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CASE SERIES

Abnormal Ultrasonography Overcomes NIPT's Inherent Limitations: Revealing Two Cases of NIPT False Negatives Caused by Trisomy 21 Mosaicism and a Literature Review

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Background: Non-invasive prenatal testing (NIPT) is widely adopted for its high sensitivity in detecting chromosomal abnormalities, but its accuracy for placental and fetal mosaicism is limited due to reliance on placental DNA, leading to false results and missed diagnoses. Prenatal ultrasound may signal chromosomal risks via soft markers. This study investigates ultrasound's diagnostic value for mosaicism in NIPT false-negative cases.

Materials and Method: A retrospective analysis was conducted on two unique cases of trisomy 21 diagnosed at our hospital's prenatal diagnosis center. Case One: At 16+2 weeks of gestation, the pregnant woman's NIPT result was negative. However, ultrasound later revealed fetal cardiac abnormalities. Amniocentesis was performed for fetal karyotype analysis and chromosome copy number variation sequencing (CNV-Seq) and whole-exome sequencing (WES) were conducted. Multiple samples from the umbilical cord and placenta, retained after induced labor, were collected for CNV-Seq detection. Concurrently, peripheral blood chromosome analysis, CNV-Seq, and WES were performed on the fetus's parents. Case 2: At 18 weeks and 5 days of gestation, the pregnant woman's NIPT returned negative results. However, the subsequent ultrasound examination revealed several soft markers, prompting the performance of amniocentesis. Fetal karyotyping and chromosomal copy number variation sequencing (CNV-Seq) were subsequently performed. The pregnant woman signed the informed consent form before the test. This study received review and approval from the Ethics Committee of Suining Central Hospital (No.: KYLLMC20240020).

Results: In Case One, karyotype analysis of amniotic fluid cells revealed 46,X? psu idic(21)(q22.3), while all placental tests returned normal results. Only the umbilical cord exhibited trisomy 21 syndrome, due to inconsistent karyotypes between the fetus and placenta. In Case Two, the amniotic fluid karyotype was 47,XY,+21[14]/46,XY[50] (mosaicism ratio 22%), indicating low fetal mosaicism NIPT failed to detect both instances of placental and fetal mosaicism, because it relies on the concentration and proportion of fetal free DNA in maternal plasma.

Conclusion: Non-invasive prenatal testing (NIPT) exhibits inherent limitations in detecting placental and fetal mosaicism, whereas ultrasound soft indicators function as a "gatekeeper" in prenatal screening, thereby providing clinicians with critical evidence for decision-making.

Keywords: non-invasive DNA testing, trisomy 21, ultrasonic soft index, prenatal diagnosis limitations, mosaicism

Introduction

Non-invasive DNA testing, a prenatal screening method using high-throughput sequencing technology, shows a much higher detection rate for fetal trisomy 21, trisomy 18, and trisomy 13 syndromes than traditional serological screening.[1]

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This approach has been widely adopted in clinical practice. It works by analyzing fetal cell-free DNA (CF-DNA) found in maternal plasma. However, the accuracy of non-invasive prenatal testing (NIPT) depends heavily on the fetal concentration of CF-DNA and the karyotype of placental chromosomes. Chromosomal mosaicism involve cell lines with two or more distinct karyotypes within an individual. If abnormal cell lines are confined to the placenta (confined placental mosaicism, CPM) or are present in minor proportions, abnormal CF-DNA signals in maternal plasma may be diluted to undetectable levels, leading to "false negative" results in NIPT.[2]

Down syndrome (DS), also known as trisomy 21, is a genetic disorder characterized by the presence of an extra chromosome 21. It stands as one of the most prevalent chromosomal abnormalities globally, occurring in approximately 1 out of every 600 to 800 live births. Roughly 96% of cases involve the standard karyotype (47, XN, +21), while translocation karyotypes account for about 3–4%, mosaic karyotypes represent 1–2% of cases, and partial trisomies of chromosome 21 constitute less than 1%.[3]

Ultrasound soft indicators, such as thickened nuchal translucency (NT), unossified nasal bone, ventricular bright spots, and strong intestinal echoes, are not structural malformations but minor signs that might indicate potential chromosomal abnormalities, infections, or other pathological conditions.[4–7] The positive predictive value of these soft indicators increases significantly when multiple indicators are present simultaneously.[8] Additionally, ultrasound examinations during pregnancy can detect structural abnormalities in the fetus, especially multiple structural malformations, which may suggest underlying chromosomal abnormalities.

This article discusses two cases where non-invasive prenatal testing (NIPT) initially yielded negative results, but trisomy 21 was later confirmed through prenatal diagnosis. This diagnosis was initiated after ultrasound revealed multiple abnormal soft markers and structural malformations. The false negatives were attributed to karyotype discrepancies between the placenta and the fetus, as well as a low level of fetal mosaicism. Furthermore, one fetus exhibited a rare variant of trisomy 21. The study aims to comprehensively examine the underlying causes, diagnostic methods, and management strategies from a clinical perspective, thereby improving clinicians' understanding and awareness of such cases. We present this article in accordance with the CARE reporting checklist.

Research Subject

Case One: Pregnant woman A is 35 years old, stands 167 cm tall, and weighs 57 kg. She is G5P1, having previously given birth naturally to a full-term boy with her ex-husband. Currently in good health, she has no history of adverse pregnancy or childbirth outcomes. This pregnancy follows her remarriage, and the fetus's father is also 35 years old. It is a naturally conceived, single pregnancy, with the last menstrual period (LMP) on January 21, 2024. She has no history of cell therapy, allogeneic blood transfusion, or exposure to toxic substances, and consanguineous marriage is denied. Case Two: Pregnant woman B is 31 years old with a body mass index (BMI) of 23.23 kg/m^2 [58/(1.58 x 1.58) kg/m^2]. She has no history of cell therapy, allogeneic blood transfusion, previous medical issues, or exposure to toxic substances, and she denies consanguineous marriage. The fetus's father is also 31 years old, and this is a naturally conceived, single pregnancy. The pregnant woman signed the informed consent form before the test. This study received review and approval from the Ethics Committee of Suining Central Hospital (No.: KYLLMC20240020).

Methods

NIPT-Plus Detection

A 10 mL sample of peripheral blood was collected from pregnant women, and plasma was obtained through centrifugation. Free DNA extraction and library construction were performed using the Fetal Chromosomal Aneuploidy Detection Kit (Beijing, Berry and Kang Biotechnology Co., LTD). Sequencing was conducted with the Illumina NextSeq CN500 sequencer, followed by bioinformatics analysis to calculate the Z value for each chromosome. The normal Z value range is −3 to 3, with values exceeding 3 or falling below −3 indicating a high risk of chromosomal abnormalities.

B-Ultrasound Examination

A diagnostically qualified ultrasound physician employed the GE Voluson E8 ultrasound diagnostic instrument, utilizing a probe frequency of 2–5 MHz, to assess a fetus at 24 weeks of gestation.

