has been used as the gold standard diagnosis of chromosome abnormalities for decades. However, it is time-consuming and labor-intensive, largely dependent on cell culture, and has a low chromosome resolution of 5 ~ 10 Mb. In recent years, the application of high-resolution chromosome micro-array analysis (CMA), which can detect abnormal chromosome number, micro-deletions/duplication, uniparental disomy, has revolutionized the testing methodology of prenatal diagnosis. It has been suggested in some studies that CMA can be solely used instead of cytogenetic karyotyping in prenatal diagnosis laboratories with limited human resources [7]. Next-generation sequencing (NGS) now offers an alternative methodology to CMA, named copy number variation sequencing (CNV-Seq) with a resolution of 0.2 Mb for the detection of clinically significant chromosomal abnormalities. CNV-Seq has uniform sequencing coverage and relatively low price and has been gradually used in prenatal diagnosis [8]. However, more studies are required to further verify the efficiency of CNV-Seq in prenatal diagnosis.

Traditional karyotyping has characteristics of low-cost and covering the whole genome, including abnormal chromosome number and structural variation of specific regions, such as euchromatic and heterochromosomal regions. It highly depends on the experience of technicians to recognize these regions using different banding techniques under the microscope, which provide information about the frequency and location of these variations. Due to the morphologic similarity between chromosomes, karyotyping is difficult to accurately distinguish subtle structural variations. Genome copy number variation (CNVs) refers to structural variations of DNA sequence of more than 0.2 Mb. Karyotyping by conventional chromosome banding technology cannot distinguish these subtle variations. Compared with karyotyping, CNV-Seq which based on next-generation sequencing technology and comparative genomics has high resolution, high throughput, and simple laboratory operations. However, CNV-Seq also has limitations such as short read lengths and not covering the whole genome, and it cannot detect balanced translocations, polymorphism, marker chromosomes, and other genetic variations out of the detection range, and it cannot accurately detect polyploidy and low-proportion chromosome mosaic. Karyotyping and CNV-Seq are two different technologies, and the combination of them in prenatal diagnosis may make up for each other’s shortcomings and verify each other’s results to improve the accuracy of prenatal diagnosis. Therefore, in this study, we comparatively analyzed the difference between cytogenetic karyotyping and CNV-Seq for the same fetal samples, evaluated the value of adding CNV-Seq in traditional prenatal diagnosis, analyzed the performances of maternal age, maternal serum screening, NIPT and fetal ultrasound scanning for the screening of pathogenic fetal CNV and investigated whether combined application of these prenatal screening methods could improve the sensitivity and specificity for fetal pathogenic CNV.

Methods
Study patients
All methods were carried out in accordance with relevant guidelines and regulations. The proposal of this study had been approved by the Ethics Committee of Institutional Research Board (IRB), First People’s Hospital of Yunnan Province before this study was conducted. The study patients were 9452 singleton pregnant women who received invasive prenatal diagnosis after informed consent forms were signed, including allowance of data management and consent for manuscript publication, in the First People’s Hospital of Yunnan Province, China, from January 2018 to December 2019. Among the study patients, 3582 (37.9%) women were served by our hospital for their prenatal care, and 5870 (62.1%) were transferred from the other hospitals because we are the provincial prenatal diagnosis center. The maternal age calculated by the expected date of confinement was 31 (27–36) years. The gestational age at prenatal diagnosis was 20 (19–21) weeks. Invasive prenatal diagnosis
Prenatal screening
Four types of prenatal screenings were involved in this study: (1) combined screening in 11 ~ 13 + 6 weeks that comprised of fetal NT measurement + maternal serum screening using pregnancy associated plasma protein-A (PAPP-A), placental growth factor (PLGF) and free human chorionic gonadotropin beta unit (β-HCG), with or without NIPT; (2) maternal serum screening in 16 ~ 20 + 6 weeks using alpha fetoprotein (AFP), β-HCG and unconjugated estriol (uE₃), with or without NIPT; (3) NIPT only if gestational weeks at screening ≥ 21 weeks, and (4) all study patients had fetal ultrasound scanning in our department. The cases with high risk of Down Syndrome (DS), high risk of Edwards Syndrome (ES), or high risk of both by maternal serum screening were all classified as high-risk cases. Fetal ultrasound scanning was classified into five grades based on the severity of abnormalities: grade 0: without abnormal findings; grade 1: fetuses with subtly ultrasound abnormalities other than grade 2, e.g. gallbladder was not detected; grade 2: soft markers that were closely associated with chromosome aneuploidy, such as thickened nuchal fold (NF), nuchal translucency (NT) ≥ 3.0 mm, absence and/or dysplasia of nasal bone, mild to moderate ventriculomegaly, aberrant subclavian arteries, fetal growth restriction (FGR), short limb bones length, micrognathia, and acromphalus; grade 3: mild to moderate structural fetal malformations; grade 4: severe structural fetal malformations or lethal abnormalities. Maternal age ≥ 35 years at the expected date of confinement was defined as advanced maternal age.

