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A gradual change of chromosome mosaicism from placenta to fetus leading to T18 false negative result by NIPS

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ABSTRACT

Background: Noninvasive prenatal screening (NIPS) has higher sensitivity and specificity compared to traditional prenatal screening. Nevertheless, the discordant results between the NIPS and prenatal diagnosis were occasionally reported. In current study, we investigated the genetic basis of a T18 fetus with a discordant trisomy 5 (T5) positive and trisomy 18 (T18) negative NIPS result.

Methods: NIPS was used to detect fetal DNA in maternal circulating plasma based on semiconductor sequencing platform. The aneuploidies of the fetus and different part of placental tissues were investigated by copy number variation sequencing (CNV-seq) and chromosome microarray analysis (CMA).

Results: The positive result of T5 was detected for the pregnant woman in NIPS, while T18 was found in the fetal karyotyping analysis after amniocentesis. Furthermore, placental mosaicism of T5 and T18 was found by CNV-seq and CMA, which revealed the mosaic ratio of T5 was gradually increased from umbilical cord to the placenta center, while that of T18 was gradually decreased.

Conclusion: For the reason of cell-free fetal DNA (cff DNA) in the maternal circulation originates from trophoblast cells of placenta, the level of placental mosaicism could cause false negative NIPS result in multiple aneuploidies. The present study proved that a discordant T5 positive and T18 negative NIPS result was caused by placental mosaicism. This study highlights placental mosaicism as a significant risk factor for discordant NIPS results. The result will be helpful for genetic counseling and clinical management of such pregnant woman.

1. Introduction

Since cell-free fetal DNA (cffDNA) fragments could be isolated and analyzed from the blood of pregnant women by Y M Dennis Lo [1], noninvasive prenatal screening (NIPS), aligning and counting DNA fragments floating in the plasma of pregnant women based on massively parallel sequencing, had gradually become integrated into conventional prenatal screening [2–4]. Till now, NIPS has been successfully applied to routine prenatal screening for fetal aneuploidy due to its current acceptable price, non-invasive and high accuracy [5–8].

NIPS is considered more reliable and accurate than traditional prenatal screening [9]. Several studies have validated that NIPS is well evidenced for detecting trisomy 21, 18 and 13 [10,11]. In a meta-analysis, the detection rate was found to be 99.0% with a 0.08% false positive rate for T21, 96.80% with 0.15% for T18, and 92.1% with 0.20% for T13 [12]. Nevertheless, NIPS is still considered as a screening technology closely to diagnosis since the small chance of false positive or false negative results. Several significant contributing factors , resulting in discordant results between NIPS and prenatal diagnosis of amniocytes , have been identified, which include confined placental mosaicism (CPM) [3,6–8], placental mosaicism [9,11,13], fetal mosaicism and maternal mosaicism [14]. The inconsistent karyotype between the placenta and the fetus is an important cause affecting the accuracy of NIPS results. One previous study on clinical application of NIPS suggested that two of 28,739 patients were reported as false negatives of T21 [11]. In a case, low level of T18 placental mosaicism was the cause of false negative NIPS result [13]. Another case of the false negative result in NIPS was caused by placental trisomy 18 mosaicism [14]. As false negatives are more likely to cause clinical misdiagnosis, there is significant meaning to study the cause of false negatives.

In present study, we describe an unusual pregnant woman who was detected T5 by NIPS while the fetal karyotyping revealed T18 result

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after amniocentesis. And the biological basis of this discordant NIPS results was further investigated by CNV-seq and CMA. Furthermore, relevant literatures in recent years were searched and analyzed. This study provided a new literature and evidence to prove that placental mosaicism is a significant risk factor for false negative NIPS results. Moreover, the result will be helpful for genetic counseling and clinical management of such pregnant woman.

