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Original Articles

Non-invasive prenatal screening for fetal aneuploidy in twin pregnancies by cell-free DNA analysis

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Summary

Objective: To evaluate the efficacy of Cell-free DNA (cfDNA) based non-invasive prenatal testing (NIPS) in detecting fetal chromosomal aneuploidies among twin pregnancies. *Materials and Methods:* A cohort of 384 women with twin pregnancies were recruited for chromosomal aneuploidies testing through NIPS. cfDNA was extracted from maternal blood serum and sequenced by massively parallel sequencing (MPS). *Result:* Two cases of trisomy 21 (T21), one case of trisomy 13 (T13), and two cases of sex chromosomal aneuploidies (SCA) in twin pregnancies were correctly identified through MPS and confirmed their discordant fetal karyotypes (one normal and the other trisomy) by karyotyping; 378 twin pregnancies cases with negative NIPS results were confirmed through postnatal phone follow-up. NIPS detection rate and positive predictive value for T21, T13, and SCA were 100%, respectively, in twin pregnancies; sensitivity and specificity towards T21 and T13 in twin pregnancies were both 100%. *Conclusion:* cfDNA based NIPS for fetal chromosomal aneuploidy have shown a satisfactory clinical performance in twin pregnancies.

Key words: Twin pregnancy; Fetal chromosomal aneuploidy; Non-invasive prenatal screening; Sex chromosomal aneuploidy.

Introduction

With the introduction of assisted reproductive technology (ART) and advance maternal age, the incidence of multiple gestation is rising steadily worldwide. North America, Europe, and East Asia reported a one-third to two-fold increase in multiple birth rate over the last decade [1, 2]. Multiple pregnancies, compared with singleton pregnancy, are more likely to be involved with complications such as low birth weight, learning disability, cerebral palsy, congenital anomaly, incomplete separation (conjoined twins), premature birth, and stillbirth [3]. Risks of chromosomal abnormalities had also been proven to be higher in twining, especially in dizygotic twins [4]. Therefore, multiple pregnancies required extra cautions in their prenatal screening and diagnosis. Non-invasive prenatal screening for fetal aneuploidy by cell-free DNA in maternal plasma had its clinical performance systematically validated by large cohorts of singleton pregnant women [5, 6]. It performed significantly better than traditional ultrasound and blood serum combined screening method. Previous studies with small sample sizes suggested that NIPS can also achieve a high accuracy in twin pregnancies [7, 8]. A recent study demonstrated a high success rate (99.1%) of NIPS for twin pregnancies and a 100% detection rate for T21 [9]. Yet independent experience with large sample size of twin pregnancy is useful to evaluate the validity of NIPS for T21 and other aneuploidies.

Here the authors report their NIPS experience with 384 twin pregnancies involving T21, T13, and sex chromosome aneuploidies (SCA). This study provides practical information on the performance of cfDNA-based NIPS in assessing multiple pregnancy fetal aneuploidy risk.

Materials and Methods

From May 2013 to February 2015, a cohort of 384 women with twin pregnancies (median gestational week 18, range from 12 to 35 weeks) was recruited for chromosomal aneuploidies testing through NIPS. The study was approved by the ethics committee of the Maternity and Child Health Hospital of Guangxi Zhuang Autonomous Region, Nanning, China. All participants had genetic counselling before the test and a written informed consent was collected from each of them.

From each pregnant participant, 10 ml blood was collected into ethylene diamine tetra-acetic acid, EDTA-K_2, vacuum tubes. Within seven hours of collection, the maternal blood samples were firstly centrifuged at 1,600 g for ten minutes at 4ºC and the resulting supernatant then again centrifuged at 16,000 g for ten minutes at 4ºC. The separated plasma stored at -80ºC as maternal blood serum samples. cfDNA extraction and library construction through PCR-free approach were perform by non-invasive pre-

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Table 1. — *Demographic and clinical characteristics of the 384 participants.*

Maternal age (years)	*Value*
Median (range)	31 (18-44)
Less than 35 years, n (%)	269 (70.2%)
35 years and beyond, n (%)	114 (29.8%)
Gestational age (weeks)	
Median (range)	18 (12-35)
Body mass (kg)	
Median (range)	55 (39-88)
Body height (cm)	
Median (range)	157 (145-170)
Chorionicity	
Monochorionic-monoamniotic (MCMA), n (%)	9 (2.3)
Monochorionic-diamniotic (MCDA), n (%)	65 (17.0)
Dichorionic-diamniotic (DCDA), n (%)	199 (52.0)
Unknown, n (%)	110 (29.7)
Type of conception	
None-assisted reproductive technology (ART), n (%)	245 (64.0)
ART, n (%)	128 (33.4)
Unknown, n (%)	10 (2.6)