Amniotic Fluid Collection

At 25 weeks of pregnancy, women undergo amniocentesis guided by B-ultrasound. Initially, 1–2 mL of amniotic fluid is aseptically extracted and discarded to eliminate the possibility of maternal cell contamination. Subsequently, approximately 20 mL of amniotic fluid is aseptically collected for fetal karyotype analysis, chromosome copy number CNV-seq detection, and whole exome sequencing analysis.

Karyotype Analysis of Chromosomes

Following the standard culture and preparation of amniotic fluid cells, karyotype images were captured using a Zeiss fully automatic microscope scanner after G, C, and N banding. Two analysts independently examined 30 karyotypes and counted 100 mitotic phases. For the rare karyotype found in Case one, additional karyotype analysis was conducted on the peripheral blood chromosomes of the fetal parents. Using conventional methods for culture, chromosome preparation, and G-banding analysis, 30 cells were counted, and 8 karyotypes were analyzed. Chromosomes were named according to the International Nomenclature System for Human Cytogenetics (ISCN 2020).

CNV-Seq Detection and Analysis

Following genomic DNA extraction, we constructed the library using the CNV-seq kit from Beijing, Berry and Kang Biotechnology Co.LTD. Sequencing was conducted on the Illumina NextSeq CN500 sequencer, achieving a sequencing depth of approximately 01×. The sequencing reads were aligned and analyzed against the human reference genome hg19 using Burrows-Wheeler Aligner (BWA) software. The clinical significance of the identified CNVs was assessed according to the ACMG rating guidelines,[9] with a resolution exceeding 0.1 Mb.

Whole Exome Sequencing Analysis

Case One: Due to fetal heart malformations, fetal whole exome sequencing analysis was conducted. Amniotic fluid and peripheral blood from the fetal parents were collected and sent to Beijing Berry and Kang Biotechnology Co., Ltd. for trio whole exome sequencing (Triose-WES). The human whole exome was sequenced using the Illumina Novaseq 6000 platform. Post-sequencing, the BWA tool aligned the data with the human reference genome GRCh38. Variant identification, including the detection and statistical analysis of SNPs and Indels, was performed using Verita Trekker®. Enliven® software annotated variants by retrieving information from external databases, while Sprinkle software conducted CNV predictive analysis based on exon data. In the familial in vitro analysis, the fetus's sequencing data were compared with the parents' data for SNP locus linkage analysis to explore potential genetic patterns.[10,11]

Placental Biopsy

Case One: Following the termination of pregnancy by pregnant woman A, eight tissue samples, each about the size of a soybean, were collected for CNV-seq verification testing. These samples were taken from the middle segment of the umbilical cord, the root of the umbilical cord, the center and edge of the placenta on the maternal side, and two sites each from the center and edge of the placenta on the fetalside.[12]

Results

NIPT-Plus Testing

Case One: Pregnant woman A underwent her initial test at 16+2 weeks of gestation. The NIPT-plus results indicated no abnormalities in chromosomal aneuploidy or pathogenic CNV variations within the test parameters, with a Z value of −0.74 for chromosome 21. Pregnant woman B was first tested at 18+5 weeks of pregnancy, and her NIPT results showed a Z value of 0.111 for chromosome 21 (Table 1).

Table 1 NIPT Test Results Table

	Gestational Weeks	Total Reads (Mb)	UniMap Reads (Mb)	Chr21 Content	Chr21 Z-value	Chr13 Z-Value	Chr18 Z-Value	Fetal (%)
Pregnant Woman A	16^{+2}	17.67	11.75	1.2932	−0.74	−0.03	−0.95	12.13
Pregnant Woman B	18^{+5}	18.72	12.15	1.3411	0.111	2.244	−0.910	13.21

B-Ultrasound Results

Case One: At 17 weeks of gestation, an ultrasound from another hospital revealed enhanced intestinal echoes in the fetal abdomen, absence of gastric bubbles, a low-lying placenta, and calcification foci on the placenta-fetal surface. By 23 +3 weeks of pregnancy, a subsequent ultrasound indicated abnormal fetal heart development, suggesting a complete atrioventricular septal defect (Figure 1).

Case Two: An ultrasound conducted at 24+3 weeks of pregnancy revealed an absence of significant ossification in the fetal nasal bone and bright spot in the left ventricle (Figure 2).

Results of Karyotype Analysis

In Case One, karyotype analysis of G band in amniotic fluid indicates that the fetus has a rare translocation trisomy 21 syndrome. The karyotype result is 46,X? psu idic(21)(q22.3). Meanwhile, the karyotypes of the fetal parents' peripheral blood chromosomes were normal (Figure 3).

Case Two: The amniotic fluid G-banding results reveal a karyotype of 47,XN,+21[14]/46,XN[50], indicating trisomy 21 syndrome of the mosaic type (Figure 4).

Case One: CNV-Seq Results and Whole Exome Sequencing Results

Case One: The amniotic fluid karyotype revealed a rare new-onset trisomy 21 in the fetus. To verify this, CNV-seq and whole exome sequencing were conducted on both parents. The amniotic fluid CNV-seq results confirmed trisomy 21 syndrome in the fetus, along with a 0.23Mb deletion (copy number: 1) in the 21q22.3 region. The CNV-seq tests of the parents showed no abnormalities. Whole exome sequencing identified a 40.31Mb duplication in the fetal amniotic fluid within the 21p12-q22.34 region, seq[GRCh38]21p11.2q22.3(6118256_46428394)×3, and a 0.23Mb deletion in the 21q22.3 region, seq[GRCh38]21q22.3(46431300_46664394del)×1. No abnormalities were detected in the parents'

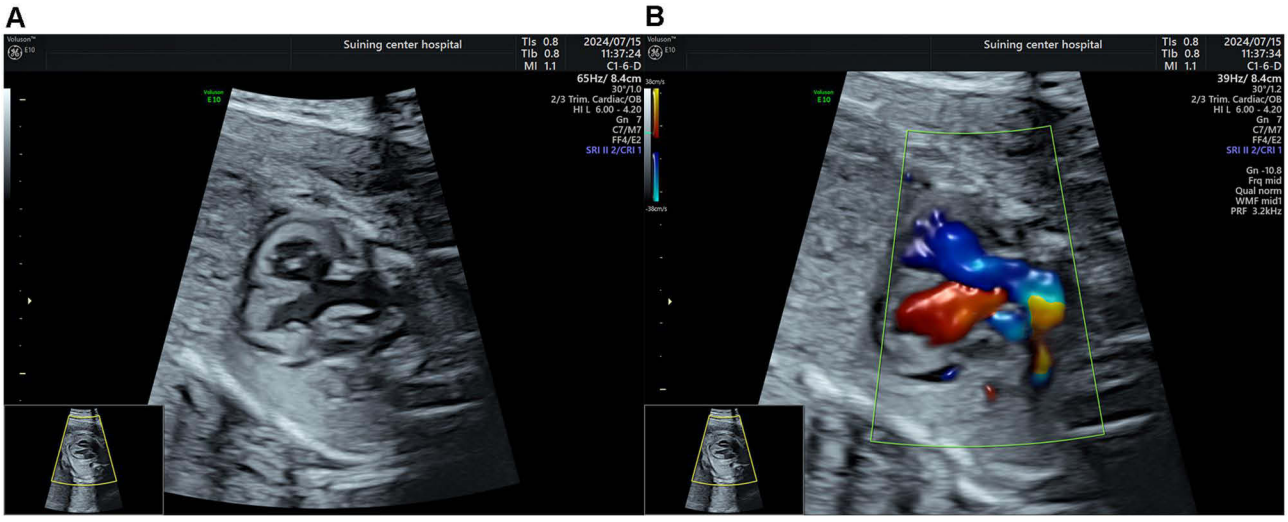

Figure 1 Shows complete atrioventricular septal defect. (**A**) Aortic override; (**B**) Blood from the left and right ventricles flows into the aorta.

