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Noninvasive prenatal screening for fetal common sex chromosome aneuploidies from maternal blood

Bin Zhang, Bei-Yi Lu, Bin Yu, Fang-Xiu Zheng, Qin Zhou, Ying-Ping Chen and Xiao-Qing Zhang

Abstract

Objective: To explore the feasibility of high-throughput massively parallel genomic DNA sequencing technology for the noninvasive prenatal detection of fetal sex chromosome aneuploidies (SCAs).

Methods: The study enrolled pregnant women who were prepared to undergo noninvasive prenatal testing (NIPT) in the second trimester. Cell-free fetal DNA (cffDNA) was extracted from the mother's peripheral venous blood and a high-throughput sequencing procedure was undertaken. Patients identified as having pregnancies associated with SCAs were offered prenatal fetal chromosomal karyotyping.

Results: The study enrolled 10 275 pregnant women who were prepared to undergo NIPT. Of these, 57 pregnant women (0.55%) showed fetal SCA, including 27 with Turner syndrome (45,X), eight with Triple X syndrome (47,XXX), 12 with Klinefelter syndrome (47,XXY) and three with 47,XYY. Thirty-three pregnant women agreed to undergo fetal karyotyping and 18 had results consistent with NIPT, while 15 patients received a normal karyotype result. The overall positive predictive value of NIPT for detecting SCAs was 54.54% (18/33) and for detecting Turner syndrome (45,X) was 29.41% (5/17).

Conclusion: NIPT can be used to identify fetal SCAs by analysing cffDNA using massively parallel genomic sequencing, although the accuracy needs to be improved particularly for Turner syndrome (45,X).

Prenatal Diagnosis Laboratory, Changzhou Woman and Children Health Hospital affiliated with Nanjing Medical University, Changzhou City, Jiangsu Province, China

Corresponding author:
Bin Yu, Prenatal Diagnosis Laboratory, Changzhou Woman and Children Health Hospital affiliated with Nanjing Medical University, 26 Bo Ai Road, Changzhou 213003, Jiangsu Province, China.
Email: ybcz0519@163.com

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Introduction

Birth defects have become a major public health problem for children's health and they have affected the quality of life of the affected population of newborns.[1] Chromosomal abnormalities are one of the most serious birth defects. Due to the lack of effective treatment, they often cause serious damage to the fetus. For example, it is well known that aneuploidies are the most common chromosomal abnormalities, including trisomy 21 (Down's syndrome), trisomy 18 (Edwards' syndrome), trisomy13 (Patau's syndrome), and sex chromosome aneuploidies (SCA).[2] SCAs are caused by the presence of an abnormal number of sex chromosomes (X or Y) in a cell, and they include 45,X (Turner syndrome), 47,XXX (Triple X syndrome), 47,XXY (Klinefelter syndrome) and 47,XYY. The main features of 45,X, 47,XXY and some cases of 47,XXX are sex development retardation or abnormality, and infertility. Some SCAs, especially 45,X and 47,XXX may show intellectual disability.[3] Once the patient has clinical symptoms, there are no effective treatments available. Early intervention services such as physical therapy, occupational therapy, and individualized education plans can make a huge difference in the outcome of patients with SCAs.[3,4] Additionally, early hormonal therapy and hormonal replacement therapy have been shown to improve the outcomes for babies with 45,X syndrome or 47,XXY syndrome.[5] Having the correct diagnostic information about SCAs in the newborn baby is critical for the introduction of early therapy. Earlier screening leads to a better prognosis for many SCA cases.[6]

Currently, the most common prenatal screening method for determining the risk of trisomy 21 (Down's syndrome) is based on measuring the maternal serum levels of alpha-fetoprotein (AFP), unconjugated estriol (uE3), and the free beta subunit of human chorionic gonadotropin (fβhCG) combined with the maternal age in the second trimester.[7,8] The rate of detection of trisomy 21 is 75% with a 5% false-positive rate using this screening programme.[9] The detection rate is much lower in some developing countries if there is not good quality control.[9–11] Therefore, screening efficiency is currently unsatisfactory.

