ORIGINAL ARTICLE

Noninvasive prenatal testing of trisomies 21 and 18 by massively parallel sequencing of maternal plasma DNA in twin pregnancies

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ABSTRACT

Objective The objective of this study is to assess the performance of noninvasive prenatal testing for trisomies 21 and 18 on the basis of massively parallel sequencing of cell-free DNA from maternal plasma in twin pregnancies.

Method A double-blind study was performed over 12 months. A total of 189 pregnant women carrying twins were recruited from seven hospitals. Maternal plasma DNA sequencing was performed to detect trisomies 21 and 18. The fetal karyotype was used as gold standard to estimate the sensitivity and specificity of sequencing-based noninvasive prenatal test.

Results There were nine cases of trisomy 21 and two cases of trisomy 18 confirmed by karyotyping. Plasma DNA sequencing correctly identified nine cases of trisomy 21 and one case of trisomy 18. The discordant case of trisomy 18 was an unusual case of monozygotic twin with discordant fetal karyotype (one normal and the other trisomy 18). The sensitivity and specificity of maternal plasma DNA sequencing for fetal trisomy 21 were both 100% and for fetal trisomy 18 were 50% and 100%, respectively.

Conclusion Our study further supported that sequencing-based noninvasive prenatal testing of trisomy 21 in twin pregnancies could be achieved with a high accuracy, which could effectively avoid almost 95% of invasive prenatal diagnosis procedures. © 2013 John Wiley & Sons, Ltd.

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Conflicts of interest: None declared

INTRODUCTION

The incidence of multiple pregnancies has increased since the 1980s in many countries.[1–3] In Canada, the rate of multiple births increased from 2.2% to 3.0% between 1995 and 2004.[4] Multiple gestations have become more common because of the expanded use of assisted reproductive techniques (ART). It is well known that twin gestations are associated with higher incidence of fetal structural abnormalities as well as fetal Down syndrome mainly because of the overall higher maternal age amount those with ART.[5,6] Conventional screening methods for fetal Down syndrome using second trimester biochemistry or first trimester combined ultrasound and biochemistry have been widely used for decades in singleton pregnancy. However, their applications in twin pregnancy have been limited by their lower sensitivity and specificity in this group of patients.[7–9] Therefore, there is an urgent need to develop a better screening test for twin pregnancies, which are at higher risk of fetal aneuploidies.

The discovery of cell-free fetal DNA in maternal plasma opened a new direction for noninvasive prenatal testing.[10] The rapid development of massively parallel sequencing (MPS) technology has made the dream of a highly accurate noninvasive prenatal testing (NIPT) a reality. In the past 5 years, several research groups have reported that the sensitivity and specificity in detecting fetal trisomies 21 and 18 using sequencing-based NIPT in singleton pregnancies were over 99%.[11–15] Three published studies also reported the potential of this new approach in twin pregnancies.[16–18] However, only one of these three reports was performed in a nonexperimental study setting with small sample size. There is a need for larger studies to confirm the accuracy of this new approach in twin pregnancy.

In this double-blind study, we recruited 189 pregnant women carrying twins to assess the accuracy of sequencing-based NIPT for trisomies 21 and 18. Maternal blood was taken before invasive sampling, and all sequencing-based tests for trisomies 21 and 18 were performed in an independent clinical laboratory. The sensitivity and specificity of sequencing-based test in twin pregnancies were calculated by comparing with karyotyping results. Our study may provide useful information and experience to clinicians who are interested in the application of this technology.

METHODS

Samples collection and overall design

In this study, 189 participants from seven medical centers, who opted to have an invasive procedure for prenatal diagnosis, were recruited for this study. The inclusion criterion was twin pregnancies that required invasive prenatal diagnosis by amniocentesis, chorionic villus sampling, or cordocentesis. The indications for invasive tests included abnormal maternal serum screening, abnormal sonographic signs, or maternal anxiety. Women with intrauterine fetal demise at the time of sampling or without fetal karyotype results were excluded from this study.

Approvals were obtained from the institutional review board of BGI-Shenzhen. Informed written consent was obtained from each participant.