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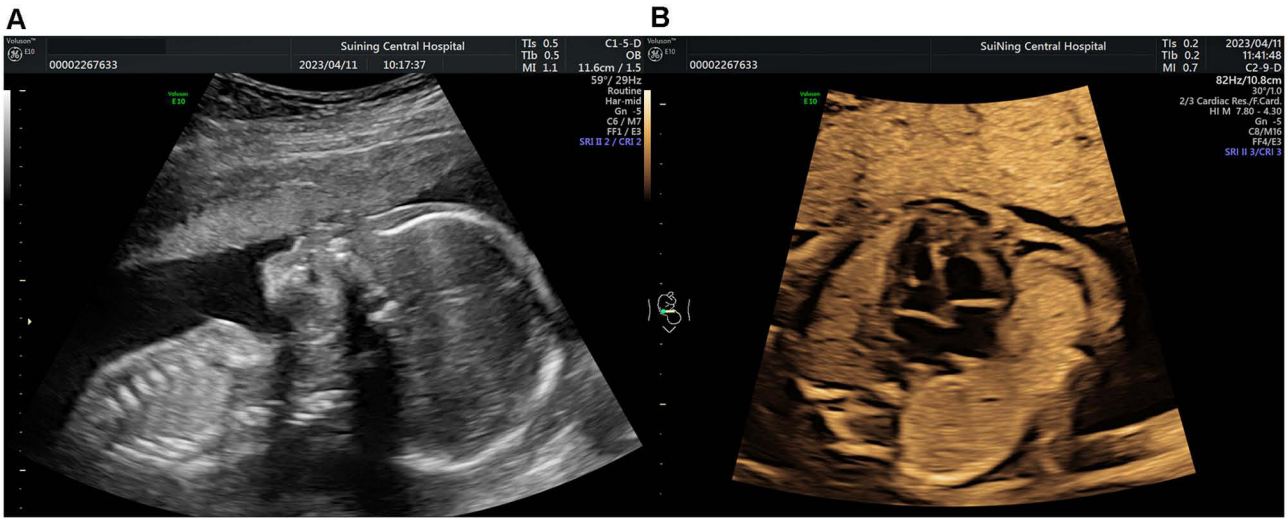

Figure 2 Shows unossified nasal bone and a bright spot in the left ventricle. (**A**) An absence of significant ossification in the fetal nasal bone; (**B**) bright spot in the left ventricle.

external examinations (Figure 5). Linkage analysis of single nucleotide polymorphisms (SNP) loci in the family revealed that the fetus inherited multiple chromosome 21 copies from the father, and both chromosome 21 copies from the father were identical (Figure 5).

Case Two: Results of Amniotic Fluid CNV-Seq

Case Two Amniotic fluid CNV-seq:seq[hg19]dup(21)(q11.2q22.3) chr21:g.14300000_48129895dup, 33.83Mb. The result was a mosaic karyotype of 47,XN,+21[25%]/46,XN[75%] (Figure 6).

Case One: Test Results of CNV-Seq in Placenta

Case One: The amniotic fluid karyotype revealed a rare new trisomy 21 in the fetus. Following the termination of the pregnancy, multiple placental tissue samples were collected (Figure 7). Four points were sampled from the fetal side: Point 1 at the middle segment of the umbilical cord, Point 2 at the root of the umbilical cord, Point 3 at the edge of the placenta, and Point 4 at the center of the placenta. Two points were sampled from the maternal side: Point 5 at the center of the placenta and Point 6 at the edge. No significant abnormalities were found at either the center or edge of the fetal and maternal sides of the placenta. Trisomy 21 was detected in both the middle segment and root of the umbilical cord, aligning with the CNV-seq results from the umbilical amniotic fluid (Figure 8). Analysis of the six loci confirmed no abnormalities in the placental tissue, while the umbilical cord and fetus exhibited trisomy 21. The discrepancy between the placental and fetal karyotypes accounted for the false negative result in the NIPT-plus test.

Literature Data Analysis

In this study, Case 1 represents a newly identified, rare 21q;21q chromosomal rearrangement responsible for a false-negative NIPT result. Employing our search strategy, we retrieved 13 additional documented cases of 21q;21q-induced false negatives for trisomy 21 in NIPT/NIPT-Plus, bringing the total to 14 cases, including this one (see Table 2). Placental analysis was conducted in 7 cases, revealing various forms of mosaicism in every instance. Notably, the literature consistently reports such cases as NIPT-negative; however, prenatal diagnosis prompted by ultrasound soft indicators ultimately confirmed mosaicism.

Discussion

The two cases presented in this study highlight an important but often overlooked clinical scenario in prenatal screening: a negative NIPT does not entirely exclude the possibility of chromosomal abnormalities, particularly mosaicism.