Invasive prenatal diagnosis
Three types of surgeries for invasive prenatal diagnosis were used in this study. Amniocentesis: Twenty mL of amniotic fluid was collected by aspiration for cell culture and cytogenetic karyotyping, and 5 mL for CNV-Seq. If amniotic fluid was contaminated by adherent amniocytes after cell culture were used for CNV-Seq. Cordocentesis: Five mL of amniotic fluid was collected at first for CNV-Seq, and then 1.5 mL of cord blood for cell culture and cytogenetic karyotyping, and 0.5 mL of cord blood for hemoglobin electrophoresis to exclude maternal blood contamination. In prenatal diagnosis, the standard method to exclude maternal blood contamination should be linkage analysis of DNA polymorphism. We had used the method of STR polymorphism linkage analysis. In recent years, we used hemoglobin electrophoresis instead because STR polymorphism linkage analysis was much more time-consuming and labor-intensive. Chorion villus sampling: a small amount of villous tissue was sampled for CNV-Seq directly. Before 2019, we were inexperienced in villus cell culture techniques. Therefore, the cases received chorion villus sampling were those with severe fetal structural abnormalities. No villus cell culture was applied for those cases, and only CNVs was provided.

Laboratory testing
Cell culture and cytogenetic karyotyping
Amniotic fluid and umbilical blood samples were set up for cell culture following the standard protocols. Chromosome preparations were G-banded using trypsin-Giemsa staining for cytogenetic karyotyping after a series of standard protocols including colchicine treatment, hypotonic treatment, fixation and centrifugation. Karyotypes were diagnosed according to the international system for human cytogenetic nomenclature (ISCN, 2009) [9, 10]. The classification and abbreviations of abnormal karyotypes in this study were as follow: DS, ES, Patau syndrome (PS), super female syndrome (XXX), super male syndrome (XYY), Klinefelter syndrome (XXY), Turner syndrome (Turner), abnormal sex chromosome number mosaic (Sex A Mosaic), autosomal aneuploid mosaic (Auto A Mosaic), possibly balanced mutual translocation (Translocation), chromosome polymorphism (Polymorphism), triploid, chromosome fragment duplication/deletion, subtle structural variations such as inv. (21), inv. (4), dup (21), inv. (Y), inv. (1), inv. (5), inv. (12), inv. (8), inv. (19), inv. (Y), inv. (10), inv. (16). A total of 52 cases had only CNV-Seq results, but no karyotyping results. Among them, 44 cases who received chorion villus sampling, since our center cannot provide villus cell culture during that time; 8 cases encountered amniotic fluid cell culture failure. The maximum and minimum gestational weeks of amniotic fluid cell culture failure were 31 and 20 weeks, respectively. It should be noted that none of the 52 cases with missing results were used in the data analysis of this study.

CNV-Seq and result interpretation
Nextseq 550AR platform (Illumina, San Diego, CA) was used for DNA sequencing, with an average sequencing depth of 0.08×, following the Q30 sequencing quality standard. The amount of fetal DNA used for CNV-Seq was 10 ~ 50 ng for each prenatal sample. AnnoroadPD software (Annoroad Gene Technology Co., Ltd., Beijing, China) was applied to analyze the sequencing data referring to the human reference genome GRCh37/hg19. The identified fetal CNV were interpreted [11] and classified into five categories: pathogenic (P-), likely pathogenic (LP-), uncertain significance (VUS-), likely benign (LB-) and benign (B-), according to the standards and guidelines that were jointly developed by the American College of Medical Genetics and Genomics (ACMG), the
Association for Molecular Pathology (AMP) and the College of American Pathologists (CAP) in 2015. To conveniently show the CNV-Seq results, we used “P-” as abbreviation for pathogenic chromosome microdeletion/duplication, “None” for no copy number variation found, “auto A” for autosomal aneuploidy, “sex A” for abnormal sex chromosomes number, “auto AM” for autosomal aneuploidy mosaic, and “sex AM” for abnormal sex chromosomes number mosaic. Cytogenetic karyotyping was the diagnostic method for numerical and structural chromosome abnormalities, and high-throughput sequencing for CNV. For LP- and VUS-, family (parents and fetuses) CNV-Seq tests, fluorescence in-situ hybridization (FISH) or multiplex ligation probe amplification (MLPA) were used for further verification.

Statistical analysis
The data were analyzed statistically using IBM SPSS Statistics (version 22.0, IBM Corp., Armonk, NY, USA). Continuous variables (for example, age and gestational weeks at prenatal diagnosis) were expressed as “median [lower quartile, upper quartile]”, and analyzed using Kruskal-Wallis one-way analysis of variance. Categorical variables are represented by “n (%)” and analyzed using Chi-square test for two-way disordered R × C table. Calculation for sensitivity and specificity: sensitivity = true positive / (true positive + false negative) *100%; specificity = true negative / (true negative + false positive) *100%. Paired chi-square test was used to test the difference between CNV-Seq and various prenatal screening methods and prenatal diagnosis results (P < 0.01 was considered statistically significant). Missing items were not applied in data analysis.

Results
Basic characteristics of study patients
Basic information of 9452 cases of prenatal diagnosis was listed and statistically analyzed in Table 1. Among study patients, 9452 (100%) had received one to two times of fetal ultrasonography in our center, 5688 (60.2%) had maternal serum screening, 1409 (14.9%) had NIPT, 551 (0.58%) had both NIPT and maternal serum screening, and 3142 cases (33.2%) were in advanced maternal age. The results showed that only 1165 (12.3%) of patients received invasive prenatal diagnosis due to high risk of NIPT. Other indications for prenatal diagnosis included advanced maternal age, abnormal fetal ultrasound scanning, high risk of maternal serum screening, adverse reproductive history, family history of single-gene genetic diseases, or others.

