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Original Article

Maternal mosaicism of sex chromosome causes discordant sex chromosomal aneuploidies associated with noninvasive prenatal testing

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ABSTRACT

Objective: To investigate the clinical efficiency of noninvasive prenatal test (NIPT) identifying fetal chromosomal aneuploidies.
Materials and methods: In the present study, 917 women with high-risk pregnancies were invited to participate in an NIPT trial based on an Illumina HiSeq massively parallel sequencing platform. Abnormal cases in NIPT were validated by karyotyping and fluorescence *in situ* hybridization (FISH) analysis. All of the participants' infants were examined clinically and followed up for at least 6 months.
Results: A total of 35 (3.82%) high-risk pregnancies were detected with abnormal results in NIPT, which included 25 cases (2.73%) of trisomy 21 (Tri21), four cases (0.44%) of trisomy 18 (Tri18), four cases (0.44%) of Turner syndrome (45, X), one cases (0.11%) of Klinefelter's syndrome (47, XXY), and one cases (0.11%) with lower X chromosome concentration. Further validation indicated that one case of Tri18 and the case with lower X chromosome concentration were false positive results (0.22%) in NIPT. Furthermore, it was found that the false positive case with lower X chromosome concentration in NIPT was caused by maternal sex chromosomal mosaicism (45, X and 46, XX).
Conclusion: Our findings indicated that maternal mosaicism of sex chromosome could cause discordant sex chromosomal aneuploidies associated with NIPT. We highly recommended that maternal karyotype should be confirmed for the cases with abnormal results in NIPT.

Introduction

Chromosome aneuploidies, mostly characterized by trisomy 21 (Tri21), trisomy 18 (Tri18), trisomy 13 (Tri13), and monosomy X [1], lead to medical conditions among neonates requiring specialized medical care and result in emotional and financial challenges to families [2]. The aneuploidies usually occur in one out of every 160 live births and account for 6–11% of all stillbirths and newborn deaths [3,4]. Moreover, the risk of giving birth to a child with chromosomal abnormalities, especially Down syndrome, increases with maternal age [5]. Early diagnosis during the course of pregnancy may inform the family about the potential for a fetus with chromosomal aneuploidy. Therefore, early prenatal screening and diagnosis to detect the most common trisomy are indispensable.

Chromosome aneuploidies are traditionally verified through invasive diagnostic procedures including amniocentesis and umbilical cord blood or chorionic villus sampling [5,6]. These invasive diagnostic procedures require skilled techniques and carry an approximately 0.5–1% risk of miscarriage [5]. In addition, the procedures for conventional prenatal diagnosis take lengthy waiting periods (usually about 14 days). With a culture failure rate of ~1%, many pregnant women dread the sampling and waiting periods

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[7,8]. Earlier, fetal cell free DNA (cfDNA) was detected in maternal circulating blood [9], which led to the development of noninvasive prenatal test (NIPT) based on analyzing fetal cfDNA in the mother's blood. NIPT is a new platform for prenatal screening and diagnosis of trisomy syndromes with high accuracy and low risk [10–12].

Recently, the use of fetal cfDNA had been reported for the prenatal diagnosis of achondroplasia and myotonic dystrophy, determination of fetal sex, and genotyping of fetal rhesus D [13–16]. However, several problems had restricted the clinical use of the analysis of fetal cfDNA, such as the low concentration of fetal cfDNA in the maternal circulation and the difficulty in distinguishing fetal from maternal chromosomes [17,18]. Moreover, a significant number of false positive results from NIPTs had underlain the biological reasons, including confined placental mosaicism (CPM), maternal mosaicism, co-twin demise, and maternal malignancy [19]. Therefore, more information about NIPT, as well as improvements in its effectiveness, should be made available to pregnant women.

Since 2012, NIPT have been offered as an additional option in our hospital for high-risk pregnant women needing to confirm chromosome aneuploidies. Up until now, 917 high-risk pregnant women from our hospital have participated in an NIPT trial and two discordant results were found. These findings will provide useful information about NIPTs for further improvements.