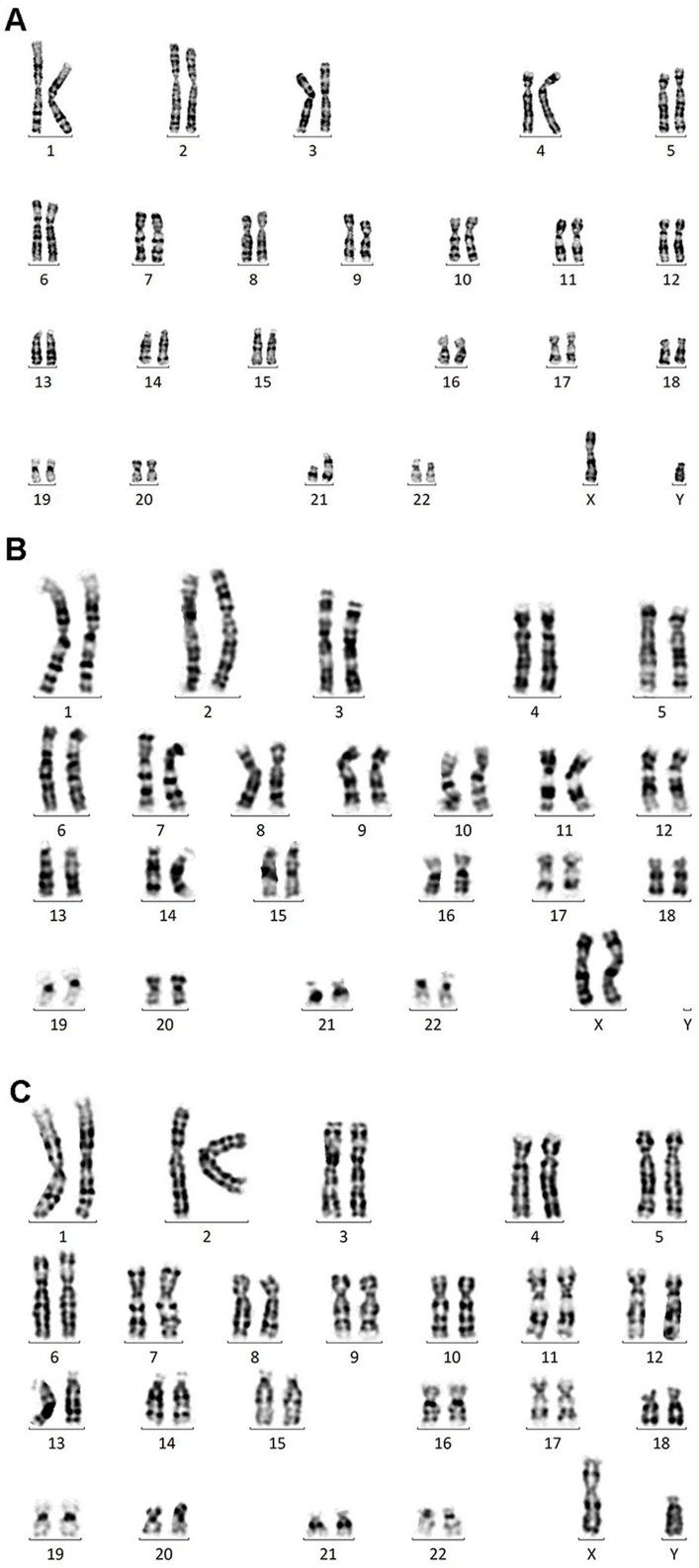

Figure 3 Shows the karyotype of Case One. (**A**) The karyotype of amniotic fluid G banding: 46,XY, psu idic(21)(q22.3); (**B**) the karyotype of the fetal mother; (**C**) the karyotype of the fetal father.

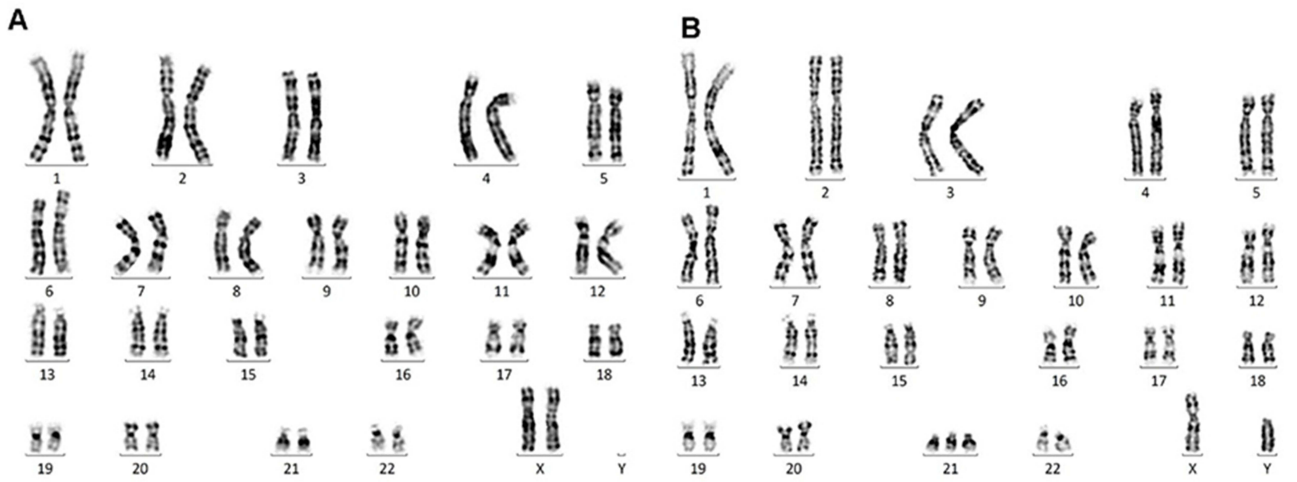

Figure 4 Shows the amniotic fluid karyotype of the fetus in Case Two: 47,XN,+21[24]/46,XN[50]. (**A**) The amniotic fluid G-banding results reveal a karyotype of 46,XN; (**B**) The amniotic fluid G-banding results reveal a karyotype of 47,XN,+21.

Mechanism of Limitations in NIPT Detection

NIPT involves high-throughput sequencing of cell-free DNA (cfDNA) in the plasma of pregnant women using large-scale parallel sequencing technology. When the sequencing volume is sufficiently high and the proportion of each chromosome falls within a specific range, bioinformatics methods can determine the proportion of each chromosome in the genome. By assessing whether the proportion of each chromosome deviates from the standard, the Z value for each chromosome is calculated. A Z value of ≥3 or ≤-3 suggests a chromosomal duplication or deletion in the fetus. The cfDNA in the plasma includes DNA from the degradation of maternal cells and "cell-free fetal DNA" (cffDNA) primarily from the apoptosis of placental trophoblast cells.[23] NIPT-Plus enhances sequencing data volume and optimizes bioinformatics algorithms based on NIPT. It expands the screening range from trisomies 21, 18, and 13 to include other rare sex chromosome aneuploidies, chromosomal deletions, and aneuploidy micro/repeat syndromes (microdeletion/microduplication syndrome, MMS). This method boasts high sensitivity and specificity,[24] but due to its detection principles and clinical practice, it remains a screening tool rather than a diagnostic one, with some false negatives and positives.[25] Factors such as restrictive placental mosaicism,[2] vanishing twin syndrome,[26] chromosomal abnormalities in pregnant women, chromosomal copy number variations, and malignant tumors with abnormal karyotypes can lead to false positives in NIPT/NIPT-Plus tests. Low concentrations of fetal cfDNA (as seen in twin or multiple pregnancies, IVF, early gestational age, maternal obesity, etc)., fetal mosaicism, discrepancies between fetal and placental karyotypes, or statistical fluctuations in Z values can result in false negatives for NIPT/NIPT-Plus.

The Limited Mechanism of NIPT in Detecting Mosaicism

The false negatives in Non-Invasive Prenatal Testing (NIPT) arise from its underlying technical principle of placental localized mosaicism (CPM). In this condition, abnormal cell lines may be present only in placental trophoblast cells, while the fetus remains normal or the mosaicism proportion is extremely low. NIPT primarily detects cell-free DNA (CF-DNA) from apoptotic placental trophoblast cells. Consequently, NIPT might indicate a high risk, yet amniocentesis results could be normal, leading to a "false positive". Conversely, if abnormal cell lines are present in the fetus rather than the placenta, or exist in very low proportions, this can result in "false negatives". Postnatal or postoperative verification of these scenarios often reveals true mosaicism. However, if the proportion of abnormal cells in the placenta is low or unevenly distributed, the weak abnormal DNA signals may not be captured by NIPT. When the proportion of abnormal cell lines in the placenta falls below the detection limit of NIPT technology (generally below 20–30%), their DNA signals are overwhelmed by those from normal cells, making effective identification difficult.