2. Material and methods

2.1. Non-invasive prenatal screening

Blood sample was analyzed using a sequencing approach based on semiconductor sequencing platform [12]. Briefly, cell-free DNA from 600 μL of maternal plasma was extracted using the QIAamp DSP DNA Blood Mini Kit (Qiagen) following the blood and body fluid protocol. DNA from maternal plasma was used for library construction according to the Ion Plus Fragment Library Kit (Life Technologies), and semiconductor sequencing was performed using an Ion Proton sequencer at 400 flows according to the manufacturer's instructions (Life Technologies). After GC-content was normalized for Chr13, 18, 21 and ChrX, uniquely mapped reads were counted and *Z*-scores were calculated for each chromosome [15,16]. We judged chromosomal aneuploidy according to the normal range of Z-scores,-3.0 < Z < 3.0 [17].

2.2. Karyotype analysis

Amniocytes samples were used for karyotype analysis. According to the protocols, trypsin digestion and cell harvesting, hypotonic swelling, fixing, and slide preparation were carried out sequentially. Metaphase karyotypes were banded by trypsin digestion followed by Wright's staining and G-banded analysis. The pregnant woman and her husband's Peripheral blood lymphocytes were harvested after cultured for 72 h in medium according. Then, Metaphases were dropped in a Termotron chamber and baked for 1 h and 30 min at 100 °C. G-banding was performed according to standard cytogenetic methods using trypsin and Leishman stain [16]. Thirty GTL-banded metaphase cells were evaluated.

2.3. Chromosome microarray analysis

Genomic DNA was extracted using Blood Genomic DNA Extraction kit (Qiangen, America) according to the manufacturer's instructions. A CytoScan 750K array (Affymetrix CytoScan 750K Array, America) was used for chromosomal aneuploidy analysis. The procedures for DNA digestion, amplification, segmentation, labeling and hybridisation with the arrays were performed according to the manufacturer's standard protocols (Affymetrix, America). The results were analyzed using Chromosome Analysis Suite software (ChAS). For the interpretation of the data, the following public databases were used: database of genomic variants (DGV; http://projects.tcag.ca/variation/), database of chromosomal imbalance and phenotype in humans using ensembl resources (DECIPHER; http://decipher.sanger.ac.uk/), online mendelian inheritance in man (OMIM; http://www.omim.org), ISCA search (http://dbsearch.clinicalgenome.org/search/) and ClinVar of NCBI (https://www.ncbi.nlm.nih.gov/clinvar/).

2.4. Copy number variation sequencing

Copy number variation sequencing was performed according to a previous study [18]. Briefly, fifity nanograms of genomic DNA was fragmented to an average size of 300 bp. After sequencing libraries prepared, HiSeq 2000 platform (Illumina Inc.) was used to sequence the libraries to generate approximately 8 million 36-bp single-end reads representing 0.1-fold genome coverage. Then, all the sequences were aligned to the unmasked hg19 genome using the Burrows-Wheeler algorithm. A minimum of 20 batched test samples were internally compared with each other as reference using the data processing and analysis algorithms. For each chromosome, we plotted the mean $\log_2$ values of the normalized sequencing reads versus the number of sequential 5-kb sliding 60-kb sequencing bins. The mean $\log_2$ value was then calculated along the length of each chromosome. The theoretical $\log_2$ value for a duplication (three copies) is log2 [1.5] = 0.58 and for a deletion (one copy) is log2 [0.5] = −1.0. Cutoff copy number values used to call duplications were set at > 2.8 ($\log_2$ [1.4] = 0.49), and those used to call deletions were set at < 1.2 ($\log_2$ [0.6] = −0.74). Chromosome representation (CR) of Chr5 or Chr18 in each sample was calculated by Chr5 or Chr18 sequence reads/total sequence reads. Levels of trisomy mosaicism were subsequently determined using the formula: CR Chr5/Chr18 in the test sample/mean CR of Chr5/Chr18 in the reference sample × 100%.

2.5. Literature search

We performed a review of literature published between 1st. of January 2000 and 31th. of December 2018 in the online PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/?term=). The search combination used was (NIPT/NIPS [all fields] OR (Non-Invasive Prenatal Testing)) AND (False negative [all fields] OR discordant [all fields] AND T18 [all fields] OR Trisomy 18). Only manuscripts written in English were included.