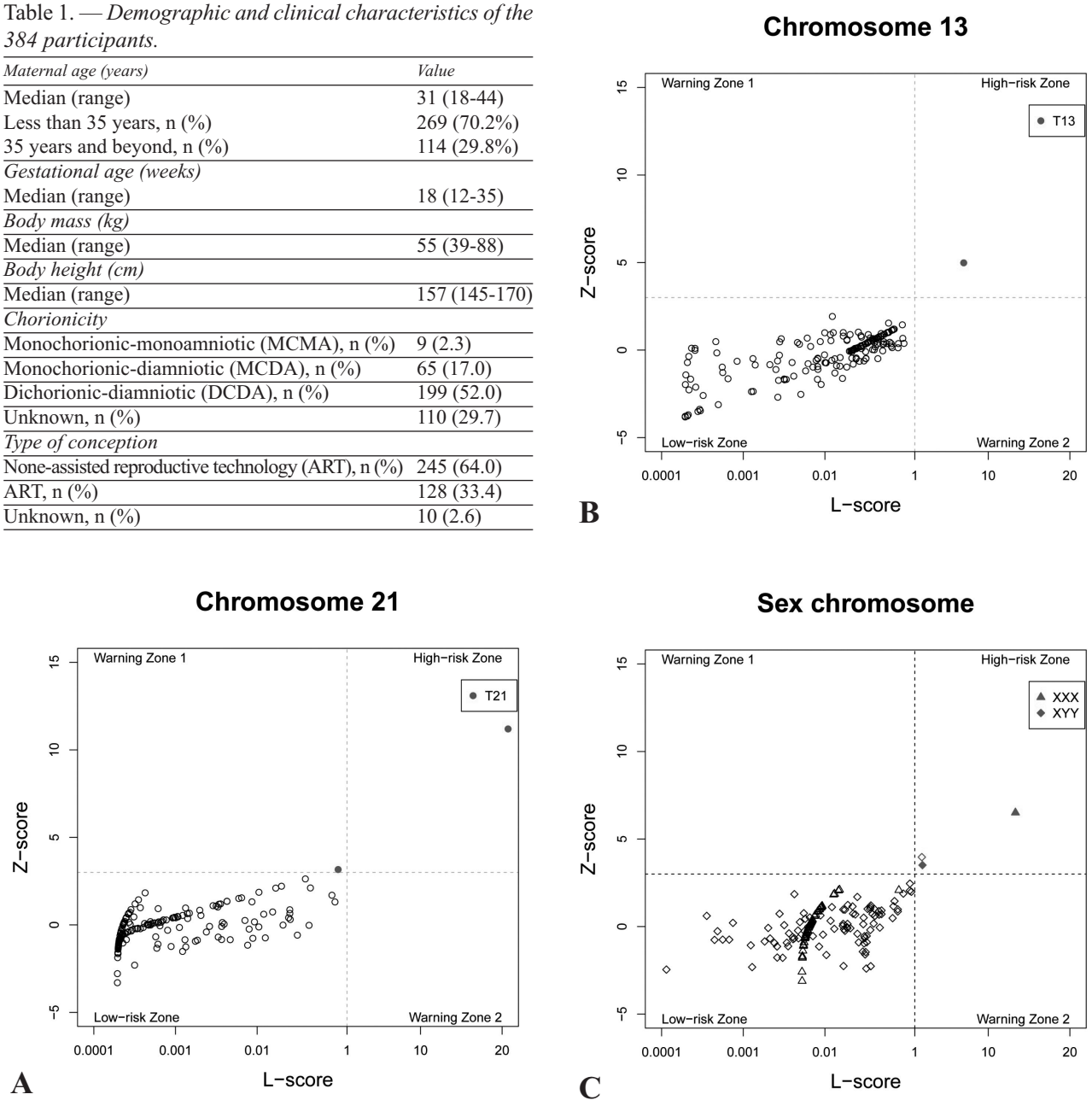

Figure 1: Fetal aneuploidies in twin pregnancies identified through MPS-based NIPS, risk assessment including L-score (x-axis) and Z-score (y-axis). Figures 1A, B, and C demonstrate T21, T13, and SCA detection performance in the current study. Cut-offs for high-risk zone were defined as Z-score > 3 and L-score > 1. In (A) and (B), hollow circular black dots represent low-risk negative samples and solid red dots are positive samples and confirmed aneuploidies by karyotyping. In (C), triangle dots represent samples with no detection of Y chromosome, and diamond dots are samples containing Y chromosome signal. Red solid polygons are NIPT positive cases and cytogenetically confirmed SCAs; the red hollow diamond dot is a NIPT positive case that refused to perform invasive karyotyping.

natal test library prep kit. DNA libraries were quantified and qualified by SYBR fast qPCR kit before pooling. Pooled libraries were then massively parallel sequenced in Nextseq-500.