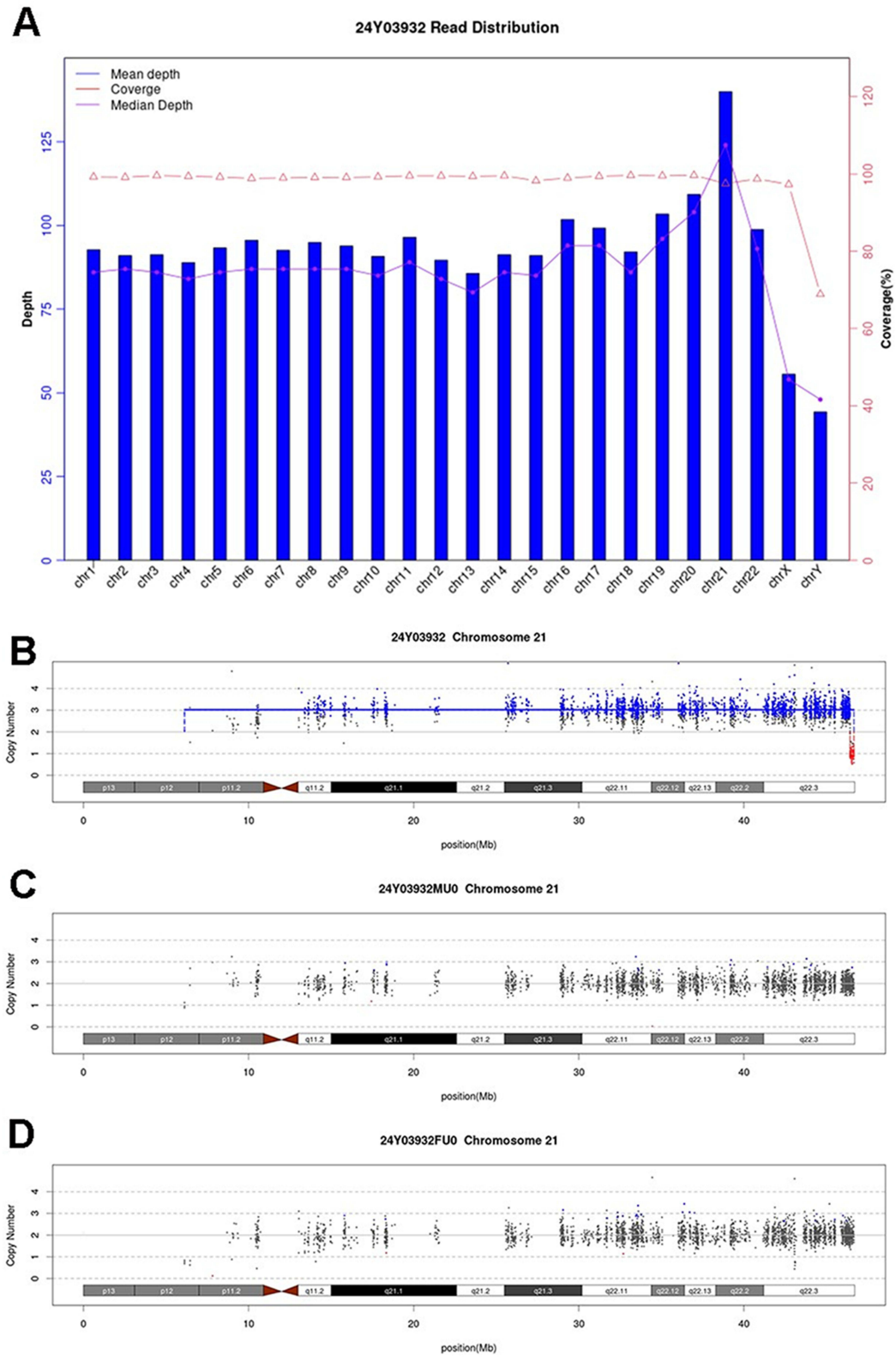

Figure 5 WES: 40.31Mb of duplication in the pediatric 21p12-q22.3 (chr21:6118256_46428394, hg38) area. (**A**) seq[GRCh38]21p11.2q22.3(6118256_46428394)×3. (**B**) a 40.31Mb duplication in the 21p12-q22.34 region, seq[GRCh38]21p11.2q22.3(6118256_46428394)×3, and a 0.23Mb deletion in the 21q22.3 region, seq[GRCh38] 21q22.3(46431300_46664394del)×1. (**C**) The normal whole exome sequencing data for chromosome 21 of mother. (**D**) The normal whole exome sequencing data for chromosome 21 of father.

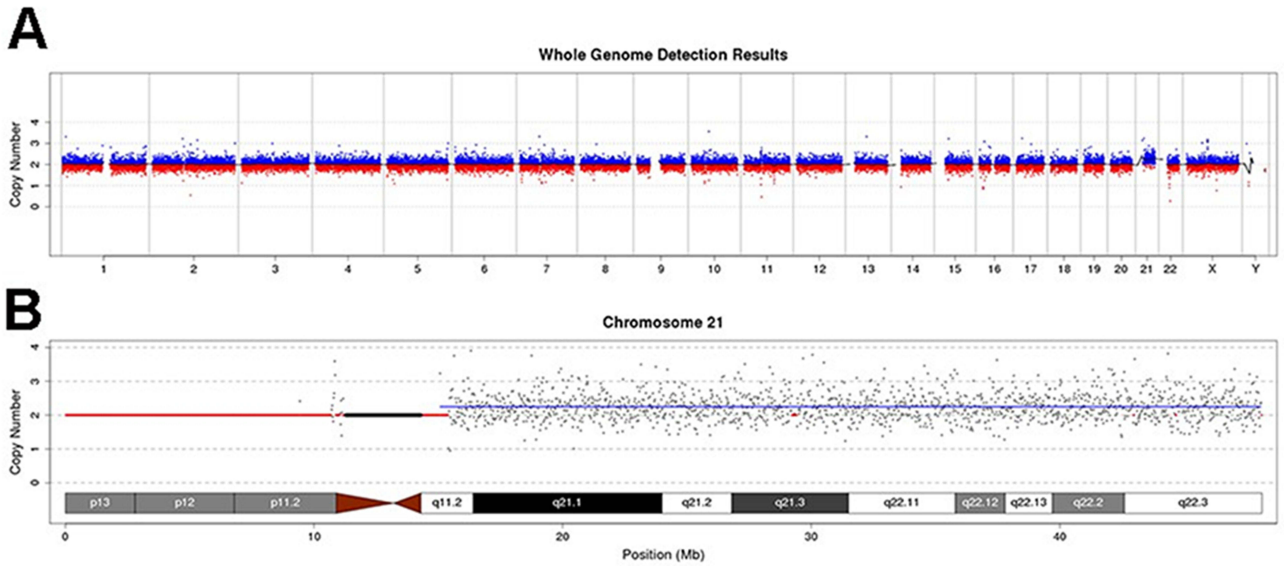

Figure 6 CNV-seq: dup(21)(q11.2, q22.3) (chr21:g.14300000_48129895, hg19) 33.83Mb, 47,XN,+21[25%]/46,XN[75%] mosaic. (**A**) All autosomal CNV-seq sequencing results of fetal amniotic fluid; (**B**) the chromosome 21 results in the fetal amniotic fluid.