Patients and methods

Patients

From our prenatal clinics, pregnant women with gestational ages between 14 weeks and 26 weeks meeting any one of the following conditions were considered as high-risk pregnancies: (1) abnormal maternal screening of AFP and free beta-human chorionic gonadotropin; (2) advanced maternal age ($\geq$ 35 years); (3) abnormal ultrasound findings; (4) abnormal amniotic fluid volume; (5) adverse pregnancy history obtained from medical records; and (6) single umbilical artery. Within a 2-year period (January 2012 to December 2013), a total of 917 high-risk pregnancies were identified, and all of them agreed to participate in the NIPT trial. After childbirth, their infants were examined clinically and followed up for at least 6 months. This project was approved by the Hospital Ethics Committee and informed consent was obtained from each participant.

NIPT

Approximately 10 mL blood from each high-risk pregnant woman was collected into a purple-top tube containing EDTA. The blood sample was immediately centrifuged at 1600*g* for 10 minutes at 4°C. The plasma portion was centrifuged at 16,000*g* for 10 minutes to minimize any additional release of maternal DNA. The plasma specimens were frozen on dry ice and sent to a commercial lab that specialized in NIPT (www.berrygenomics.com). The NIPT procedures, including DNA extraction, library construction, whole-genome sequencing, and data analysis, were carried out according to protocols published elsewhere [10]. In brief, plasma DNA was extracted from 1 mL of the plasma using the QIAamp Circulating Nucleic Acid kit from Qiagen (Hilden, Germany). Then, the resulting plasma DNA was used as the input DNA to make a library for sequencing. Plasma DNA libraries were indexed using 6 bp indexing oligos, quantitated by Kapa SYBR fast qPCR kit from Kapa Biosystems (Woburn, MA, USA), pooled, and loaded into one lane in a v2 Illumina HiSeq2000 flow cell (Illumina, USA). Clustering and sequencing were conducted according to Illumina's instruction, using the single-ended 43 bp sequencing protocol. Finally, the sequences were binned for each sample according to the index and mapped to the unmasked human genome sequence (hg19) using the software SOAP2 (obtained from soap.genomics.org.cn/), and the z-score for each chromosome was calculated to judge abnormality referencing to the normalized chromosome representation.

Karyotyping and fluorescence in situ hybridization

For each abnormal woman in NIPT, approximately 20 mL of amniotic fluid and 10 mL of maternal peripheral blood were collected. Conventional karyotyping analysis and fluorescence *in situ* hybridization (FISH) were performed for further validation. Karyotyping was processed using a conventional Giemsa banding (G-binding) method [20], and FISH was performed according to the method previously established [21].

Results

NIPT

A total of 917 high-risk pregnant women were recruited for the NIPT trial. Their ages ranged from 18 years to 46 years with the following distribution: 18–25 years (299, 22.86%), 26–35 years (318, 40.00%), and 36–46 years (300, 37.14%). Among them, the number of women with advanced maternal age, abnormal maternal serum screening, abnormal ultrasonic graphic findings, and other conditions (with abnormal amniotic fluid volume, adverse pregnancy history, or single umbilical artery) were 300 (32.72%), 521 (56.82%), 6 (0.65%), and 90 (9.81%), respectively (Table 1). After NIPT, 34 (3.71%) high-risk pregnancies were found with fetal aneuploidies, which included 25 cases (2.73%) of Tri21, four cases (0.44%) of Tri18, four cases (0.44%) of Turner syndrome (45, X), and one case (0.11%) of Klinefelter's syndrome (47, XXY) (Table 1). Furthermore, one woman (0.11%) was found with lower X chromosome concentration than expected. This finding could have been due to fetal Turner syndrome, but this was considered to be

Table 1
Noninvasive prenatal test (NIPT) result of the high-risk pregnancies.