Bioinformatics analysis of sequencing results was accomplished based on the methodology for singleton pregnancy as previously published [7, 10]. The authors' bioinformatics approach assessed the twin pregnancy as a whole, rather than two individual fetuses. Two major parameters involved in this analysis were *Z*-scores and *L*-scores for target chromosomes (21, 18, 13, and sex chromosomes). *Z*-score for a specific chromosome, for example chromosome 21 (Chr21), represents how the fetal percentage of Chr21, based on sequencing data, is deviated from the

Table 2. — *Clinical details of the five cases of NIPT high-risk twin pregnancies.*

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Maternal demographics						
Age (year)	30	34	33	34	36	36
Gestational age (week)	20	22	20	18	18	30
Chorionicity	DCDA	DCDA	DCDA	DCDA	DCDA	DCDA
Type of conception	Natural	IVF	IVF	IVF	IVF	ICSI
NIPT Result (t/L-score)	T13 high-risk (4.99/7.00)	T21 high-risk (11.20/21.27)	T21 warming (3.17/0.92)	XYY high-risk (4.57/12.66)	XYY high-risk (4.65/15.42)	XXX high-risk (6.15/13.33)
Fetal karyotype	Normal/T13	Normal/T21	Normal/T21	Normal/XYY	n/a	Normal/XXX
Invasive test	Amniocentesis	Amniocentesis	Amniocentesis	Amniocentesis	n/a	Amniocentesis
Sonographic markers for affected fetus	Holoprosencephaly, and persistent truncus arteriosus	Nuchal thickening	Nuchal thickening, and hypolastic nasal bone	No reported sonographic marker	n/a	Fetal bowel dilatation
Pregnancy decision and outcome						
Decision	MFPR to the affected fetus	MFPR to the affected fetus	MFPR to the affected fetus	Continue pregnancy	Continue pregnancy	Continue pregnancy
Outcome	Miscarriage after MFPR	Delivered one healthy baby	Miscarriage after MFPR	Preterm birth, lost both twins	Preterm birth, lost both twins	Neonatal death of XXX due to meconium ileus; the normal karyotype fetus survive.

group of reference, which is a set of euploid Chr21. When a sample fetal Chr21 percentage significantly deviates from the mean of the reference set, that sample contains at least one fetus which recognized as trisomy high risk in Chr21. *L*-scores are a logarithmic likelihood ratio that used to evaluate whether a fetus is trisomy high risk [10]. Samples with calculated *Z*-score equal or above 3 and its *L*-score equal or above 1 (located in high-risk and Warning Zones) all considered 'high riskers' for pregnancy with at least one aneuploidy; however, the authors could not differentiate which one or if one or more fetuses were affected. Samples in Warming Zone 1, 2 (*Z*-score > 3 and *L*-score < 1; *Z*-score < 3, and *L*-score >1) were likely affected but due to the presence of mosaicism, partial trisomy, or inadequate fetal fraction, respectively [7]. Samples with *Z*-score < 3 and *L*-score < 1 were considered low risk pregnant with aneuploidies.

Individuals with high-risk in aneuploidy by NIPS were all provided with consultation regarding follow-up invasive karyotyping test. All except one individual with suspected XYY in one or two of her twins, refused to conduct invasive procedure; the remaining of individuals with high risk were tested by G-banding karyotyping. The invasive tests were performed at three to four weeks after the NIPS. Individuals with low risk for aneuploidies were also follow-up by phone interview to inquire information regarding ultrasound findings, pregnancy outcomes, and any signs of abnormal morphological features.

Results

For this cohort of 384 twin pregnancy women, the median maternal age was 31 (Table 1). It ranged from 18 to 44 years; 70% of them are older than age 35. The median gestational week was 18, ranging from 12 to 35 weeks, at the time of NIPS sampling; 200/384 (52.1%) twins were dichorionic-diaminiotic (DCDA), 65/384 (16.9%) monochorionic-diaminiotic (MCDA) 9/384 (2.3%) mono- chorionic-monoamniotic (MCMA), and 110/384 (29.7%) unknown; 129/384 (33.4%) of the twin pregnancies achieved through ART, by *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) and the remaining were natural conception.

With the present bioinformatics approach, six positive cases were identified from the 384 participants, including four at high-risk zone and one at warning zone (Figure 1). The chorionicity of the six positive cases were all DCDA. Five out of the six positive cases were products of ART. The median maternal age was 34 (range 30-36) years and median gestational age was 20 (range 18-22) weeks. One high-risk participant with potential XYY in at least one of her twins (case 5 in Table 2) refused to undergo invasive karyotyping; the other five underwent invasive testing through amniocentesis for both fetuses. The karyotypes confirmed that all five twins had discordant karyotypes, that is one fetus was normal and the other had aneuploidy. The three autosomal trisomy cases (cases 1, 2 and 3 in Table 2) show ultrasound findings consistent with trisomy, such as nuchal thickening and hypoplastic nasal bone. Details related to ultrasound findings, pregnancy decisions, and outcomes of the positive cases of aneuploidies detected by NIPS are summarized in Table 2. After genetic counselling, the families with autosomal trisomy decided to have multiple fetal pregnancy reduction (MFPR) for the affected fetus, whereas the remaining (cases 4-6) opted to

continue their pregnancies.