Comparison of results between CNV-Seq and cytogenetic karyotyping
The results of 9452 cases of cytogenetic karyotyping were listed in Table 2: a total of 704 (7.5%) cases of fetal chromosome abnormalities, 171 (1.8%) chromosomal polymorphism, 20 (0.2%) subtle structural variations, 74 (0.7%) mutual translocation (possibly balanced), 52 (0.6%) without karyotyping results, and 8431 (89.2%) normal karyotypes were detected.

The results of CNV-Seq in Tables 2, 8354 fetuses with CNV-Seq findings were included as None, B-, and LB-, cytogenetic karyotyping showed that except for 2 cases of triploid, the rest 271 cases of abnormal karyotypes had good prognosis. A total of 530 cases of fetal aneuploidies (DS, ES, PS, XXY, XYY) were diagnosed, and the results of karyotyping and CNV-Seq were consistent. The details for 60 cases of pathogenic microdeletion/duplication detected by CNV-Seq were shown in Table 3. CNV-Seq detected 1 case of chromosomal aneuploidy and 2 cases of mosaic in fetuses with normal cytogenetic karyotypes. Furthermore, 2 cases (No.29 and 30) of pathogenic microdeletion/duplication were detected in 9 fetuses with marker chromosomes, and 1 case (No.38) of pathogenic microdeletion/duplication were detected in fetuses with mutual translocations (Seen in Table 4). Therefore, we may conclude that the combination of the two methodologies significantly improved the accuracy of prenatal diagnosis for fetal pathogenic CNV and was helpful to assess fetal prognosis. Due to its detection limitations, for example, two cases of triploid by karyotyping had normal CNV-Seq results, CNV-Seq could not replace karyotyping at present stage but might be an effective complement.

Performances of maternal age, maternal serum screening, NIPT and fetal ultrasound scanning for pathogenic CNV-Seq results and pathogenic karyotypes
The target diseases of maternal serum screening are common chromosomal aneuploidies. For fetuses with pathogenic microdeletion/duplication, the prognosis is mostly poor. NIPT is the ideal prenatal screening method for pathogenic microdeletion/duplication, but the cost may limit its clinical use to a certain extent. If NIPT was unavailable, we wondered whether other screening methods could recognize pathogenic microdeletion/duplication. In this study, we retrospectively analyzed the results of maternal age, maternal serum screening, NIPT, and fetal ultrasound scanning for women with pathogenic fetal CNV-Seq results, including pathogenic microdeletion/duplication and Auto A, Sex A, Auto AM, Sex AM, as shown in Table 5. NIPT missed one case of Auto AM (CNV-Seq) and Triploid (karyotypes), shown in Table 6. In maternal serum screening, 55.2% of pathogenic microdeletion/
duplication (CNV-Seq) and 42.1% of unbalanced fragment deletion/duplication (karyotypes) showed high risks results. As to abnormal ultrasound findings (grades 2–4), there was significant difference between CNV-Seq of auto A and pathogenic microdeletion/ duplication (73.6% versus 43.2%, \( P < 0.001 \)). Regarding to pathogenic karyotypes, NIPT missed one case of triploid, whose maternal serum screening and fetal ultrasound were abnormal. Only 42.2% of severe chromosomal abnormalities (aneuploidy, unbalanced fragment deletion/
duplication, triploidy) were screened out by advanced maternal age. The detection rate of abnormal fetal ultrasound findings (grade 2–4) in fetuses with abnormal karyotypes were DS 69.9%, ES 85.5%, PS 91.7%, unbalanced fragment deletion/duplication 56.8%, triploid 100%, XXX 14.3%, XYY 24.2%, and XXY 18.1%. We speculated that there might be a dose-effect between fetal ultrasound abnormalities and chromosomal diseases, and a difference between autosomal and sex chromosomal abnormalities. Therefore, maternal age, maternal serum screening, fetal ultrasound scanning and NIPT all had certain predictive values for pathogenic CNV-Seq results and pathogenic karyotypes (chromosomal aneuploidy, unbalanced segment deletion/duplication, and triploidy).

As seen in Table 7, for pathogenic fetal CNV-Seq results, NIPT had the highest sensitivity of 1.00 (0.99–1.00) but lowest specificity of 0.22 (0.18–0.26), while maternal serum screening had higher sensitivity of 0.63 (0.57–0.68) and lower specificity of 0.37 (0.35–0.39). The sensitivity and specificity of fetal ultrasound scanning were 0.69 (0.66–0.72) and 0.59 (0.57–0.60), respectively. Advanced maternal age had a specificity of 0.68 (0.67–0.70) and a sensitivity of 0.39 (0.35–0.42).

| Table 2 Comparison of results between cytogenetic karyotyping and CNV-Seq |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| cytogenetic karyotyping | CNV | B | LB | VUS | LP | P-del/dup | Auto A | Auto A M | Sex A | Sex A M | Total |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| None (n = 4851) | 4666 (96.2) | 36 (38.3) | 3355 (66.8) | 289 (55.1) | 22 (45.1) | 60 (50.8) | 0 (0.0) | 1 (5.6) | 1 (0.6) | 1 (3.0) | 843 (17.3) |
| B (n = 38) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| LB (n = 3465) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| VUS (n = 304) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| LP (n = 118) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| P-del/dup (n = 118) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Auto A (n = 446) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Auto A M (n = 18) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Sex A (n = 156) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Sex A M (n = 33) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Total (n = 9452) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

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Correlation between fetal CNV-Seq and indications for prenatal diagnosis

According to the number of indications for prenatal diagnosis, the 9452 women were divided into four groups: (1) single indication, (2) positivity for any two or (3) three or (4) four indications. Indications included high risk in NIPT, high risk in maternal serum screening, fetal ultrasound abnormalities (Grade 2–4), advanced maternal age, and other indications such as adverse childbearing history except monogenic diseases. The sensitivity and specificity of different prenatal diagnostic indications for the screening of pathogenic microdeletion/microduplication were listed in Table 8. Single indication had the highest sensitivity and the lowest specificity. In a similar trend, four indicators had the lowest sensitivity and the highest specificity. Accordingly, to achieve the optimal sensitivity and specificity, a prenatal screening program that combines two methods could be considered.