Groups	Number						
	Participating patients (%)	Abnormal patients in NIPT (%)	Abnormality discovered by NIPT				
			Tri21 (%)	Tri18 (%)	Turner syndrome (45, X) (%)	Klinefelter's syndrome (47,XXY) (%)	Lower X chromosome concentration (%)
Abnormal maternal serum screening	521 (56.82)	10 (1.09)	5 (0.55)	1 (0.11)	3 (0.33)	0 (0)	1 (0.11)
Advanced maternal age	300 (32.72)	13 (1.42)	9 (0.98)	2 (0.22)	1 (0.11)	1 (0.11)	0 (0)
Abnormal ultrasonic finding	6 (0.65)	1 (0.11)	1 (0.11)	0 (0)	0 (0)	0 (0)	0 (0)
Abnormal amniotic fluid volume, adverse pregnancy history, or single umbilical artery	90 (9.81)	11 (1.20)	10 (1.09)	1 (0.11)	0 (0)	0 (0)	0 (0)
Total	917 (100)	35 (3.82)	25 (2.73)	4 (0.44)	4 (0.44)	1 (0.11)	1 (0.11)

unlikely because the z-score was not compatible with previous cases of fetal Turner syndrome. Therefore, the laboratory suggested that the most likely cause was maternal mosaicism. All of the 35 women (3.82%) with abnormal findings above included nine with advanced maternal age, 14 with abnormal maternal serum screening, one with an abnormal ultrasound finding, and 11 with other conditions (5 with abnormal amniotic fluid volume, 3 with adverse pregnancy history, and 3 with single umbilical artery).

For the 35 women with abnormal results in NIPT, full karyotyping analysis of their amniotic fluid was performed for further validation. The karyotyping results of 33 cases were consistent with NIPT results. However, one case identified as Tri18 in NIPT was detected as normal in karyotyping analysis (Figure 1A). Additionally, the case with lower X chromosome concentration in NIPT was detected as normal in karyotyping analysis. For the remaining 882 normal cases in NIPT, no visible chromosomal abnormality of babies was detected in the subsequent clinical follow up (once/month) from birth to 6 months.