Recently, noninvasive prenatal testing (NIPT) for fetal aneuploidies has been shown to be a better prenatal screening method than blood biochemical screening; with NIPT detecting cell-free fetal DNA (cffDNA) obtained from the maternal plasma using massively parallel sequencing technology.[12] Currently, NIPT is widely used to screen for trisomy 21, trisomy 18, and trisomy 13 fetal aneuploidies because of its high accuracy and sensitivity. For example, a detection rate of 99.2% with a false-positive rate of 0.09% was reported for trisomy 21, a detection rate of 96.3% and a false-positive rate of 0.13% for trisomy 18, and a detection rate of 91.0% and a false-positive rate of 0.13% for trisomy 13.[13] Some professional medical organizations have issued guidelines about NIPT, such as the American College of Obstetricians and Gynecologists, the International Society for Prenatal Diagnosis, and the American College of Medical Genetics.[14–16] NIPT is regarded to be the best technology for screening for the commonest autosomal trisomies, such as trisomy 21, trisomy 18,

and trisomy 13, at present.[17] Research has also investigated the use of NIPT to screen for fetal SCAs.[13] However, there are few publications on the use of NIPT for SCAs and little clinical experience.[18]

The present study investigated the feasibility of using NIPT to screen for fetal SCAs using cffDNA in maternal plasma.

Patients and methods

Patient population

This retrospective study recruited consecutive pregnant women who attended Changzhou Woman and Children Health Hospital affiliated with Nanjing Medical University, Changzhou City, Changzhou, Jiangsu Province, China for prenatal screening and diagnosis between October 2012 and October 2016. The inclusion criteria were as follows: (i) pregnant women aged 18–50 years; (ii) gestational age of 13–27 weeks. Gestational age was determined using the date of the last menstrual period and data from the first ultrasound. After prenatal screening in the second trimester, women were recruited to undergo NIPT.

The study was approved by the Institutional Review Board of Changzhou Woman and Children Health Hospital and each study participant provided written informed consent prior to participation.

Routine prenatal screening in the second trimester

After the demographic characteristics and medical history were recorded, a maternal blood sample (5 ml) was withdrawn from the cubital vein and collected into a BD Vacutainer sample tube (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) containing spray-coated silica and a polymer gel for serum separation. The serum was separated by centrifugation as soon as possible and stored at −20°C. The concentrations of AFP, uE3, and fβhCG were measured using DELFIA® time-resolved fluorescence assays (PerkinElmer, Gaithersburg, MD, USA). Combined with maternal age, gestational age, body weight, and presence of type 2 diabetes mellitus, the risk of trisomy 21 and trisomy 18 was calculated using Lifecycle 4.0 software (PerkinElmer). A high-risk score for trisomy 21 was $\geq 1/300$ and for trisomy 18 was $\geq 1/350$; an intermediate risk score for trisomy 21 was 1/301–1/1000 and for trisomy 18 was 1/351–1/1000. A risk value less than 1/300 or 1/350 was considered as low risk for trisomy 21 and trisomy 18, respectively. Advanced maternal age was defined as ≥ 35 years.

Laboratory methodology for NIPT

A sample of whole blood (5 ml) was collected from all study participants into EDTA-K2 spray-dried Vacutainers (EDTA tubes; Becton, Dickinson & Co.). Whole blood samples were refrigerated or stored on wet ice and were processed to plasma within 6 h of collection. The maternal blood samples were centrifuged using an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany) at 1600 ***g*** for 10 min at 4°C, and the plasma was collected. The plasma was then centrifuged in an Eppendorf 5424 centrifuge (Eppendorf) at 1600 ***g*** for 10 min at 4°C and immediately stored frozen at −70°C until DNA extraction. The plasma DNA was extracted from 1 ml plasma for each study participant using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA). If the concentration of total free DNA was > 0.7 ng/μl, then it would not be possible to construct DNA libraries, so a second blood sample was collected. The resulting plasma DNA was used to make DNA libraries for sequencing using the modified ChIP-Seq protocol as described previously.[19] DNA libraries from 12 plasma samples were indexed using 6 nt barcodes and quantified with a KAPA SYBR® FAST qPCR kit (Kapa Biosystems,

Wilmington, MA, USA). These libraries were then pooled and loaded. One lane of an Illumina NextSeq 500 flow cell (Illumina China, Shanghai, China) was used to perform the sequencing using a single-ended 43-base pair sequencing protocol following the manufacturer's instructions.

The sequences from each library were split according to their unique indexes. The split sequences were then mapped to the unmasked human genome sequence (hg19). SOAP2 mapping algorithm was used to obtain the results as previously described.[20] The sequences of each sample that were mapped to each chromosome were counted, and the guanine-cytosine (GC) content was calculated. Normalized chromosome representation and GC correction were used to generate a Z-score as previously described.[20] Each pair of chromosomes was defined as increased if their Z-score was > 3 and decreased if its Z-score was < -3. Samples with a fetal fraction $< 4\%$ of the total cell-free DNA were considered inappropriate for further analysis as there were insufficient data to analyse.