Discussion

Combination of cytogenetic karyotyping and CNV-Seq can prenatally diagnose more fetal pathogenic microdeletion/duplication and provide comprehensive prenatal information

In addition to traditional cytogenetic karyotyping, CMA and CNV-Seq have gradually been used in prenatal diagnosis. CNV-Seq is worth applying in prenatal diagnosis due to its lower cost and uniform sequencing coverage. Fetuses with normal karyotype/ chromosome polymorphism usually have good prognosis, but pathogenic microdeletion/duplication cannot be excluded. In this study, among fetuses with normal karyotypes, CNV-Seq diagnosed 60 (0.6%) cases of pathogenic CNV, and fortunately the births of 44 fetuses with poor prognosis were avoided. A complex case was also prenatal diagnosed with CNV-Seq result of 47,XXY and karyotyping result of 46,XX. SRY gene detection indicated a male gender, but all 6 loci on AZF gene were missing. Fetal ultrasound showed male external genitalia. Taking fetal ultrasound findings together, the fetus might have sexual reversal and risks of abnormal reproductive system development in puberty. The parents were fully informed the advantages, disadvantages and limitations of karyotyping and CNV-Seq, and to make clear that the two results cannot be denied by each other but be mutually complementary. The couple chose to continue the pregnancy. The boy is now 1.5 years old and is generally healthy. Follow-up and etiological examination were recommended. On the other hand, we should make clear that not all fetuses with structural chromosomal abnormalities by karyotyping have poor prognosis. For example, mutual translocation (paternal/maternal/de novo) with normal CNV-Seq and fetal ultrasound is very much likely that the fetuses have good prognosis. The combination of karyotyping and CNV-Seq enables mutual verification of the results in prenatal diagnosis and helps to avoid misdiagnosis and provide more information for comprehensive evaluation of fetal prognosis. However, the combined application of CNV-Seq and karyotyping may lead to increased economic burden. It needs further verification about whether the cost-effectiveness is worth promoting. We need to choose an appropriate prenatal diagnosis program based on our own characteristics.

Combined several prenatal screening significantly improves the specificity but reduces the sensitivity for fetal pathogenic CNV

Maternal serum screening detects 70 ~ 80% of DS, at a false positive rate of 5% [12]. It was reported that a small
Table 4  Forty five cases with inconsistent karyotyping and CNV-seq results