The Limited Mechanism of NIPT in Detecting Mosaicism

This case exemplifies an additional factor that contributes to false - negative results in non - invasive prenatal testing (NIPT), namely, a low proportion of mosaicism. In Case 2, fetal karyotype analysis indicated a low proportion of chimeric cells, thereby confirming the existence of fetal mosaicism. The NIPT technology does not directly detect the fetus; instead, it detects cell - free DNA (cfDNA) that is released into the maternal blood subsequent to the apoptosis of placental trophoblast cells. In such circumstances, the cfDNA detected by NIPT predominantly reflects the genetic composition of the placenta. When the proportion of abnormal cells in the placenta is extremely low (eg, below 30–40%), the analysis of cfDNA in maternal blood may fail to detect an adequate number of signals to confirm a positive result, thus resulting in false - negative outcomes. As a screening rather than a diagnostic tool, NIPT has a limited detection capacity for abnormal cell mosaicism ratios below approximately 30–40%. When the proportion of abnormal cells in the placenta is low, the abnormal DNA signals released into the maternal blood become exceedingly weak and challenging to identify. In this particular case, the proportion of abnormal cells was approximately 21.9%, which is just below the current stable detection threshold of NIPT technology, making it readily classified as “low - risk”. Such situations are highly susceptible to missed diagnoses. In contrast, ultrasound screening for fetal structural abnormalities provides soft indicators that can serve as reliable evidence for further prenatal diagnosis. Therefore, ultrasound screening plays a crucial role in prenatal care.

Rare Trisomy 21 Karyotype Leads to Non-Invasive False Negative Analysis

Case 1: The analysis of the amniotic fluid cell karyotype, CNV-seq, and WES of the pregnant woman’s fetus revealed a homozygous 46,XY,psu idic(21)(q22.3) karyotype, with no evidence of fetal mosaicism. The pregnant woman was healthy, and no abnormalities were detected in the G-banding, CNV-seq, or WES analyses of her peripheral blood chromosomes, indicating no maternal chromosomal interference. Furthermore, the quality control for the three NIPT-Plus test analyses was maintained, with no fluctuations in Z values observed. It is hypothesized that the false negative result of the NIPT-Plus was due to a discrepancy between the fetal and placental karyotypes. Consequently, CNV-seq testing was conducted on multiple placental samples. The CNV-seq results showed that only the fetal umbilical cord and amniotic fluid had a consistent trisomy 21 CNV-seq. At the fetal face placental center point 1 (sampling point 3), the results were 45,XY,-21[8%]/46,XY[92%], while at center point 2 (sampling point 4), the results were 46,XY. The duplication of 21q22.3 measured 2.44Mb, and the deletion was 0.26Mb. No significant abnormalities were found in other placental tissues, suggesting that the farther from the umbilical cord, the more normal the placenta appears. As previously

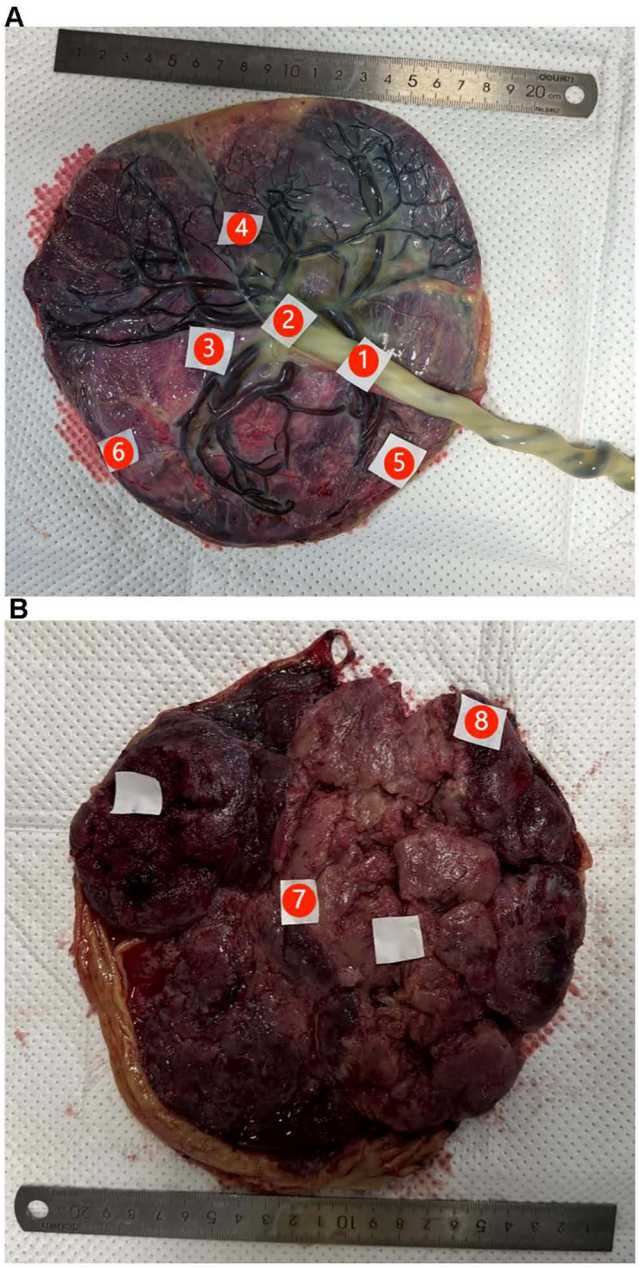

Figure 7 Placenta sampling site labeling diagram. (**A**) Fetal surface of the placenta; (**B**) Maternal surface of the placenta.

mentioned, the primary source of fetal cell-free DNA detected by NIPT-Plus is placental trophoblast cells. Similar to chorionic villus sampling, the low risk indicated by NIPT-Plus may reflect only the cytotrophoblast rather than the fetal karyotype. Thus, the degree and regionalization of placental mosaicism may reduce the effective amount of fetal DNA entering the maternal circulation, keeping it below the NIPT detection threshold and resulting in false negatives. Huijsdsen-van Amsterdam et al[18] found that among 646 cases diagnosed with trisomy 21 syndrome, 9 had false negative NIPT results, with 5 involving 21q; 21q rearrangement. Combined with other literature, the proportion of 21q; 21q rearrangement in NIPT false negatives is as high as 28%, significantly higher than the 2% in live births of children with Down syndrome. Literature review shows that false negatives of trisomy 21 NIPT due to 21q; 21q rearrangement have been reported in 13 cases worldwide. Including this case, there are now 14 cases (see Table 2). Of these, 7 placentas were analyzed, revealing varying degrees and forms of mosaicism. Research indicates that trisomy 21 with 21q rearrangement is more likely to cause false negative NIPT results than standard trisomy 21. The standard trisomy 21 arises from the