Maternal blood was sampled before invasive procedures and delivered to the clinical laboratory of BGI Health immediately as a clinical sample. Then the plasma DNA was isolated. The sequencing-based test and analysis were completed in seven working days once a sample was received. The sequencing-based test and full karyotyping analysis were performed in the clinical laboratory of BGI Health and the local medical centers, respectively. The karyotyping result and the sequencing result were kept confidential to the respective analyzing institution until the final analysis. The sensitivity and specificity of the sequencing-based test in detecting fetal aneuploidies were calculated by comparing with karyotyping. The overall design was shown in Figure 1.

Maternal plasma DNA sequencing

In this study, 5 mL of peripheral blood was obtained from each participant in an ethylene diamine tetraacetic acid-anticoagulated tube before invasive procedures, and plasma was separated within 8 hours following a double-centrifugation protocol.

All subsequent procedures, including cell-free DNA isolation, library construction, and sequencing, were performed at an ISO/IEC 17025 certified clinical laboratory in Shenzhen, following previously reported workflow.[19]

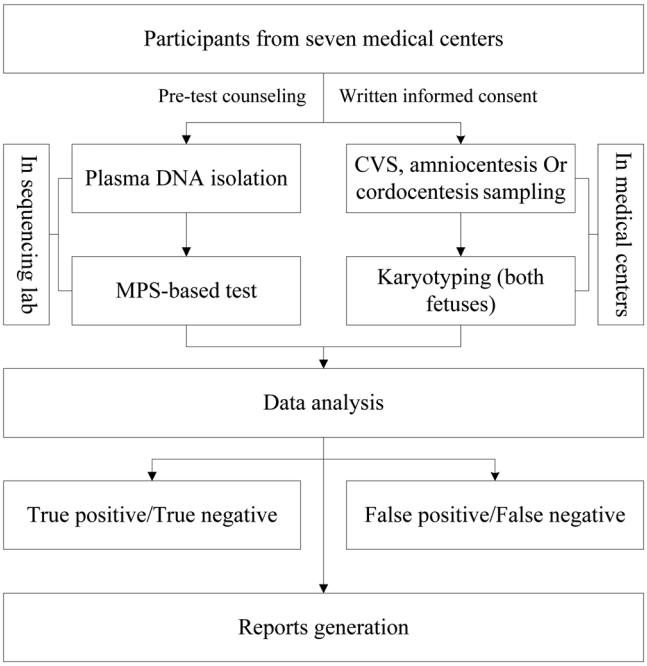

Figure 1 Overall design of this study. A double-blind study was designed. In this study, 189 participants were recruited from seven medical centers with pre-test counseling and written informed consent. Maternal blood was obtained before invasive sampling. Sequencing-based test and karyotyping analysis were simultaneously performed in the sequencing laboratory and local medical centers, respectively. The sequencing-based results of all cases were used to calculate the sensitivity and specificity, comparing with karyotyping results

Bioinformatics analysis for the detection of trisomies 21 and 18

Analysis of the sequencing data and detection of fetal aneuploidies were according to previously reported methodology for singleton pregnancy.[20] Briefly, a binary hypothesis *t*-test and logarithmic likelihood ratio, *L*-score between the two *t*-tests were used to classify whether the fetuses had trisomies 21 or 18 or not. If both the *t*-score were >2.5 and the *L*-score was >1, the sample was in the high-risk zone. If either the *t*-score was >2.5 or the *L*-score was >1, the sample was in the warning zones. If the *t*-score was <2.5 and the *L*-score was <1, the sample was in the low-risk zone.

According to our method, mosaicism and deletion/duplication could also be detected. If a sample result was in the 'Low-risk Zone', the pregnancy was normal. If a sample result was in the 'High-risk Zone', the pregnancy was affected. Cases falling in the 'Warning Zone 1' were classified as affected but usually because of the presence of mosaicism or partial trisomy. Such cases were reported as high-risk but supplemented with appropriate comments. Cases in 'Warning Zone 2' were likely affected pregnancies but with inadequate fetal DNA concentration. If clinically allowed, a repeat blood sampling and sequencing experiment would be repeated. Otherwise, a high-risk report was issued. This classification was used in both singleton and twin pregnancy in the laboratory. Of course, mosaic cases with high percentage of the abnormal cell line might fall into the high-risk zone, whereas those with very low-level mosaicism might be classified as low risk.

Further details of the methodology are included in the Supporting Information.

Karyotyping analysis

Invasive sampling for each case was performed after the peripheral blood was drawn. The metaphase chromosome G-banding karyotyping was performed at a level of 320 to 400 bands. The results of karyotyping were used as the gold standard to calculate the sensitivity and specificity of sequencing-based NIPT. In this study, 95% confidence intervals were evaluated on the basis of standard normal distribution.