Based on this cohort of 384 twin pregnant women, the sensitivity and specificity for T21 and T13 were 100% (95%CI 100%) respectively, and the positive predictive value for T21, T13, and SCA were all 100%; 378 participants with negative NIPS results (low-risk for aneuploidies) who responded to phone interview reported no trisomy 21, 18 or 13. Yet among the negative cases, six miscarriages, three preterm births, two still births, and one neonatal death due to tracheobronchial stenosis to one or both of the twins were reported. None of the fetal and perinatal mortalities (12/378, 3.17%) had their karyotypes examined. The authors also received claims for one case of hydrocephaly and one case of congenital heart disease in one of the twins from the NIPS-negative group (2/378, 0.53%).

Discussion

Non-invasive screening for trisomies for twin pregnancies is more complicated, thus more technically challenging than for singleton pregnancy. Its clinical sensitivity and specificity requires a thorough evaluation. A small number of studies on cfDNA analysis in twin pregnancies, with restricted cases, had reported a high detection rate of 95%, 86% and 100% for T21, T18, and T13, and a false positive rate of 0% [7, 11-13]. The present study contributes to extend the data group for validating the performance of NIPS in twin pregnancies. This study yielded a detection rate of 100% for T21 and T13, respectively, which is consistent with previous studies and further strengthens the clinical implementation of non-invasive maternal blood serum screening in twin pregnancies for conventional autosomal trisomy detection.

With detection of XYY and XXX in twin pregnancy, this study also demonstrates a feasibility of applying MPS-based NIPS in twin pregnancies containing SCA. Previous MPSS based singleton NIPS study revealed that XYY has the highest positive predictive value in the method of NIPS compared to XXY, XXX, and XO [14]. Except for the lethal type of monosomy X, phenotypes of SCA are generally mild with substantial physical and cognitive deficits which usually manifest later in life. XXX or XYY individuals were difficult to identify through questionnaire-based phone follow-ups; cytogenetic testing results are required to unveil the true SCA cases. Therefore, in this study, the authors were only able to report predictive positive value for NIPS towards XYY and XXX in twin pregnancies. Although they achieved a predictive positive value of 100% for SCA, larger sample size which contains various types of SCA and precise follow-up for the NIPS negative cases are needed to further evaluate the performance of NIPS in identifying twin pregnancies with SCAs.

From the present NIPS negative population (n=378), the authors received 13 mortality reports from neonatal death, still birth, preterm birth, and miscarriage; furthermore, one case each of congenital heart disease and hydrocephaly. This minor group of lethality and deficiency may generate by chromosomal microdeletion and microduplication, which were not detectable by the current NIPS method. One common syndrome related to congenital heart disease is the DiGeorge syndrome, 22q11 deletion; with an estimated prevalence of one in 4,000 in the population [15]. Unfortunately the authors could not confirm the karyotype of these lethal or deficient cases; therein they may raise false negative cases. A more stringent experimental design will involve karyotype analysis results for all NIPS negative cases validation. Another limitation of the study is the random whole genome-wise analysis of cfDNA by MPS, this approach evaluates the twin pregnancy as a whole but not two individual fetuses. Moreover, it cannot distinguish triploidy after standardization of all autosomal chromosomes, neither can it identify vanishing twins. A higher cost method, single nucleotide polymorphism (SNP)-based sequencing of cfDNA could overcome these drawbacks [16]. Vanishing twin is a potential cause for false positive in NIPS.

Sonography is routinely used during pregnancy to identify harmful conditions in mothers and babies. Depending on the chorionicity and conditions, after first trimester scan, women with twin pregnancies were recommended for ultrasound exam every two to four weeks [17]. The three affected autosomal trisomy cases all showed corresponding sonographic markers. Studies for NIPS performance in twin pregnancies, including the current one, demonstrated and support that common autosomal trisomy (T21 and T13) screening is a reliable approach. This study also expended the usage of cfDNA analysis in SCA (XYY, and XXX) identification among twin pregnancies prenatal screening. Yet verification of the clinical performance of NIPS in twin pregnancies still required larger numbers of participants and stringent follow-ups. At the same time, factors other than autosomal trisomy and SCA can affect fetal development; hence despite a negative result from cfDNA analysis, women with twin pregnancies, as well as singletons, shall always continue with their prescript routine ultrasound check up to ensure fetal and maternal health.

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