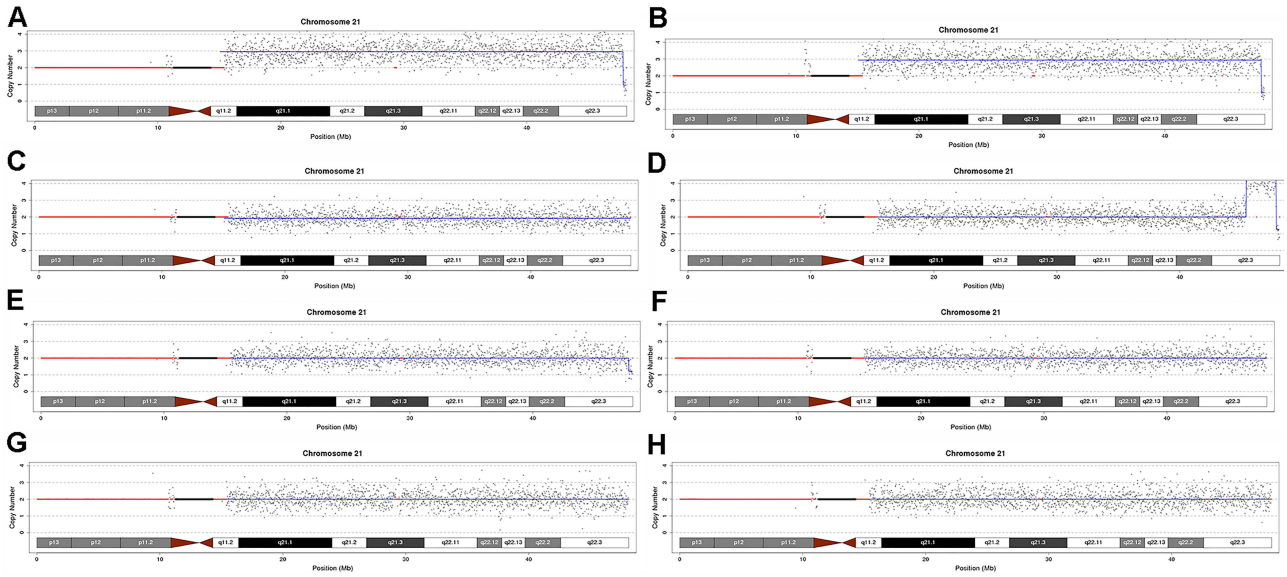

Figure 8 Placenta sampling and CNV-seq test results. (**A**) 47,XY,+21, del(21q22.3) 0.26Mb. (**B**) 47,XY,+21, del(21q22.3) 0.26Mb. (**C**) 45,XY,-21[8%]/46,XY[92%]. (**D**) 46,XY, dup(21q22.3) 2.44Mb, del(21q22.3) 0.26Mb. (**E**) 46,XY, del(21q22.3) 0.26Mb. (**F**) 46,XY. (**G**) 46,XY. (**H**) 46,XY.

nondisjunction of chromosome 21 during gamete meiosis, forming a trisomy 21 zygote that differentiates into a placenta and fetus with a trisomy 21 karyotype. In contrast, 21q; 21q rearrangement often results from centromere misdivision or U-shaped exchange of sister chromatids during zygote post-mitosis. This variation can occur at different stages of early embryonic development, leading to an inconsistent distribution of normal and abnormal cells, which may result in

Table 2 Case Characteristics of 14 Cases with 21q;21q Rearrangement Leading to False-Negative Results in NIPT/NIPT-Plus

Case No.	Author & Year	Method	Gestational Weeks	Risk/ Screening	Ultrasound Findings	Karyotype Results	Placental Findings
1	Wang Y et al[13]	MPSS (2013)	Serological screening revealed a T21 risk of 1/370	32	18w	15.60%	2.04
2	Zhang H et al[14]	MPSS (2015)	Serum screening revealed a T21 risk of 1/590	26	25w	13.4%	low risk
3	Willems et al[15]	tMPS (2016)	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded
4	Oepkes et al[16]	MPSS (2016)	Serological screening revealed a T21 risk of 1/140	34	13w+5d	10.5%	0.68
5	Wen Ping et al[17]	Not recorded (2017)	Serum screening revealed a T21 risk of 1/55	27	18w	Not recorded	Low risk
6	Huijsdens-vanAmsterdam et al[18]	MPSS (2018)	10w+6d	7% fetal fraction	No abnormalities at 19w+2d	46,XX,der(21;21)(q10;q10), +21q rearrangement	Not studied
7	Huijsdens-vanAmsterdam et al[18]	MPSS (2018)	12w+3d	T21 risk 1/300-1/70	No abnormalities at 19w+4d	46,XX,i(21)(q10)	Not available

(*Continued*)

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Table 2 (Continued).

Case No.	Author & Year	Method	Gestational Weeks	Risk/ Screening	Ultrasound Findings	Karyotype Results	Placental Findings
8	Huijsdens-vanAmsterdam et al[18]	tMPS (2018)	23w	17.2% low-risk	Short nasal bone, short femur, borderline Doppler	46,XX,der(21;21)(q10;q10), +21q rearrangement	Not available
9	Huijsdens-vanAmsterdam et al[18]	tMPS (2018)	13w	12.7% low-risk	NT thickening, tricuspid regurgitation	46,XX,der(21;21)(q10;q10), +21q rearrangement	Not available
10	Xu HH et al[19] (2020)	MPSS	17w+5d	T21 risk 1/592	Not recorded	46,XY,der(21;21)(q10;q10),+21	Not recorded
11	Feresin A et al[20] (2022)	MPSS	10w+5d	8% fetal fraction	NT=3.35mm, continued increase	46,XX,+21,der(21;21)(q10;q10)	50-60% embedded T21
12	Zhao Q et al[21] (2023)	MPSS	28w	T21 risk 1/529	Pleural effusion, nasal hypoplasia	46,XX,+21,der(21;21)(q10;q10)	13-88% T21 mosaic
13	Sun Weijia et al[22] (2023)	Not recorded	31w	Not recorded	Duodenal obstruction, vascular abnormalities	46,XN,i(21)(q10)	Maternal surface 21%, subplacental 9%
14	This study (2024)	MPSS	16w+2d	Advanced maternal age (35y)	Cardiac abnormalities, absent gastric vesicle	46,XY,psu dic(21)(q22.3)	Focal abnormalities, umbilical cord trisomy 21

Abbreviations: MPSS, Massively Parallel Signature Sequencing; tMPS, targeted Massively Parallel Sequencing.

mosaicism of normal or abnormal karyotypes in the placental trophoblast, or even completely normal karyotypes, while the fetus is abnormal. This discrepancy causes inconsistency between fetal and placental karyotypes.[13–22]