3. Results

3.1. The discordant prenatal diagnosis results for an unusual pregnancy

A 36-year old pregnant woman came for prenatal counseling. She and her husband had unremarkable personal and family histories. After genetic counseling, the patient declined amniocentesis due to the associated risks, and she opted for NIPS to exclude chromosomal abnormalities. The sequencing result showed that the fetal risk of trisomy13, 18 and 21 were all low (*Z*-score for T21 = −1.132, T18 = 0.684 and T13 = −0.817), but the fetus was highly suspected with T5 (Z-score = 6.327) at 20^{+4} weeks' gestational age. After further genetic counseling, ultrasound examination was performed for the fetus but no abnormality was found. Then the woman agreed to adopt amniocentesis for fetal karyotyping at 22 week's gestational age. The karyotyping analysis of the amniocytes revealed a T18 karyotype (Fig. 1). The karyotyping analysis of the pregnant woman and her husband's peripheral blood lymphocytes were also preformed which revealed normal karyotypes. After careful genetic counseling, the parents elected to terminate pregnancy at 26 week's gestational age.

3.2. Placental mosaicism analysis of T18 and T5 in different samples

In order to investigate the reasons for the inconsistency between NIPS and karyotype analysis of amniocytes, tissue samples from umbilical cord (UC), and placenta center (PC), four different edges of the placenta (PE-1, PE-2, PE-3, PE-4) at the maternal side (Fig. 2) were collected for further examination. Placental mosaicism of T18 and T5 was detected by CNV-seq based on the DNA extracted from the tissue collected above. The different mosaic ratios of the different tissues were listed in Table 1. The mosaic ratios of T18 in UC, PC, PE-1, PE-2, PE-3, PE-4 were 100%, 18%, 37%, 96%, 75% and 34% respectively. And the mosaic ratios of T5 in UC, PC, PE-1, PE-2, PE-3, PE-4 were 0%, 82%, 63%, 4%, 25% and 66% respectively. Similar results were also detected by CMA (Table 1). According to the Whole Genomic View of different samples detected by CMA (Fig. 3), the mosaic ratio of T5 was gradually increased from umbilical cord to placenta center while that of T18 was gradually decreased.