RESULTS

Study population

A total of 189 pregnant women from seven medical centers were recruited in this study. Basic characteristics of the study population are shown in Table 1. The maternal age ranged from 22 to 44 years with a median of 31, and 33.9% (64/189) were 35 years old or older. The median gestational age at the time of blood sampling was 19 weeks. There were 31 monochorionic diamniotic (MCDA) twins (16.4%), two monochorionic monoamniotic (MCMA) twins (1.1%), and 152 dichorionic diamniotic (DCDA) twins (80.4%). Chorionicity was not unknown in four cases (2.1%). Method of conception was available in 183 participants, among which, 59.8% (113/183) were from ART.

Karyotyping result

Karyotyping result was obtained in all cases. The method of invasive test was chorionic villus sampling in four cases (2.1%), amniocentesis sampling in 178 cases (94.2%), and cordocentesis sampling in seven cases (3.7%). There were nine cases of trisomy 21 and two cases of trisomy 18, with seven cases in DCDA pregnancies, one case of trisomy 18 in a MCDA pregnancy, and three cases without information of chorionicity (Table 2). Only one of the two fetuses was affected in all positive cases.

Table 1 Basic characteristics of the 189 participants

Maternal age	
Median (year)	31
Range (year)	22–44
Advanced maternal age (≥35 year)	64 (33.9)
18–24 year (*n*, %)	13 (6.9)
25–29 year (*n*, %)	57 (30.2)
30–34 year (*n*, %)	55 (29.1)
35–39 year (*n*, %)	54 (28.6)
≥40 year (*n*, %)	10 (5.3)
Gestational age	
Median (week)	19
Range (week)	11–36
9–12 week (*n*, %)	4 (2.1)
13–16 week (*n*, %)	15 (7.9)
17–20 week (*n*, %)	94 (49.7)
21–24 week (*n*, %)	46 (24.3)
25–28 week (*n*, %)	21 (11.1)
≥29 week (*n*, %)	6 (3.2)
Unknown gestational age (*n*, %)	3 (1.6)
Chorionicity (*n*, %)	
MCDA	31 (16.4)
MCMA	2 (1.1)
DCDA	152 (80.4)
Unknown chorionicity (*n*, %)	4 (2.1)
Type of pregnancy (*n*, %)	
Natural pregnancy	70 (37.0)
Assisted pregnancy	113 (59.8)
Unknown type of pregnancy	6 (3.2)

Identification of fetal trisomies 21 and 18 by noninvasive prenatal testing

Using our bioinformatics analysis approach, there were a total of ten screened positive cases with a gestational age ranged from 13 to 28 weeks with a median age of 18 weeks. All nine cases of trisomy 21 and one case of trisomy 18 were correctly identified. Seven of them were results of assisted reproduction. There was one discordant case (Positive Case11 in Table 2) for trisomy 18 at a gestational age of 25 weeks, with a *t*-score of 0.04 and *L*-score of 0.02 (Figure 2 and Table 2).

In this cohort, the sensitivity and specificity for fetal trisomy 21 by NIPT were both 100% and for fetal trisomy 18 were 50% and 100%, respectively.

Table 2 **Clinical details of the 11 cases with fetal trisomies 21 and 18**

Sample	Maternal age (years)	Gestational age (weeks)	Placentation	Conception	NIPT result (*t*/*L*-score)	Karyotyping	Invasive test
Positive Case1	38	18	DCDA	ART	High risk for T21 (6.51/1.99)	Normal/T21	Amniocentesis
Positive Case2	38	18	DCDA	ART	High risk for T21 (9.03/19.89)	Normal/T21	Amniocentesis
Positive Case3	27	23	DCDA	NP	High risk for T21 (2.63/0.22)	Normal/T21	Amniocentesis
Positive Case4	42	18	DCDA	ART	High risk for T21 (7.62/0.11)	Normal/T21	Cordocentesis
Positive Case5	30	18	–	ART	High risk for T21 (4.27/4.23)	Normal/T21	Amniocentesis
Positive Case6	36	28	–	ART	High risk for T21 (12.87/5.27)	Normal/T21	Cordocentesis
Positive Case7	32	14	–	ART	High risk for T21 (5.72/18.18)	Normal/T21	Amniocentesis
Positive Case8	41	13	DCDA	NP	High risk for T21 (5.27/0.59)	Normal/T21	Amniocentesis
Positive Case9	41	13	DCDA	NP	High risk for T21 (6.25/22.56)	Normal/T21	Amniocentesis
Positive Case10	32	18	DCDA	ART	High risk for T21 (3.39/0.25)	Normal/T18	Amniocentesis
Positive Case11	30	25	MCDA	NP	Normal (0.04/0.02)	Normal/T18	Amniocentesis