| No. | Indications for prenatal diagnosis | Abnormal karyotypes | CNV-seq results | Fetal ultrasound | Pregnancy outcomes |
|-----|-----------------------------------|---------------------|-----------------|------------------|--------------------|
| 1   | Advanced maternal age             | 69,XXX              | 46,XX           | Fetal growth restriction, tethered spinal cord, ankle joint reflexion, diaphragm expansion | Termination of pregnancy |
| 2   | Maternal serum screening ES high risk, NIPT low-risk | 69,XXX | 46,XX | Fetal trunk is significantly smaller than the head, left lung absent, double Outlet Right Ventricle | Termination of pregnancy |
| 3   | NIPT high-risk                     | 47,XXX              | 46XX(20%)/47XXX(80%) | Normal | Gave birth to a girl |
| 4   | NIPT high-risk                     | 45,X[18]/46,X,i(X)(p10)[34] | 45,X,del[(11.21-p22.33)52.45mb(73%)/46,XX(27%)] | Thickened nuchal folder | Continue pregnancy |
| 5   | NIPT high-risk                     | 45, X               | 46XX(11%)/45.X(89%) | Fetal growth restriction | Termination of pregnancy |
| 6   | NIPT high-risk                     | 45,X[53]/47,XXX [5] | 45,X | Ventricular septal defect | Termination of pregnancy |
| 7   | Advanced maternal age              | 45,X[18]/46,X,+mar[24] | 45X with possible X structure abnormality | Mild bilateral renal hydrops, bilateral ventriculomegaly, slightly larger right heart | Termination of pregnancy |
| 8   | NIPT high-risk                     | 45,X[19]/46,XY [16] | Turner mosaic | Bilateral renal pelvis separation | Termination of pregnancy |
| 9   | NIPT high-risk                     | 45,X[35]/46,XX [9] | 45,X | Normal | Termination of pregnancy |
| 10  | Maternal serum screening high risk | 47,XYY [7]/46, XY[56] | Y chromosome duplication(16.85 Mb) | Normal | Gave birth to a boy |
| 11  | NIPT high-risk                     | 45,X[22]/46,XY [8] | Yq11.221-q11.223 deletion(VUS) | Bowel echo enhancement | Termination of pregnancy |
| 12  | Maternal serum screening high risk | 45,X[37]/46,XY [7] | 4p15.33- , Yq11.222-q11.223 deletion(VUS) | Normal | Termination of pregnancy |
| 13  | Childbearing history of gastrodialysis | 45,X [10]/46, XX[42] | Likely benign variation | Left nasal bone dysplasia, right Nasal bone absent | Gave birth to a girl |
| 14  | NIPT high-risk                     | 45X [5]/46XX | Likely benign variation | Retract chin and lower lip | Continue pregnancy |
| 15  | NIPT high-risk                     | 45,X [8]/46,XX[92] | Likely benign variation | Normal | Termination of pregnancy |
| 16  | NIPT high-risk                     | 45,X [4]/46,XX[51] | Likely benign variation | Normal | Continue pregnancy |
| 17  | Couples are thalassaemia carrier   | 45,X [10]/46, XY[28] | Normal | Normal | Termination of pregnancy |
| 18  | Advanced maternal age              | 47,XXY[10]/46, XY[45] | Normal | Bilateral renal pelvis separation, bowel echo enhancement | Gave birth to a boy |
| 19  | NIPT high-risk                     | 45X[34]/47, XXX[26] | Normal | Bilateral renal pelvis separation | Termination of pregnancy |
| 20  | Couples are thalassaemia carrier   | 45,X [5]/46,XY[40] | Normal | The fetus is smaller 8 days than gestational week | Gave birth to a boy |
| 21  | NIPT high-risk                     | 47,XN,+ 21[18]/46, XN[31] | 47,XN,+ 21[58%] | Small humerus and femoral length, small head circumference | Termination of pregnancy |
| 22  | NIPT high-risk                     | 47,XX,+ 21[27]/46, X[8] | DS | Normal | Termination of pregnancy |
| 23  | NIPT high-risk                     | 47,XX,+ 18[20]/46XX [5](GTG) | 47,XN,+ 18[78%]/46,XN[22%] | Incontinuity of lower part of cerebellar vermis, complete endocardial cushion defect | Termination of pregnancy |
| 24  | NIPT high-risk                     | 47,XX,+ 18[20]/46XX [5](GTG) | 47,XN,+ 18[78%]/46,XN[22%] | Incontinuity of lower part of cerebellar vermis, complete endocardial cushion defect | Termination of pregnancy |
| 25  | NIPT high-risk                     | 47,XN,+ 15[4]/46, XN[51] | Trisomy 15 mosaic (50%) | Single umbilical artery | Termination of pregnancy |
| 26  | NIPT high-risk                     | 47,XY,+ 5[15]/46, XY[47] | CNVs benign variation | FGR, ventricular septal defect, thickened right ventricular wall, Aorta straddle, enhanced echo of the aortic valve, tricuspid valve and intestinal echo | Termination of pregnancy |
portion of sex chromosome abnormalities showed abnormal findings in maternal serum screening [13]. It is unknown whether maternal serum screening is abnormal for pathogenic CNV. The findings of this study indicated that maternal serum screening can detect 55.2% of fetal pathogenic chromosomal microdeletion/duplication in fetuses with normal or abnormal karyotype. The intrauterine phenotype of fetuses with pathogenic microdeletion/duplication lacks specificity, so prenatal ultrasound scanning is difficult to identify. In this study, fetuses with autosomal aneuploidy had the most severe ultrasound abnormalities, followed by pathogenic microdeletion/duplication with a large variability that some fetuses had completely normal ultrasonography. Fetuses with abnormal sex chromosome number and structure and the mosaic had mild ultrasound abnormalities. Fetal ultrasound scanning had higher sensitivity for autosomal aneuploidy. However, if other prenatal screening

| No. | Indications for prenatal diagnosis | Abnormal karyotypes | CNV-seq results | Fetal ultrasound | Pregnancy outcomes |
|-----|-----------------------------------|---------------------|----------------|------------------|-------------------|
| 27  | NIP high-risk                      | 47,XY,+ 13 [5]/46, XY [63] | CNVs-           | Normal           | Gave birth to a healthy boy |
| 28  | Advanced maternal age             | 47,XY,+ 18 [5]/46, XY [90] | CNVs(-)         | Polyhydramnios   | Gave birth to a healthy boy |
| 29  | NIP high-risk                     | 47,XN,mar           | 12p12.1-p13.33 and 21q11.2-q22.11 duplication (pathogenic) | Normal           | Termination of pregnancy |
| 30  | Maternal serum screening high risk | mos46,X, +mar[23]/45,X [14] | Xp11.21-p22.33 deletion 56.8mb and Xq21.31-q28 deletion 64.6mb (pathogenic) | Short humerus and femoral length, Ventricular Septal Defect | Termination of pregnancy |
| 31  | Advanced maternal age             | 47,XN,mar           | 2q11.1-q11.2 duplication (VUS) | Bilateral choroid plexus cysts, enhanced bowel echo | Lost to follow-up |
| 32  | Advanced maternal age             | 47,XX,mar           | 5q21.2-q21.3 duplication, VUS | Normal           | Continue pregnancy |
| 33  | Amniotic fluid 46, XN[38]/47,XN, +mar[22] | Cord blood 47,XX, +mar[17]/46,XX [17] | dup(8q24.22) Likely benign variation | Normal           | Continue pregnancy |
| 34  | NIP high-risk  abnormal chromosome 3 number | 47,XX,mar | Likely benign variation | Short nasal bone | Gave birth to a healthy girl |
| 35  | Fetal acromphalus                 | 47,XY,+mar [11]/46,XY[31] | Likely benign variation | Acromphalus, edema | Termination of pregnancy |
| 36  | Thalassaemia?                     | 47,XX,mar [13]/46,XX[62] | Likely benign variation | Normal           | Gave birth to a healthy girl |
| 37  | Advanced maternal age             | 47,XY,+mar [7]/46,XY[33] | Normal           | Normal           | Gave birth to a healthy boy |
| 38  | NIP high-risk                     | 46,XN(1;13)(q25; q22) de novo | 13q14.3-q21.33 deletion 23.1mb (pathogenic) | Normal           | Termination of pregnancy |
| 39  | Childbearing history of deaf children | 46,XY, dup(1)(q21.2)? | 1q52.12 duplication (VUS) | Normal           | Gave birth to a healthy boy |
| 40  | NIP high-risk                     | 46,Xdel(Y)(q11)? | Xp22.31-p22.33 duplication VUS | Fetal right ventricular wall has strong echo and was thickened | Lost to follow-up |
| 41  | NIP high-risk                     | 46,XN, inv(9)(p12q13)[79] | DS mosaic (20%) | Normal           | Lost to follow-up |
| 42  | Maternal serum screening high risk, Advanced maternal age | 45,Xdel(13Y; 13)[q11.2; p10][26]/45,X [5] | Xdel(Y)[75%]/XO(25%) | FGR?             | Termination of pregnancy |
| 43  | Maternal serum screening high risk | 46,XY[45] | XY(60%)/XXX(40%) | Right aortic arch | Continue pregnancy |
| 44  | NIP high-risk                     | 46,XX[40] | XXY Gene detection SRY existed, AZF all missing | Male genitalia | Gave birth to a healthy boy, 2 years-old |
| 45  | NIP high-risk                     | 46,XY[83] | 47,XN,+ 2[23%]/46,XN[77%] | Normal           | Termination of pregnancy |
### Table 5: Performances of karyotyping over CNV-seq in each indication for prenatal diagnosis