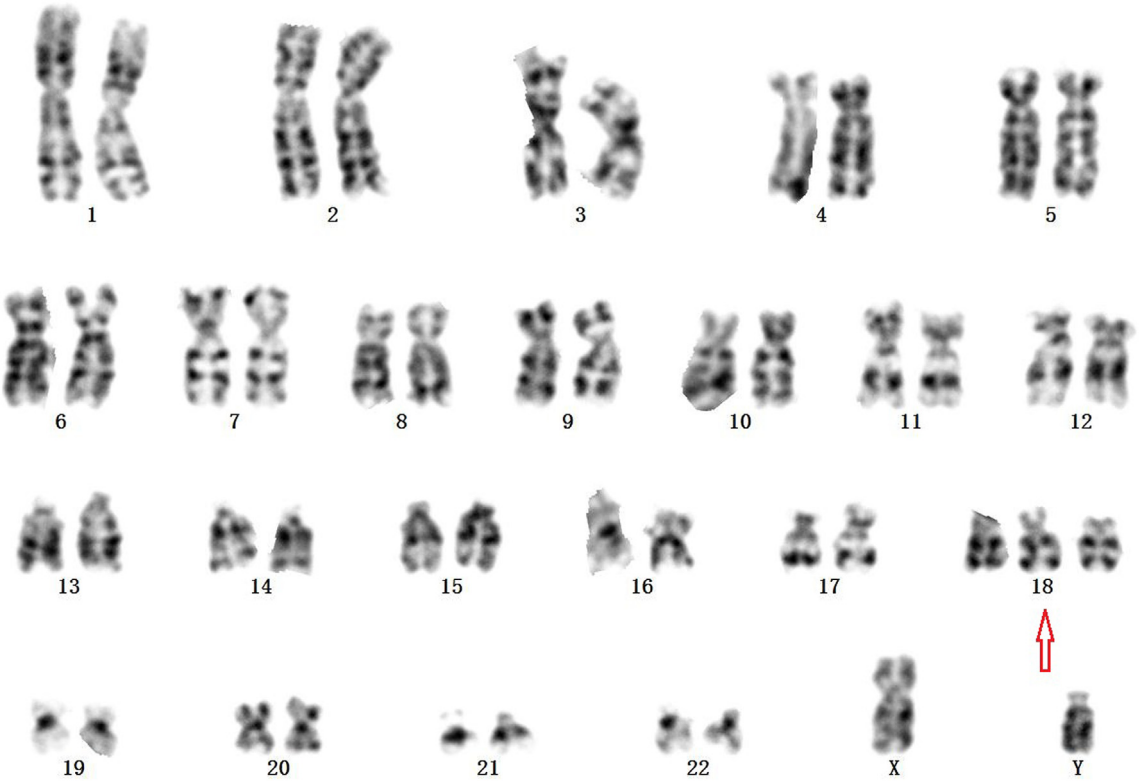

Fig. 1. G-banded karyotype analysis of cultured amniocytes shows the fetal karyotype as 47, XX, +18 (trisomy 18).

3.3. Literature search

Relevant literatures, published between 1st. of January 2000 and 31st. of December 2018 in the online PubMed database, were searched and analyzed. A total of eight articles involving ten negative T18 cases in NIPS were identified in PubMed database (Table 2). Among the ten cases, five were detected as normal in NIPS, two were high risk of T21, and three were high risk of sex chromosome abnormalities (45, X or 47, XXX). For the karyotype result of the ten cases, seven were proved to be T18, and other three were showed T18 accompanied by other chromosomal abnormalities. An overview of reasons for T18 false negative results in NIPS were summarized in Table 2. Placental mosaicism was frequently involved and was the most common cause of T18 false negative result in NIPS. In our case, a discordant NIPS result in a T18 pregnancy was caused by T5 and T18 placental mosaicism, which was never been reported in previous studies.

4. Discussion

NIPS was established as an additional pregnancy test for detecting the fetal T21, T18 and T13, which was rapidly becoming a common clinical practice in recent years [9,19]. However, false positive and false negative results till exist, as cffDNA originates from apoptotic placental trophoblast cells [20]. Thus, NIPS is still an aneuploidy screening test

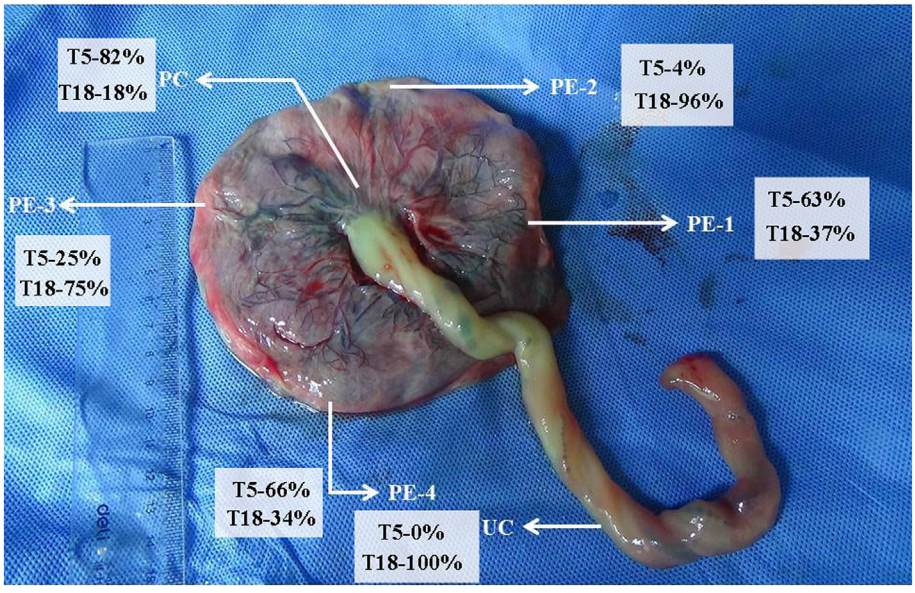

Fig. 2. Placental samples at different positions were collected from the maternal side of placenta for CMA and CNV-seq analysis. The position and the corresponding chimeric ratios were marked and shown in the figure, respectively. UC: Umbilical cord, (T5, 0%; T18, 100%); PC: Placenta center (T5, 82%; T18, 18%); PE-1: Placenta edge 1, (T5, 63%; T18, 37%); PE-2: Placenta edge 2, (T5, 4%; T18, 96%); PE-3,: Placenta edge 3, (T5, 25%; T18, 75%); PE-4: Placenta edge 4, (T5, 66%; T18, 34%).