Fetal chromosome karyotype analyses

Patients identified as having pregnancies associated with SCAs were offered prenatal fetal chromosomal karyotyping. All prenatal samples were cultured following standard protocols.[21] Amniocytes were cultured with BIO-AMF™-2 medium (Biological Industries, Kibbutz Beit-Haemek, Israel) and Chang Medium® D (Irvine Scientific, Santa Ana, CA, USA). Cord blood cells were cultivated with peripheral blood lymphocyte medium (Xiangya Gene Technology, Hunan, China). At least 20 G-banded metaphases from each sample were analysed using the Wright's staining method.[22]

Statistical analyses

All statistical analyses were performed using the SPSS ® statistical package, version 18.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Data are presented as mean ± SDs. Analysis of variance was used to compare the differences between different groups. A P-value < 0.05 was considered statistically significant.

Results

After prenatal screening during the second trimester, a total of 10 275 pregnant women agreed to undergo NIPT. Of these, 6118 pregnant women agreed to prenatal biochemical screening prior to NIPT and 4157 agreed to undergo NIPT without prior prenatal biochemical screening. Figure 1 shows the flow of study participants through this study. The baseline demographic and clinical characteristics of the patients are shown in Table 1. Blood was collected from all patients at gestational ages of 13–27 weeks. A total of 3585 of 10 275 (34.89%) patients were of advanced maternal age (≥ 35 years). A total of 2591 of 10 275 (25.22%) patients were considered at high risk of second trimester prenatal screening and 2010 of 10 275 (19.56%) were at intermediate risk.

Using massively parallel sequencing technology, 57 patients (0.55%) demonstrated positive NIPT results for fetal SCA, including 27 patients positive for 45,X (Turner syndrome), eight for 47,XXX (Triple X syndrome), 12 for 47,XXY (Klinefelter syndrome) and three for 47,XYY (Figure 1). As a result of these genetic findings, 33 of 57 pregnant women underwent fetal karyotyping, which included 30 patients who underwent amniocentesis, two who underwent cordocentesis and one newborn baby who underwent neonatal blood karyotyping. The remaining women either refused to undergo any further tests ($n = 21$) or the pregnancy was lost ($n = 3$). A comparison of the outcome of the NIPT with the karyotyping results demonstrated that the fetal SCA of 18 of 33 patients was validated by