| Indication for prenatal diagnosis | P-del/dup (n = 118) | Auto A (n = 446) | Auto A M (n = 18) | Sex A (n = 156) | Sex A M (n = 33) | Total (n = 771) | P value |
|----------------------------------|--------------------|-----------------|------------------|----------------|----------------|----------------|---------|
| NIPT High-risk                   | 30 (25.40)         | 272 (61.00)     | 11 (61.00)       | 125 (80.10)    | 21 (63.60)      | 459 (59.53)    | < 0.001 |
| NIPT Low-risk                    | 0 (0.00)           | 0 (0.00)        | 1 (6.00)         | 0 (0.00)       | 0 (0.00)        | 1 (0.13)       |         |
| Absent                           | 88 (74.60)         | 174 (39.00)     | 6 (33.00)        | 31 (19.90)     | 12 (36.40)      | 311 (40.34)    |         |
| Maternal serum screening         |                    |                 |                  |               |                |                |         |
| Maternal serum screening High-risk| 32 (27.12)         | 129 (28.90)     | 6 (33.33)        | 27 (17.31)     | 12 (36.36)      | 188 (24.40)    | < 0.001 |
| Maternal serum screening Low-risk | 26 (22.03)         | 42 (9.40)       | 4 (22.22)        | 5 (15.15)      | 6 (18.20)       | 111 (14.40)    | < 0.001 |
| Absent                           | 60 (50.85)         | 275 (61.70)     | 8 (44.44)        | 115 (73.70)    | 14 (42.40)      | 472 (61.20)    |         |
| Fetal ultrasound                 |                    |                 |                  |               |                |                |         |
| fetal ultrasound (0)             | 47 (39.83)         | 82 (18.00)      | 9 (50.00)        | 88 (56.41)     | 13 (39.40)      | 239 (31.00)    | < 0.001 |
| fetal ultrasound (1)             | 20 (16.95)         | 36 (8.00)       | 5 (28.00)        | 27 (17.31)     | 17 (13.00)      | 100 (13.00)    | < 0.001 |
| fetal ultrasound (2)             | 15 (12.71)         | 177 (40.00)     | 1 (5.50)         | 18 (11.54)     | 5 (15.15)       | 216 (28.00)    |         |
| fetal ultrasound (3)             | 20 (16.95)         | 78 (18.00)      | 2 (11.00)        | 13 (8.33)      | 2 (6.06)        | 115 (14.90)    |         |
| fetal ultrasound (4)             | 16 (13.56)         | 73 (16.00)      | 1 (5.50)         | 10 (6.41)      | 1 (3.03)        | 101 (13.10)    |         |
| Maternal age                     |                    |                 |                  |               |                |                |         |
| Advanced maternal age            | 22 (18.60)         | 210 (47.10)     | 7 (38.90)        | 49 (31.40)     | 12 (36.40)      | 300 (38.90)    | < 0.001 |
| maternal age < 35 years old      | 96 (81.40)         | 236 (52.90)     | 11 (61.10)       | 107 (68.60)    | 21 (63.60)      | 471 (61.10)    | < 0.001 |
| Indication for prenatal diagnosis |                    |                 |                  |               |                |                |         |
| DS (n = 358)                     | 231 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| ES (n = 55)                      | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| PS (n = 12)                      | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| XXX (n = 28)                     | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| XY (n = 33)                      | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| XXY (n = 72)                     | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| XO (n = 13)                      | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| Triploid (n = 2)                 | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| Unbalance (n = 51)               | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| Total (n = 624)                  | 236 (64.50)        | 30 (54.50)      | 7 (58.30)        | 22 (78.60)     | 65 (90.30)      | 16 (31.40)     | 407 (65.20) |
| P value                          |                    |                 |                  |               |                |                |         |

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Table 5  Performances of karyotyping over CNV-seq in each indication for prenatal diagnosis (Continued)

| Indication for prenatal diagnosis | P-del/dup (n = 118) | Auto A (n = 446) | Auto A M (n = 18) | Sex A (n = 156) | Sex A M (n = 33) | Total (n = 771) | P value |
|----------------------------------|---------------------|------------------|-------------------|----------------|----------------|----------------|---------|
| unadvanced maternal age          | 179 (50.00)         | 32 (58.20)       | 9 (75.00)         | 29 (50.00)     | 13 (100.00)    | 44 (86.30)     | 366 (58.70) |

P values were the statistical difference of constituent ratios by Chi-square test between NIPT and CNV-seq, maternal serum screening and CNV-seq, fetal ultrasound and CNV-seq, maternal age and CNV-seq.