The formation of an equal-arm pseudobicentromere chromosome with a "mirror image" duplication at the end of chromosome 21's long arm may involve telomere fusion, mutual translocation between homologous chromosomes' long arms (or sister chromatids) during meiosis, or translocation[2] between two chromosome 21s during early mitosis in 21-trisomy. This article employs SNP locus linkage analysis through familial whole exome sequencing to investigate the origin and mechanism of the rare trisomy 21 karyotype 46,XY,idic(21)(q22.3) in fetuses. The analysis of single nucleotide polymorphism (SNP) loci in the studied family reveals that the fetus possesses two homozygous 21p12-q22.3 repeat segments from the father, with a symmetry loss in the terminal region of 21q22.3. However, no abnormalities were detected in the karyotype of the fetal parents, nor through CNV-seq detection and WES analysis. Based on these findings, it is suggested that during early mitosis of the fertilized egg, a deletion occurs in the terminal region of the paternal chromosome 21's long arm, followed by the fusion of sister chromatids in this region, indicating that the 21q rearrangement in the fetus originates from one of the father's normal chromosome 21s and is a de novo variation. Variations can occur at different stages of early embryonic development, potentially resulting in mosaicism with normal or abnormal karyotypes in the placental trophoblast, leading to false negative results. Among 13 cases reviewed in the literature, all false negatives of NIPT caused by 21q rearrangement were of the synthmic fusion type 21q. The 21q rearrangement chromosome, featuring inverted fusion at the 21q22.3 band and a monomeric isocentromeric trisomy 21 at the terminal part of 21q22.3 [46,X?,idic(21)(q22.3)], represents a rare trisomy 21 karyotype. Over the years, only a few dozen cases have been documented,[27–30] and false negative NIPT results due to this karyotype have not been previously reported. To our knowledge, this article is the first to report false negative results of NIPT Plus caused by this karyotype.

The terminal deletion of the idic(21)(q22.3) karyotype, designated as 46,X?, results in a partial monomer at the terminal end. This deletion does not affect the Down syndrome critical region (DSCR), so the phenotypes of these patients generally align with those of Down syndrome caused by free trisomy 21, with only minor differences.[31,32] Cardiac abnormalities, such as ventricular septal defect (VSD), are detected in 15.9% of fetuses with trisomy 21, while

the absence of a gastric bubble is rare. The Putra M study identified a case of 46,XX,idic(21)(q22.3) with cardiac abnormalities and no gastric bubble, similar to the ultrasound findings in this study. The fetuses in this study exhibit a 21q rearrangement, likely due to telomere fusion of the sister chromatids of chromosome 21 from the father during early mitosis of the fertilized egg. This variation, occurring early in embryonic development, results in a mosaic combination of normal and abnormal karyotypes in the placental trophoblast. Consequently, the fetus with an abnormal karyotype of 46,XY,idic(21)(q22.3) can lead to false negatives in the NIPT-Plus test. This article reports the first instance of false negative NIPT-Plus results caused by the 46,XY,idic(21)(q22.3) karyotype. A literature review identified 13 other cases of 21q rearrangement, with false negative NIPT results found in 14 cases. Analysis of the placentas in 7 cases revealed placental mosaicism in all. The 21q rearrangement typically occurs post-fertilization due to incorrect centromere division or U-shaped exchange between sister chromatids, forming 21q after zygote development. This rearrangement often results in placental mosaicism, which can reduce the effective output of fetal DNA into the maternal circulation, keeping it below the NIPT detection limit and leading to false negatives. Although NIPT/NIPT-Plus technology is highly accurate for detecting common fetal aneuploidies, complex chromosomal rearrangements like 21q can result in false negatives due to biological mechanisms, not sequencing quality. Therefore, in clinical practice, if low-risk NIPT/NIPT-Plus results coincide with abnormalities in later ultrasound monitoring, timely interventional prenatal diagnosis is essential to rule out fetal chromosomal abnormalities and prevent false negatives.

Clinical Value and Decision-Making Role of Ultrasound Soft Indicators

In this study, abnormal ultrasound findings, particularly soft ultrasound indicators, emerged as crucial in addressing the "blind spot" of NIPT. Both pregnant women exhibited abnormal ultrasound results. In Case Two, the presence of multiple (≥2) abnormal soft indicators prompted clinical alerts, leading to invasive diagnostic procedures. This observation strongly aligns with numerous domestic and international guidelines, which assert that abnormal ultrasound structures or multiple soft indicators independently warrant prenatal diagnosis, irrespective of NIPT[14] outcomes. Ultrasound provides a real-time evaluation of fetal morphology, directly reflecting potential fetal issues without being influenced by placental biology. Thus, it serves as a vital tool for verifying and supplementing NIPT's limitations. Regarding prenatal diagnosis strategies and genetic counseling for mosaicism, once mosaicism are confirmed, a meticulous diagnostic process and comprehensive genetic counseling become essential. Multi-tissue sampling is recommended, with amniocentesis as the preferred initial method. However, to authenticate mosaicism and evaluate the true mosaic ratio in the fetus, umbilical cord blood puncture is often advised, as CVS may misdiagnose CPM and is not the first choice. Amniotic fluid cells primarily originate from fetal skin, urinary tract, and digestive tract cells, while umbilical cord blood reflects the fetal hematopoietic system. Combining these methods offers a more thorough assessment. Prognostic assessment poses challenges due to the wide phenotypic spectrum of trisomy 21 mosaicism, which can range from nearly normal to typical Down syndrome features. The severity is closely linked to the distribution of abnormal cells across various organ tissues complicating prenatal consultation.[15] Families must be thoroughly informed about the prognosis's uncertainty, potential outcomes, and the significance of long-term follow-up through a multidisciplinary team (MDT) consultation, involving prenatal diagnosticians, geneticists, neonatologists, and others. Postpartum verification and follow-up, as illustrated in Case One, should include karyotype analysis of multiple tissues, such as peripheral blood and skin fibroblasts, after delivery to confirm the diagnosis. Immediate initiation of long-term developmental monitoring and intervention support is crucial.

Innovation and Clinical Implications of This Study

This case report is innovative in its clinical cautionary emphasis, using typical cases to caution clinicians against over-reliance on NIPT results. A "NIPT negative" result does not guarantee the fetus's absolute safety. The report underscores the critical importance of the traditional clinical decision-making pathway: from identifying abnormal soft ultrasound indicators to genetic counseling and invasive diagnosis. This pathway remains indispensable, even in the NIPT era, and should not be neglected. Additionally, the report discusses management strategies in detail, outlining the comprehensive clinical management process from discovery and diagnosis to consultation of fetal mosaicism, offering a practical reference for colleagues dealing with similar cases.

Conclusion

Non-invasive prenatal testing (NIPT) has limitations, as illustrated by these two cases of low-proportion mosaicism or structural abnormalities. Ultrasound, particularly the detection of soft markers, plays a complementary role. When multiple abnormal soft markers are present, offering invasive prenatal diagnosis (eg, karyotyping) may be considered even after a low-risk NIPT result, as part of informed shared decision-making. Prenatal specialists should remain aware of these limitations, integrate various techniques, and provide balanced counselling to expectant mothers.

Data Sharing Statement

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

Ethics Approval and Consent to Participate

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethic committee of Suining Central Hospital KYLLMC20240020. This approval covers the publication of case details and accompanying images. The written informed consent was obtained from the participants.

Consent for Publication

Written informed consent was obtained from the patients for publication of the details of their medical cases and any accompanying images. The institutional approval to publish this case was granted by the Ethics Committee of Suining Central Hospital (KYLLMC20240020).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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