Table 1
Different mosaic ratios of T5 and T18 in different samples.

	Mosaic ratio			
	T18(%)		T5(%)	
	NGS	CMA	NGS	CMA
Placental location				
UC	100	100	0	0
PC	18	22	82	64
PE-1	37	41	63	73
PE-2	96	86	4	27
PE-3	75	64	25	38
PE-4	34	41	66	77
average	52	50.8	48	55.8
Placenta	39.25	40	60.75	58.875

which should be followed by diagnostic testing such as amniocentesis or chorionic villus sampling (CVS) for aneuploidy confirmation and clinical management of the pregnancy [21,22]. The low level of cffDNA fraction in maternal plasma can result in a false negative NIPS result. The maternal overweight can significantly decrease the fetal DNA fraction in maternal plasma, which usually leads to the false negative result in NIPS [23]. In addition, a vanishing twins, fetal mosaicism or placental mosaicism can also cause false negative results in NIPS [14,17]. Most importantly, cffDNA in maternal peripheral blood originates from trophoblastic cells of the placenta but not the fetus, which destines that NIPS cannot provide a definite diagnosis [24,25].

The Circulating cell-free (ccf) DNA fragments in maternal plasma, which are sequenced in NIPS, originate from both the mother and the placenta. The proportion of cffDNA derived from the placenta is known as the fetal fraction. The average fetal fraction in maternal plasma is ranged from under 3% to over 30% when measured between 10 and 20 gestational weeks. Generally, the cffDNA concentration > 4% are considered suitable for NIPS [26,27]. In this case, the cffDNA fraction in the maternal plasma at 20^{+4} GA was 10% which was enough for NIPS detection. However, placental mosaicism of T18 and T5 was found based on the examination result at six different placental sites by CNV-seq and CMA. Since the average mosaic ratio of T18 in placenta was approximately 40%, the effective cffDNA fraction observed by the algorithm on the T18 was about 40% × 10% = 4%. A trisomy with an actual fetal fraction of 4% is mathematically insufficient to detect the