Table 6  Cases with high risk of combined screening test but low risk NIPT

| Maternal serum screening | Fetal ultrasound | Maternal age (years) | Fetal CNV-seq results | Fetal karyotypes | Pregnancy outcomes |
|-------------------------|------------------|----------------------|-----------------------|------------------|--------------------|
| 1 N/A                   | Ventriculomegaly, cardiac malformations, pulmonary dysplasia | 28 | 47,XN, + 13(40%)/46, XN(60%) | 47,XY, + 13(12)/46,XY(74) | Termination of pregnancy |
| 2 High risk             | Imbalance of head-body ratio, double outlet right ventricle, absent left lung | 27 | Normal | 69,XXX | Termination of pregnancy |
| 3 Low risk              | Nasal bone dysplasia | 30 | Likely benign variation | 46,XX,t(11;17)(q21;q23) | Continue pregnancy |
| 4 N/A                   | Right aortic arch | 36 | Likely benign variation | 46,XN,inv(9)(p12q13) | Continue pregnancy |
| 5 Low risk              | Enhanced echo in both kidneys and intestine, pleural effusion | 31 | Likely benign variation | 46,XX,t(12;22)(q24.1;q13) | Continue pregnancy |
| 6 Threshold risk        | Right choroid plexus cyst | 28 | Likely benign variation | 46,XY,1qh+ | Continue pregnancy |
| 7 Low risk              | Normal | 40 | Normal | 46,XX, inv(19)(p13.3q13.1) | Continue pregnancy |
| 8 N/A                   | Normal | 36 | Normal | 46,XY,t(8;16)(q12; q21)mat | Continue pregnancy |
| 9 N/A                   | Duodenal atresia | 23 | Normal | 46,XX,t(2;7)(q13q22) | Continue pregnancy |
| 10 N/A                  | Nasal bone absent | 38 | Normal | 46,XY,15 ps+ | Continue pregnancy |
| 11 N/A                  | Thickened ventricular wall, small heart size, enlarged liver and spleen | 26 | Normal | 46,XY,21 ps+ | Continue pregnancy |
| 12 Low risk             | Holoprosencephaly, clearly displayed nasal bones, incontinuity of upper alveolar process | 31 | Normal | Chorionic villus sampling, no karyotyping | Termination of pregnancy |
| 13 N/A                  | Ventricular septal defect, hemivertebra, scoliosis | 26 | Normal | Normal | Termination of pregnancy |
| 14 N/A                  | Holoprosencephaly, agenesis of corpus callosum, hydrocephalus | 33 | Normal | Normal | Termination of pregnancy |
| 15 N/A                  | Absent right lung, Tetralogy of Fallot, hemivertebræ | 25 | Normal | Normal | Termination of pregnancy |
| 16 Threshold risk       | Complete endocardial cushion defect | 25 | Normal | Normal | Termination of pregnancy |
| 17 N/A                  | Left ventricular rhabdomyomas, strehenopodia | 36 | Normal | Normal | Termination of pregnancy |
| 18 Low risk             | Left microtia, nasal dysplasia, atresia of nostril | 35 | Normal | Normal | Termination of pregnancy |
| 19 N/A                  | Ventricular septal defect, pulmonary artery stenosis, missing pubic bones, hooked hands | 33 | Normal | Normal | Termination of pregnancy |
methods such as NIPT and maternal serum screening are unavailable at the same time, the ultrasonologist’s subjective assessments of fetal subtle facial features/ minor heart variations are inadequate for accurate assessment of fetal prognosis, and the clinical value is limited. NIPT has been widely used as a first-line prenatal screening method [14]. It was reported that the sensitivities of NIPT for Trisomy 21, 18, 13 are 99.1, 98.2 and 100%, respectively [14]. The detection rates of NIPT for fetal aneuploidy and CNV > 20 Mb were

| CNV          | Total | McNemar’s  | P value | Sensitivity | Specificity |
|--------------|-------|------------|---------|-------------|-------------|
| NIPT         | 1     | 459        | 798     | 334.03      | < 0.001     | 1.00 (0.99, 1.00) | 0.22 (0.18, 0.26) |
| Maternal serum screening | 1     | 188        | 1705    | 1212.5      | < 0.001     | 0.63 (0.57, 0.68) | 0.37 (0.35, 0.39) |
| Fetal ultrasound | 1     | 532        | 2117    | 991.79      | < 0.001     | 0.69 (0.66, 0.72) | 0.59 (0.57, 0.60) |
| Advanced maternal age | 1     | 300        | 1507    | 321.95      | < 0.001     | 0.39 (0.35, 0.42) | 0.68 (0.67, 0.70) |