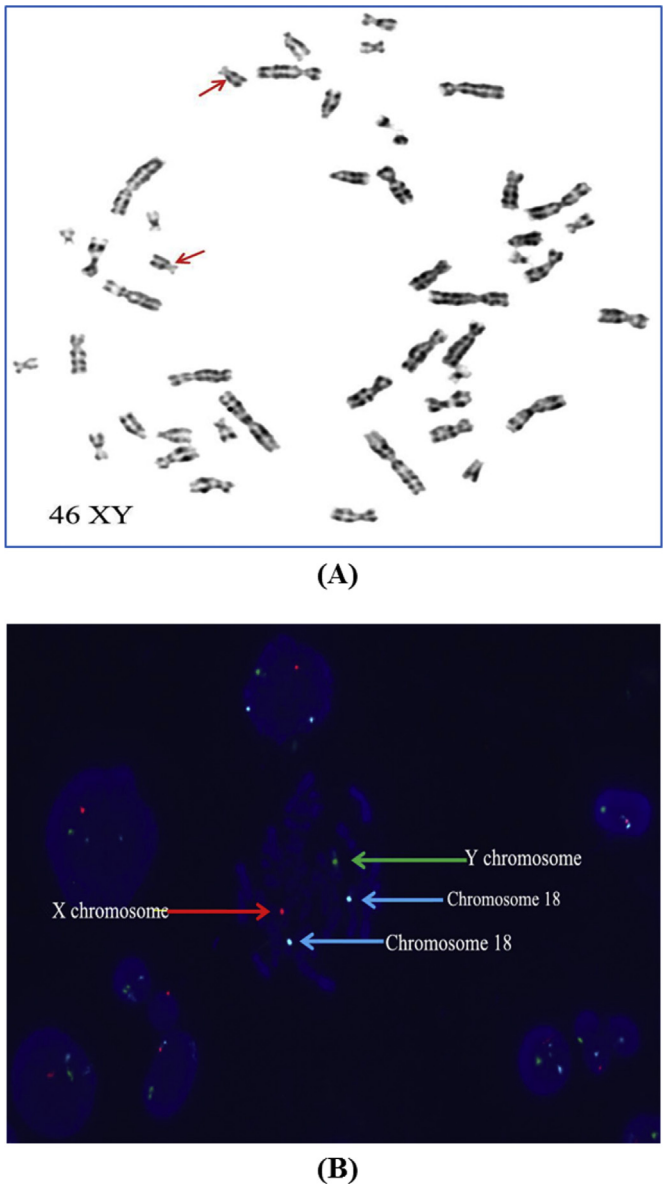

Figure 1. Validation of the false positive trisomy 18 (Tri18) in noninvasive prenatal test (NIPT) through (A) full karyotyping and (B) fluorescence *in situ* hybridization (FISH) of the amniotic fluid cell. Two chromosomes 18 were marked with red arrows in the karyotype (A). Under fluorescence microscope, chromosomes 18, X and Y were hybridized with probes labeled with blue, red and green fluorescence, respectively (B).

Further validation of two special cases

For the woman with discordant results in NIPT (Tri18) and karyotyping analysis (normal), FISH for chromosome 18 was performed using cells from the amniotic fluid. Under the fluorescence microscope, only two fluorescence probe signals for chromosome 18 were detected in each cell (Figure 1B), which indicated that the fetus of the woman was normal disomy 18. Moreover, no morphological abnormality of the fetus was detected in the following ultrasonic diagnosis. Ultimately, the woman gave birth to a full term boy, and no visible chromosomal abnormality was found in karyotyping analysis of the baby's peripheral blood (data not shown) and subsequent clinical follow up.

For the woman with lower X chromosome concentration in NIPT, full karyotyping analysis of her peripheral blood was performed for further verification. Among the 40 G-banded karyotypes randomly selected, 28 (70%) were normal (46, XX), but 12 (30%) were abnormal (45, X) (Figure 2), which indicated that the woman presented maternal mosaicism for chromosome X (45, X and 46, XX). However, the woman also gave birth to a full term boy, and no visible chromosomal abnormality was found in karyotyping analysis of his peripheral blood (data not shown) and subsequent clinical follow up.

Discussion

Since fetal cfDNA was first discovered in plasma from pregnant women in 1997 [9], NIPTs of fetal aneuploidies dependent on fetal cfDNA had attracted increasing attention. Now, fetal whole

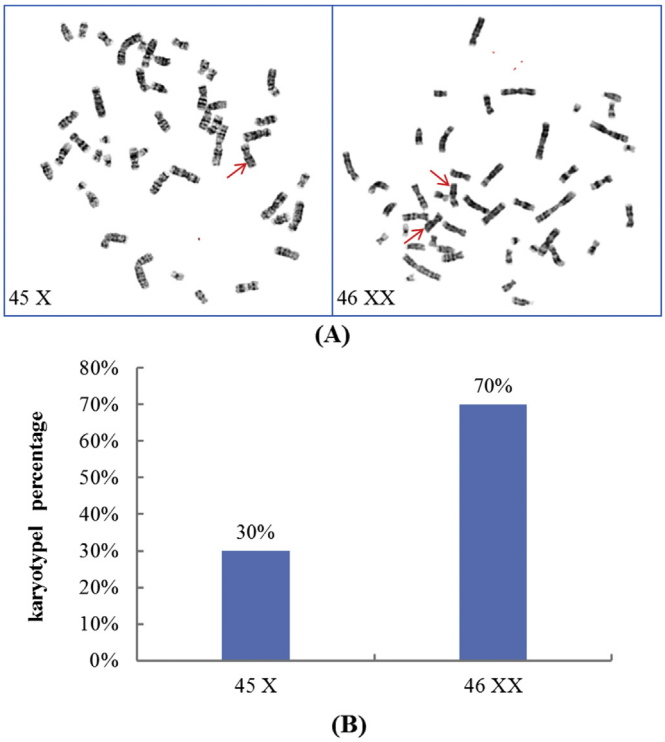

Figure 2. Validation of maternal mosaicism of sex chromosome (45, X and 46, XX) through maternal karyotyping. (A) chromosome X was marked with the red arrow in karyotypes and (B) the percentage of each karyotype was described in column graph.