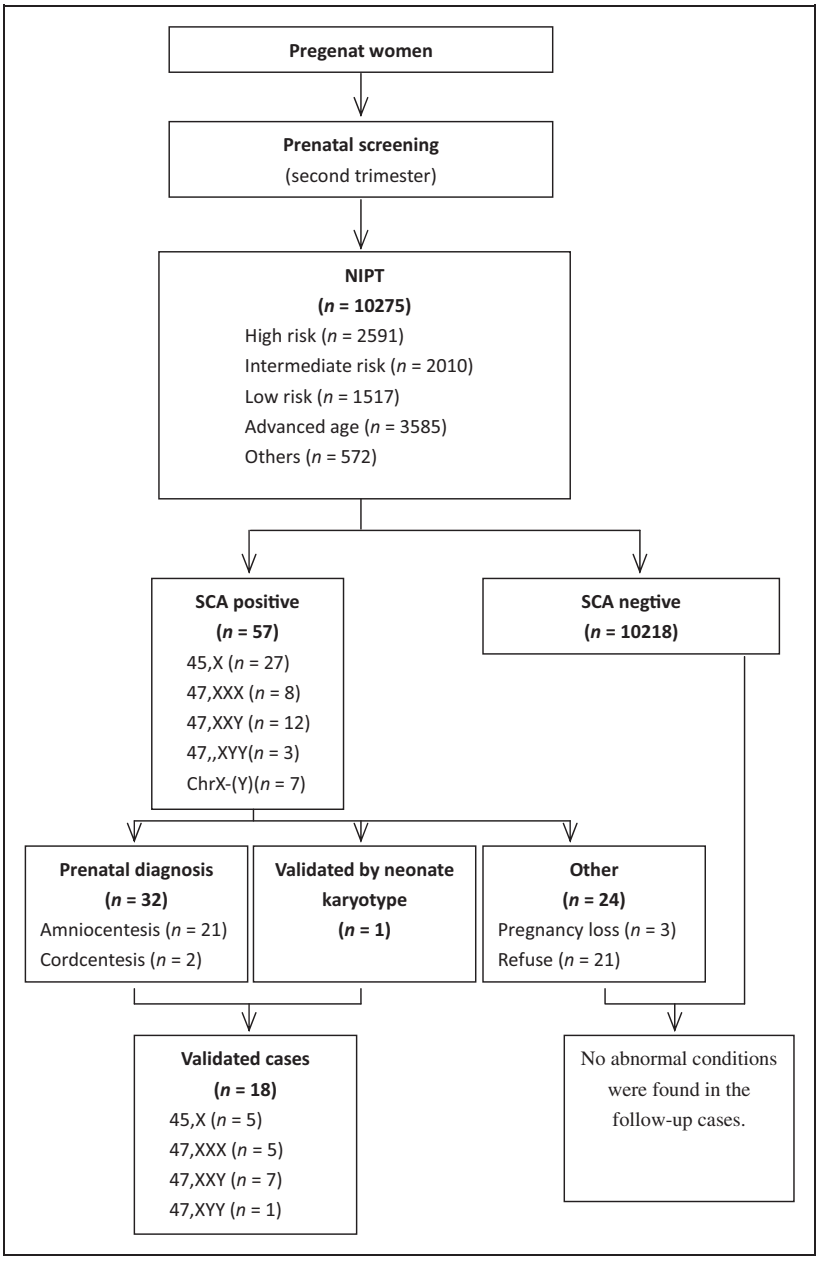

Figure 1. Flow diagram showing patient numbers at various stages of a study that investigated the use of noninvasive prenatal testing (NIPT) for screening for fetal sex chromosome aneuploidies.

karyotyping and the newborns of 15 patients were considered to have normal karyotypes. The positive predictive value (PPV) of NIPT in SCA was 54.54% (Table 2).

A comparison of the positive NIPT results for SCAs compared with karyotyping in pregnant women stratified according to the demographic characteristics of

prenatal screening risk and advanced age demonstrated that of the 2591 high-risk pregnant women, 13 (0.50%) were NIPT positive (Table 3). After karyotyping validation, seven patients were confirmed as being true positives, giving a PPV of 77.78%. For the 12 out of 2010 intermediate-risk patients who received NIPT positive results, the PPV was 37.50%. Of the 3585 women aged ≥ 35 years, 19 had a positive NIPT result and the PPV was 45.45%.

Table 1. Demographic and clinical characteristics of the pregnant women ($n = 10\,275$) enrolled in a study that investigated the use of noninvasive prenatal testing (NIPT) for screening for fetal sex chromosome aneuploidies.

Characteristic	*n* (%)
Chinese	10 275 (100.00)
Singleton pregnancy	10 275 (100.00)
Gestational age at NIPT	
13–27 weeks	10 275 (100.00)
Routine prenatal screening results	
High risk	2591 (25.22)
Intermediate risk	2010 (19.56)
Low risk	1517 (14.76)
Maternal age, years	
<35	6690 (65.11)
≥35	3585 (34.89)

When the individual SCAs were analysed, the PPVs were as follows: Turner syndrome (45,X; PPV: 29.41% [5/17]); Klinefelter syndrome (47,XXY; PPV: 77.78% [7/9]), Triple X syndrome (47,XXX; 100% [5/5]) and XYY syndrome (PPV: 100% [1/1]).

Discussion

In the past few years, NIPT has been widely applied to screen for trisomy 21, trisomy 18 and trisomy 13 using cffDNA in maternal plasma.[23] Clinicians consider NIPT to be the best way to screen for trisomy 21, trisomy 18 and trisomy 13 in the fetus during the second trimester, being better than prenatal screening in the first or second trimesters.[24] According to a 2015 meta-analysis, the detection rates and false-positive rates of NIPT in singleton pregnancies were 99.2% and 0.09%, for trisomy 21, respectively; 96.3% and 0.13% for trisomy 18; and 91.0% and 0.13% for trisomy 13.[13] Other studies have yielded similar results.[25,26]

In addition to screening for the common fetal aneuploidies described above, some research has demonstrated that NIPT could be used to identify SCA. For example, a meta-analysis showed that the detection rate was 90.3% for monosomy X and 93.0%

Table 2. Comparison of the positive noninvasive prenatal testing (NIPT) results for fetal sex chromosome aneuploidies (SCA) compared with karyotyping in pregnant women ($n = 57$) enrolled in a study that investigated the use of NIPT for screening for fetal SCAs.

NIPT positive SCA	Karyotype validated[a]				Without karyotype validated
	n	True positive	False positive	Positive predictive value (%)	
45,X	27	5	12	5/17 (29.41)	10
47,XXY	12	7	2	7/9 (77.78)	3
47,XXX	8	5	0	5/5 (100.00)	3
47,XYY	3	1	0	1/1 (100.00)	2
ChrX-(Y)	7	0	1	–	6
Total	57	18	15	18/33 (54.54)	24

[a]Tests included amniocentesis ($n = 30$), cordocentesis ($n = 2$) and neonatal karyotyping ($n = 1$).