-, means the missing information; DCDA, dichorionic diamniotic; MCDA, monochorionic diamniotic; ART, artificial reproductive technology; NP, natural pregnancy.

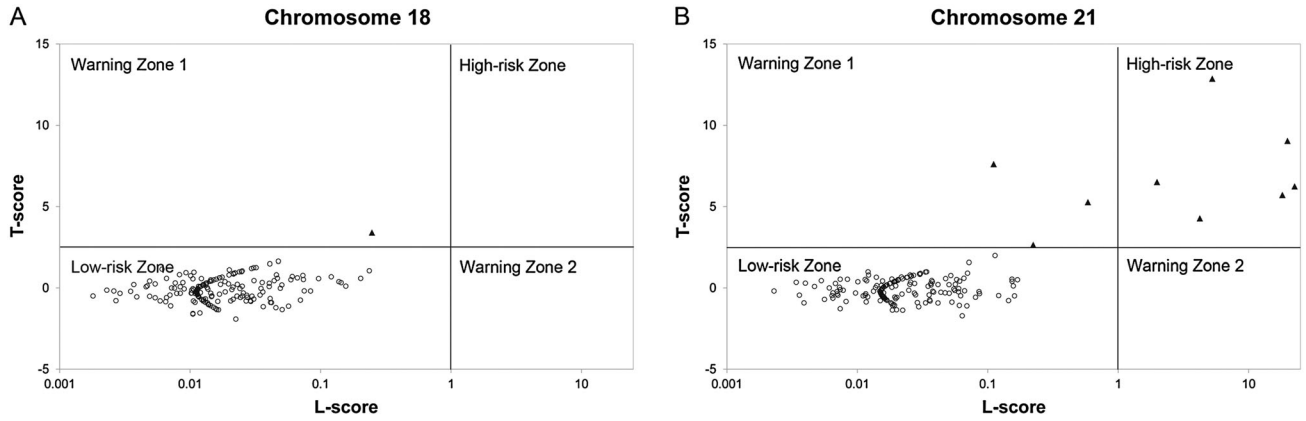

Figure 2 **Identification of fetal trisomies 21 and 18. The risk of fetal aneuploidy was described by *L*-score (*x*-axis) and *t*-score (*y*-axis). Solid triangles represented the true aneuploidies, and open circles represented the negative samples. The high-risk zone is defined by an *L*-score > 1 and a *t*-score > 2.5. Figure (A) and (B) showed the performance for trisomies 18 and 21 detection, respectively**

Follow-up investigations

Among the ten true positive cases, fetal reduction of the abnormal fetus was performed in eight cases of trisomy 21 and one case of trisomy 18. The other one case of trisomy 21 decided to continue the pregnancy, and a normal fetus and an affected fetus with typical clinical phenotypes of trisomy 21were delivered.

The MCDA case with discordant result for trisomy 18 test was recruited at 25 weeks of gestation. Full karyotyping analysis of amniocentesis, performed for sonographic anomalies, confirmed that one fetus was affected by trisomy 18, whereas the other was normal. Further DNA finger printing analysis confirmed that it was a monozygotic twin. After careful post-test genetic counseling, the pregnant woman decided to deliver the fetuses without any treatment. The patient delivered at 36 weeks. One fetus showed typical clinical phenotypes of trisomy 18 and died 2 days later. Unfortunately, placental tissue was not collected for further study.

The remaining 178 cases were classified as negative for both trisomy 21 and trisomy 18, all confirmed by karyotyping analysis. Pregnancy outcome was available in 173 cases, whereas the remaining five cases were lost to follow up. Among the 173 with pregnancy outcome, 149 participants (86.1%) had delivered two normal fetuses, 16 (9.3%) resulted in abortion, and 8 (4.6%) had fetal reduction for reasons other than fetal trisomies 18 or 21.