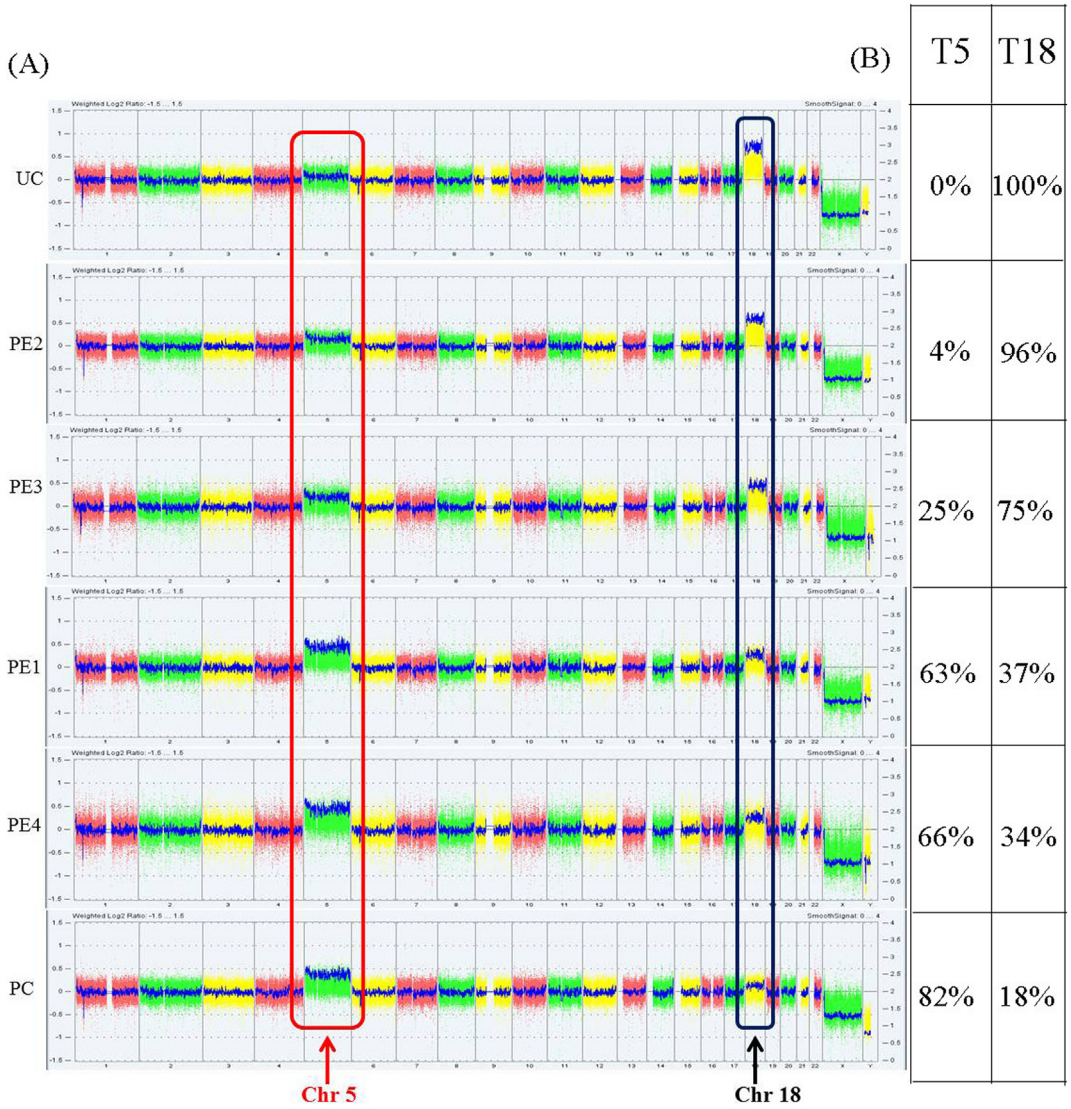

Fig. 3. Samples at different positions on the placenta were used for CMA and CNV-seq. (A) The Whole Genomic View of different sanples detected by CMA. The chimeric levels of T5 in different samples were shown in red frame and the chimeric levels of T18 were shown in black frame. (B) The corresponding chimeric ratios of T5 and T18 were detected by CNV-seq. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Published cases of false negative T18 by NIPS.

Case number	Age/pregnancy week	Clinical indications	NIPS results	Karyotype	Explanation for false negative NIPS result	References
1	39 years/12 W	Senior age of pregnant	High risk for T21	47,XY,+21/48,XY,+18,+21 in CVS	T18 and T21 placental mosaicism	Canick et al. [21]
2	22 years/17 W	Med risk for T18 by serums screening	High risk for T21	47,XX,+18 by amniocentesis	T18 and T21 placental mosaicism	Mao et al. [3]
3	24 years/17^{+4}W	High risk for T21 by serums screening	High risk for monosomy X	47,XX,+18 by amniocentesis	T18 and 45,X placental mosaicism	Pan et al. [22]
4	27 years/17^{+3}W	High risk for T13 by serums screening	Normal	47,XY,+18 in products of conception	Unknown	Zhang et al. [23]
5	29 years/19^{+4}W	Med risk for T21 by serums screening	Normal	47,XY,+18 in products of conception	T18 and tetraploidy placental mosaicism	
6	24 years/20^{+4}W	High risk for T21 by serums screening	High risk for monosomy X	47,XX,+18 by amniocentesis	T18 and 45,X placental mosaicism	
7	42 years/13^{+2}W	High risk for fetal T21	High risk for trisomy X	48,XXX,+18 by amniocentesis	47,XXX and T18 placental mosaicism	Y. Gao et al. [13]
8	32 years/15 W	High risk for fetal T21	Normal	47,XN,+18[61]/46,XN[39] of cord blood sample	T18 placental mosaicism	J. Yang et al. [14]
9	40 years/11 W	Not performed	Normal	47,XX,+18 by amniocentesis	T18 placental mosaicism	R. Hochstenbach et al. [24]
10	unknown	Not performed	Normal	47,XX,+18 by amniocentesis	unknown	R.V. Lebo et al. [25]
Current case	36 years/20^{+4}W	Senior age of pregnant	High risk for T5	47,XX,+18 in cultured amniocytes	T18 and T5 placental mosaicism	/