chromosome aneuploidy can be accurately detected from maternal blood by NIPT using massively parallel sequencing [10]. Based on massively parallel sequencing, NIPT is considered as a highly accurate protocol for the prenatal detection of Down syndrome and Tri18. This novel protocol has higher detection and lower false positive rates than traditional effective screening protocols (with false positive rates of 2–5%) based on the combination of maternal age, ultrasound examination of the fetus, and levels of various proteins or hormones in the maternal blood [22]. However, for various reasons, established NIPT is recognized as a potentially highly effective screening test, but not as a test that can replace current invasive prenatal diagnosis [23]. One of the vital reasons is false positive results found with NIPTs. For example, Norton et al [24] found one Tri21 false-positive and two Tri18 false-positive results among 2888 high-risk cases when evaluating the performance of NIPT for Tri21 and Tri18. Palomaki et al [25] found five Tri18 false-positive and 16 Tri13 false-positive results among 1688 high-risk cases. The potential explanations for NIPT fetal karyotype discordance include CPM, maternal mosaicism, co-twin demise, maternal malignancy, and even laboratory error [19]. CPM is present in 1–2% of first trimester placentas [26]. Because fetal cfDNA in maternal blood was thought to originate from placental apoptotic trophoblasts [23,27], a majority of the reported NIPT false positive or false negative results were the consequence of CPM [28,29], which were involved in Tri13, Tri18, Tri21, and other chromosomes [30–33]. In the present study, a Tri18 false positive NIPT result was identified among 917 high-risk pregnancies after validation by full karyotyping and FISH analysis. Medical records about the case during pregnancy showed that the woman had no history of co-twin demise or maternal malignancy. Considering that the karyotyping of the woman and her fetus were both normal, the false positive Tri18 was probably associated with CPM, although the placenta was not used for further validation. Taken together, our present findings and other emerged reports suggested that some pregnancies might evolve with CPM that was sufficient to cause discordant NIPT results. Therefore, the positive result in NIPT should be confirmed by conventional invasive testing and karyotyping, especially at early implementing stage.

The circulating cfDNA in maternal plasma is a combination of maternal and fetal DNA, among which the fetal fraction is only 3–6% [17]. Therefore, abnormal maternal chromosome complements may lead to discordant NIPT results. Recent studies suggested that low level maternal mosaicism resulted in variations of the maternal contribution to circulating DNA, which could impact NIPT results [34]. For example, Osborne et al [35] reported that a malignant cell population could cause abnormal maternal cfDNA that led to discordant NIPT results. Using massively parallel sequencing, Wang et al [36] also found that maternal mosaicism was a significant contributor to discordant sex chromosomal aneuploidies associated with NIPT. In the present study, NIPT findings suggested a case with lower X chromosome concentration, but the subsequent results of amniocentesis showed that the fetus was normal (46, XY). Full karyotyping analysis of the maternal peripheral blood presented the maternal mosaicism for a missing X (45, X and 46, XX), indicating that maternal mosaicism for aneuploidy could result in discordant NIPT results. Therefore, maternal mosaicism must be considered as a confounding factor in NIPT. In addition, the z-score, which was calculated to judge abnormality in NIPT [10], of the X chromosome for this case was only 60% of the normal value in the NIPT (data not shown), which was beyond the scope of the impact of fetal cfDNA. Therefore, besides fetal aneuploidies, NIPT may also be used to examine maternal mosaicism, which may represent a new application of NIPT in the future.

In conclusion, our findings indicated that maternal mosaicism of sex chromosomes could cause discordant sex chromosomal aneuploidies associated with NIPT. Therefore, we highly recommend that, for the cases with abnormal results in NIPT, maternal karyotype should be confirmed to eliminate the influence of maternal mosaicism.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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