DISCUSSION

There are ample studies confirming the accuracy of NIPT in singleton pregnancies for the prenatal testing of fetal trisomy 21, and to a lesser extent trisomy 18. However, there is still scanty relevant data for twin pregnancies, including only three small series published so far. In two of them, which included 25 and eight twin pregnancies respectively, with a total of nine Down syndrome, one trisomy 18 and one trisomy 13, were performed retrospectively on archived samples under experimental conditions.[17,18] The last and only prospective study included only 12 twin pregnancies with one trisomy 21 fetus.[16] Although the aforementioned limited data all suggested that prenatal detection of fetal trisomy 21 by NIPT is probably as good as in singleton pregnancies, confirmation by larger series is required. This study was the largest one so far, included 189 twin subjects, and the blood samples were processed as real clinic specimen, providing a real reflection of the test performance. Our study confirmed that prenatal detection of fetal trisomy 21 by NIPT is highly accurate, with 100% sensitivity and specificity.

However, the performance on trisomy 18 is less satisfactory, with a sensitivity of only 50% and a specificity of 100%. The low detection rate for trisomy 18 was probably contributed by (1) the small number of abnormal cases of two, and therefore estimations could be easily affect by sampling error, and (2) the fact that the discordant case was a rare case of discordant trisomy 18 in a monozygotic twin. This could be a result of trisomic rescue in one of the fetus from a trisomy embryo or a result of a post-zygotic event in an originally normal embryo. Because placenta is the major source of fetal DNA in maternal plasma, the success or failure of NIPT in detecting the fetal anomalies depends on the status of cells in the placenta. The proportion of abnormal cells in the placenta could be variable in a pregnancy with trisomic rescue, whereas the placenta could be totally normal if the underlying mechanism was a post-zygotic event. This low level of abnormal cells in the placenta might explain the discordant result.[21] Unfortunately, placental tissue was not available for further study in that case. Further data are required to provide a better estimation of the performance of NIPT for fetal trisomy 18.

It may be argued that NIPT is not good enough because if it is positive, we still do not know which of the two fetuses is affected. However, for the far majority of cases in which both fetuses are normal, the NIPT will enable the couple to avoid any invasive test, because of the high sensitivity and specificity. NIPT enables the selection of almost only the affected pregnancies for invasive test. There is little double that NIPT out-performs any existing alternative Down syndrome screening tests. It is therefore reasonable to make this available to our pregnant women for consideration as a clinical screening strategy for prenatal screening of fetal trisomy 21.

Recently, there were several publications suggesting that noninvasive determination of zygosity is possible.[22,23] It is therefore possible in the future to determine which of the two fetuses is affected in a screened positive case by NIPT. However, before that is feasible, all NIPT positive cases still requires confirmation by karyotyping of both fetuses, except in monochorionic twin pregnancies in which karyotyping of one sample may be adequate.

The major strengths of this study were the relative large sample size, and that all samples were processed immediately as clinical samples, and therefore the results are reflection of the true clinical setting. However, there are several limitations. First, the majority of cases were recruited in the second trimester, and only four subjects were recruited at a gestational age between 9 and 12 weeks. This was due to the relatively late medical records for pregnant women in most hospitals in China. Nonetheless, experience from singleton pregnancy by NIPT suggested that the detection rate of aneuploidy does not seem to be affected by gestational age beyond 12 weeks.[24] Further study with twin subjects in early pregnancy will provide a direct answer. Lastly, the sample size of this study was still not large enough to be conclusive. But on the basis of all available information, NIPT for fetal Down syndrome in twin pregnancy is likely to be as accurate as in singleton pregnancies.

CONCLUSION

In this study, using sequencing-based test, we successfully identified chromosome aneuploidy in twin pregnancies. This study provided further clinical date supporting the application of this new noninvasive prenatal test in twin pregnancies.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Massively parallel sequencing (MPS) has been proved to be feasible for the noninvasive prenatal testing of trisomies 21 and 18 in singleton pregnancies. However, there is still lack of studies assessing the performance of this approach in twin pregnancies.

WHAT DOES THIS STUDY ADD?

- This is a double-blind study of MPS-based noninvasive prenatal testing for trisomies 21 and 18 in twin pregnancies. We proved that the MPS-based test is highly accurate for detecting trisomy 21 in twin pregnancies.

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SUPPORTING INFORMATION

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