T18 (*Z*-score of Chr18 was 0.684) in our NIPS. In contrast, the mosaic ratio of T5 in placenta was approximately 60%, and the effective cffDNA fraction of the T5 was nearly 6% which was sufficient for NIPS detection. That was why the T5 was detected with a Z-score of 6.327 in our NIPS. Therefore, in order to avoid false negative results caused by placental mosaicism, the method of increasing the effective concentration of cffDNA or improving sensitivity and specificity of NIPS is necessary for us to explore in future.

According to the previous researches of NIPS, several false negative cases in NIPS resulted from placental mosaicism were reported. For these cases, mosaic T18 could be combined with T21 or sex chromosome abnormality in placental tissues [26,28–31], but information pertaining mosaic T18 combined with other autosomal abnormality in placental tissues was still limited. In present study, a case with T5 result in NIPS but diagnosed as a T18 fetus was reported, and placental mosaicism of T18 and T5 was found after further examination of placental tissues. Although T18 was not detected in NIPS, the T5 result in NIPS effectively helped us to recommend the pregnant woman for prenatal diagnosis which finally found T18 for the fetus in our case. Therefore, although NIPS was well evidenced for detecting T21, T18 and T13 in China [10,11], it sometimes could detect abnormalities for other chromosomes, which might be an important warning for false negative result of the T21, T18 or T13. For this reason, during clinical counseling, the clinicians should pay more attention to the abnormal result except T13, T18 and T21 in NIPS and recommend pregnant women for further prenatal diagnosis to found the abnormal fetus with T13, T18 or T21 missed in NIPS.

In our case, a gradual change of T5 and T18 mosaicism was found from placenta to fetus. Based on the CNV-seq and CMA data, the mosaic ratio of T5 was gradually increased from umbilical cord to placenta center, while the mosaic ratio of T18 changed oppositely. For T5, it was only detected in placental tissues with the average mosaic ratio of approximately 60%, but not in umbilical cord and the fetus, which was more like belonging to confined placental mosaicism (CPM). CPM can be caused by the mitotic nondisjunction (NDJ) event in the early stages of cell division or the meiotic error followed by mitotic correction [32,33]. NDJ is thought to be the major mechanism resulting in CPM , which produces a mosaic involving three cell lines (a trisomic line, a monosomic line, and a normal cell line) though the zygote is initially normal. Except the monosomic line is usually disappeared, both the trisomic and normal cell line can expand [32]. In the 32-to-64 cell stage, only three to four cells become compartmentalized into the inner cell mass to form the embryo, others become precursors of the extraembryonic tissues [34]. Therefore, for our case, the NDJ of Chr5 possibly occurred in the extraembryonic precursor cells or occurred earlier with the T5 cells segregating only to the extraembryonic tissues, which finally resulting in that T5 cells was only found in placental tissues. For T18, its mosaic ratio was changed from average approximately 40% in placenta to 100% in umbilical cord and the fetus. According to the proposed mechanisms for CPM above, it could be explained that the NDJ of Chr18 occurring before the 32-to-64 cell stage produced the T18 cells that subsequently occupied all the inner cell mass to form the embryo but only a part of the precursor cells of the placenta. In conclusion, it was possible that the different occurring time of NDJ event for Chr5 and Chr18 as well as the different developmental directions of the trisomic line in early embryo development caused the different gradual change of T5 and T18 mosaicism from placenta to fetus.

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