Table 8  Sensitivity and Specificity of single or multiple indications for fetal pathogenic CNV

| CNV                | Total | McNemar’s  | P value | Sensitivity | Specificity |
|--------------------|-------|------------|---------|-------------|-------------|
| Positive for single indication | 1     | 746        | 4061    | 3286.1      | < 0.001     | 0.99 (0.98, 0.99) | 0.10 (0.09, 0.11) |
| Positive for two indications | 1     | 746        | 4061    | 546.15      | < 0.001     | 0.69 (0.65, 0.72) | 0.70 (0.69, 0.72) |
| Positive for three indications | 1     | 180        | 268     | 356.25      | < 0.001     | 0.24 (0.21, 0.27) | 0.98 (0.97, 0.98) |
| Positive for four indications | 1     | 15         | 16      | 751.01      | < 0.001     | 0.02 (0.01, 0.03) | 1.00 (1.00, 1.00) |

CNV:
1-including pathogenic microdeletion/duplication, Auto A, Sex A, Auto AM, Sex AM
0-including likely pathogenic (LP-), uncertain significance (VUS-), likely benign (LB-) and benign (B-)
NIPT:
1-high risk.
0-low risk.
Maternal serum screening:
1-high risk.
0-low risk.
Fetal ultrasound:
1-abnormal ultrasound findings (grades 2–4).
0-normal or subnormal ultrasound findings (grades 0–1).
Advanced maternal age:
1-Advanced maternal age (≥ 35 yrs)
0-Maternal age (< 35 yrs).
reported to be 100% [15]. NIPT was an effective method for prenatal screening of fetal CNV ranging from 1 to 129 Mb, with a sensitivity of 84.2% [16]. The findings of this study indicated that NIPT was a reliable method for prenatal screening of fetal pathogenic microdeletion/duplication. However, NIPT cannot detect fetal polymorphism, polyploids, balanced translocations and other fetal structural abnormalities. Therefore, the combination of maternal age, maternal serum screening, fetal ultrasound scanning and NIPT had been recommended for prenatal screening in some studies [17]. Our findings were partially in agreement with this perspective. For pathogenic CNV, the sensitivity of single prenatal diagnosis indication was 0.99 (0.98, 0.99), but the specificity was 0.10 (0.09, 0.11). When prenatal diagnosis indications increased from two to four, the sensitivity was decreased to 0.02 (0.01–0.03) and the specificity increased to 1.00 (1.00–1.00). According to our data, the combination of two screening methods was possibly to achieve a maximal summation of sensitivity and specificity. Moreover, each combined screening program had its own advantages and limitations, which require comprehensive consideration by the couples and doctors.

In short, we would recommend that combined at least two kinds of prenatal screening could be used as the efficient program if medical resources for prenatal care are sufficient and the couples are willing to receive the screening.

Conclusions
Combination of cytogenetic karyotyping and CNV-Seq significantly improves the detection rate of fetal pathogenic chromosome microdeletion/duplication. NIPT was recommended for the screening of pathogenic chromosome microdeletion/duplication, and NIPT combining with other screening methods further improved the screening performance for pathogenic fetal CNV.

Abbreviations
ACMG: American college of medical genetics and genomics;
AMP: Association for molecular pathology; Auto A: Autosomal aneuploidy;
Auto AM: autosomal aneuploidy mosaic; B-CNV: benign copy number variations; CAP: College of American pathologists; CMA: Chromosome microarray analysis; CNV: Copy number variation; CNV-Seq: Copy number variation-sequencing; DS: Down’s syndrome; trisomy 21 syndrome; ES: Edward’s syndrome, trisomy 18 syndrome; FGR: Fetal growth restriction; ISCN: International system for human cytogenetic nomenclature; LB-CNV: Likely benign copy number variations; LP-CNV: Likely pathogenic copy number variations; NF: Nuchal fold; NIPT: Noninvasive prenatal testing; NT: Nuchal translucency; P-CNV: Pathogenic copy number variations; PS: Patau’s syndrome, trisomy 13 syndrome; Sex A: abnormal sex chromosome number; Sex AM: abnormal sex chromosome number mosaic; VUS- CNV: copy number variations of uncertain significance

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Authors’ contributions
Jinman Zhang has participated in the design and performance of the work, analysis of data and drafted the manuscript; Xinhua Tang, Jilin Hu, Guillin He and Jiajun Wang have participated in the performance of the work, analysis of data; Baosheng Zhu and Yingting Zhu have directed the design of the work, analysis of data and substantively revised this manuscript. Baosheng Zhu have also obtained funds to support this designated work. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets from the current study are available from the corresponding author upon request.

Declarations
Ethics approval and consent to participate
The proposal of this study was approved by the Ethics Committee of Institutional Research Board (IRB), First People’s Hospital of Yunnan Province, China (Committee reference number, KHLL2020-KY025). All study patients provided written informed consent, in the First People’s Hospital of Yunnan Province, China.

Consent for publication
All authors have agreed to submit this manuscript to your journal for consideration of publication. In addition, consent for publication from all individuals (in the case of children, their parents or legal guardians) involved in this study, including any individual person’s data in any form, such as any individual details, images or videos, has been obtained in written forms, available upon request.

Competing interests
No competing interests are declared from all authors.

Author details
1. Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, Kunming, Yunnan 650500, People’s Republic of China. 2. Department of Obstetrics and Gynecology, First People’s Hospital of Yunnan Province, No. 157, Jinfo Road, Xishan District, Kunming, Yunnan 650032, People’s Republic of China. 3. Shanghai Children’s Medical Center, Shanghai 200127, People’s Republic of China. 4. Research and Development Department, TissueTech, Inc., 7235 Corporate Center Drive, Suite B, Miami, Florida 33176, USA.

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