Table 3. Comparison of the positive noninvasive prenatal testing (NIPT) results for fetal sex chromosome aneuploidies (SCA) compared with karyotyping in pregnant women stratified according to the demographic characteristics of prenatal screening risk and advanced age.

Characteristic	*n*	NIPT positive	Karyotype validated[a]			Without karyotype validated
			True positive	False positive	Positive predictive value (%)	
Prenatal screening risk						
High risk	2591	13	7	2	7/9 (77.78)	4
Intermediate risk	2010	12	3	5	3/8 (37.50)	4
Low risk	1517	8	2	2	2/2 (50.00)	4
Advanced age	3585	19	5	6	5/11 (45.45)	8

[a]Tests included amniocentesis, cordocentesis and neonatal karyotyping.

for SCAs other than monosomy X.[13] However, the detection rates were different in the individual studies included in the meta-analysis and the detection rate for monosomy X ranged from 66.7% to 100%.[13] Another report showed that the analysis of maternal plasma cffDNA using a targeted assay could detect fetal SCA with a reasonably high sensitivity (92.6%) and a combined false-positive rate of less than 1%.[27] The positive predictive rate for the SCAs was reported to be 48.4% and the negative predictive value was 100% in a study using massively parallel genomic sequencing of DNA.[28] Another study using the same sequencing technique reported a PPV of 54.17% for fetal SCAs.[29] In present study, a high-throughput massively parallel genomic sequencing technique was used to screen for fetal SCAs as part of an investigation to determine the potential value of NIPT in detecting fetal SCAs in the second trimester. The overall PPV of NIPT in the present study was 54.54%, which when categorized by individual SCAs was 29.41% for Turner syndrome (45,X), 77.78% for Klinefelter syndrome (47,XXY), 100% for Triple X syndrome (47,XXX) and 100% for XYY syndrome (47,XYY). In this present study, there were 24 patients at a high risk SCAs as determined by NIPT who refused to undergo further karyotyping analysis. If these 24 patients had yielded true positive results, then the upper limit PPV of NIPT would have been 73.68 % (42/57); and the lower limit PPV of NIPT would have been 31.58 % (18/57), if these 24 patients were regarded as false positive.

Circulating cell-free DNA is derived from both maternal and placental tissues, so intrinsic biological factors such as maternal somatic mosaicism, undiagnosed maternal SCA and maternal copy-number imbalance can influence the accuracy of NIPT.[30] Cell-free fetal DNA mainly originates from the placental trophoblasts, which are often discovered to be mosaic. In a mosaic, the degree of mosaicism will impact the performance of the test because it will reduce the effective fetal fraction.[31–33] This potential for mosaicism should be considered as a limitation of NIPT. Secondly, the strongest factor associated with the fetal fraction is maternal weight; the false negative rate and rate of low fetal fractions are highest for women with high maternal weights.[31–33] These are well-known reasons for discordant results between NIPT and fetal karyotyping.

In the present study, the PPV for Turner syndrome (45,X; 29.41%) was lower than for the other SCAs. There are several reasons that might account for this: (i) there are

1098 genes on the X chromosome and 78 genes on the Y chromosome; of which 58 genes are homologous genes on both sex chromosomes, and the majority of these (29 genes) are at the ends of the X and Y chromosomes; (ii) at present, the detection length is only 36 bases, which could easily lead to sequencing positioning dislocation between the X and Y chromosomes; (iii) the non-random inactivation of the X chromosome in placental tissue might be the reason for the low PPV of Turner syndrome, with the paternal X chromosome tending to inactivate in XX female trophoblasts.[34,35]

This study had a number of limitations. First, the sensitivity, specificity and negative predictive value were not calculated due to the difficulties in screening every newborn baby by karyotype analysis. Newborns with SCA can appear normal without physical or intellectual disability, so it is difficult to confirm the presence of the SCA syndrome without karyotype analysis before adolescence. Secondly, a relatively small number of pregnant women in a single centre were enrolled into the study. Larger multicentre studies are warranted to corroborate these findings. Thirdly, there was a small number of patients with SCAs, which has an incidence of 1 in 400 newborns.

In conclusion, this present study demonstrated that NIPT can be used to identify fetal SCAs by analysing cffDNA from the mother's plasma using massively parallel genomic sequencing, although the accuracy needs to be improved particularly for Turner syndrome (45,X).

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Declaration of conflicting interests

The authors declare that there are no conflicts of interests.

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