Twin Research and Human Genetics
Volume 20 ▌ Number 3 ▌ pp. 242–249 ▌ © The Author(s) 2017 ▌ doi:10.1017/thg.2017.23

Massively Parallel Sequencing (MPS) of Cell-Free Fetal DNA (cffDNA) for Trisomies 21, 18, and 13 in Twin Pregnancies

Erqiu Du,[1,2] Chun Feng,[1,2] Yuming Cao,[1,2] Yanru Yao,[1,2] Jing Lu,[1,2] and Yuanzhen Zhang[1,2]
[1]Department of Obstetrics and Gynecology, Zhongnan Hospital of Wuhan University, Wuhan, China
[2]Hubei Provincial Key Laboratory of Developmentally Originated Disease, Wuhan University, Wuhan, China

Massively parallel sequencing (MPS) technology has become increasingly available and has been widely used to screen for trisomies 21, 18, and 13 in singleton pregnancies. This study assessed the performance of MPS testing of cell-free fetal DNA (cffDNA) from maternal plasma for trisomies 21, 18, and 13 in twin pregnancies. Ninety-two women with twin pregnancies were recruited. The results were identified through karyotypes of amniocentesis or clinical examination and follow-up of the neonates. Fluorescent in-situ hybridization was used to examine the placentas postnatally in cases of false-positive results. The fetuses with autosomal trisomy 21 ($n = 2$) and trisomy 15 ($n = 1$) were successfully detected via MPS testing of cffDNA. There was one false-positive for trisomy 13 ($n = 1$), and fluorescence in-situ hybridization (FISH) identified confined placental mosaicism in this case. For twin pregnancies undergoing second-trimester screening for trisomy, MPS testing of cffDNA is feasible and can enhance the diagnostic spectrum of non-invasive prenatal testing, which could effectively reduce invasive prenatal diagnostic methods. In addition to screening for trisomy 21, 18, and 13 by cffDNA, MPS can detect fetal additional autosomal trisomy. False-positive results cannot completely exclude confined placental mosaicism.

■ **Keywords:** twin pregnancies, trisomy, prenatal screening, massively parallel sequencing

Multiple pregnancies have gradually become common because of the expanded use of assisted reproductive techniques (ART). As a result, twin pregnancies are also related to the incidence of fetal structural anomalies and Down syndrome, mainly because of the overall higher maternal age and gene imbalance of parents using ART (Hansen et al., 2013; Odibo et al., 2003). Conventional serum screening has been widely used for singleton pregnancies in recent decades. Although first-trimester and second-trimester aneuploidy screening is available for twin pregnancies, conventional serum screening is less accurate for twin than for singleton pregnancies.

Invasive prenatal diagnosis in twin pregnancies is associated with a risk of pregnancy loss that is higher than the baseline risk of loss among twin pregnancies (Cleary-Goldman & Berkowitz, 2005; Spencer, 2000; Vink et al., 2012). Thus, there is an urgent need to develop better screening methods for twin pregnancies with a high risk of fetal aneuploidy. MPS technology has become increasingly available and has been widely used to screen for trisomies 21, 18, and 13 in singleton pregnancies. Massively parallel sequencing (MPS) testing for fetal Down syndrome has also gradually become more commonly used for twin pregnancies and has shown high sensitivity and specificity (Canick et al., 2012; Gromminger et al., 2014; Huang et al., 2014; Lau et al., 2013; Leung et al., 2013). Here, we report the screening of trisomies 21, 18, and 13 with MPS testing for twin pregnancies. All samples were prospectively collected and assayed immediately in the same manner.

Materials and Methods

Sample Collection

In this study, 92 women with twin pregnancies were recruited from January 1, 2013 to October 1, 2016. The included criteria were twin pregnancies that required invasive prenatal diagnosis using amniocentesis or clinical examination and follow-up of the neonates. An ultrasound examination was performed at 11+0 to 13+6 weeks to

RECEIVED 17 January 2017; ACCEPTED 20 March 2017

ADDRESS FOR CORRESPONDENCE: Yuanzhen Zhang, No. 169 East Lake Road, Wuchang, Wuhan, China. E-mail: zhangyuanzhen@vip.sina.com

determine gestational age based on the fetal crown–rump length (CRL), diagnose any major fetal anomalies, and measure the fetal nuchal translucency (NT) thickness. In the twin pregnancies, an ultrasound examination was used to determine the CRL of the larger fetus, and chorionicity was identified by examining the junction of the inter-twin membrane with the placenta. The measured NT was reported as the difference from the expected normal mean for gestation (delta value; Wright et al., 2008). In our study, the measured free beta human chorionic gonadotrophin (β-hCG) and pregnancy-associated plasma protein A (PAPP-A) were not used in combination to estimate the patient-specific risk for trisomies 21, 18, and 13 because of their relatively low precision in this group of patients. The indications for invasive tests included positive MPS test results, acatastatic sonographic signs, family genetic history, maternal age, and history of acatastatic fetal pregnancy. Women with intrauterine fetal demise at the time of sampling or without fetal karyotype results or clinical examination and follow-up were excluded from this study. Informed written consent was obtained from participants who had undergone extensive individual counseling and an ultrasound scan. The cffDNA was isolated from maternal peripheral blood. The results of MPS testing were presented within two weeks after the sample was received. Positive results of MPS testing of cffDNA for fetal aneuploidies were identified via karyotype analysis. Fluorescence in-situ hybridization (FISH) was used to examine the placenta postnatally in cases of false-positive results.

Maternal Plasma DNA Sequencing

In this study, 10 mL of peripheral blood was obtained from each participant and stored in EDTA-containing tubes before invasive procedures were performed. The maternal blood samples were centrifuged twice within 8 hours after collection to extract cffDNA. MPS testing was satisfactory for the fetal fractions (above 10%, see Table 1). All subsequent procedures, including cffDNA extraction, DNA library construction, and sequencing were carried out in China according to a previously reported workflow (Dan et al., 2012).

Bioinformatics Analysis for the Detection of Trisomies 21, 18, and 13

The analyses of the sequencing data and the detection of fetal aneuploidies were consistent with the methodology previously reported for singleton pregnancies. A bioinformatic analysis was performed, with z scores ≥ 3 indicating the presence of a fetal trisomy 21, 18, and 13 (Chen et al., 2011; Chiu et al., 2011; Gregg et al., 2013). In brief, the data were analyzed using Illumina software, and an effective assessment of the standard deviations beyond the central estimate (z score) identified chromosomes 21, 18, and 13. If the A-value was >3 and the T-value was >3, the sample was in the high-risk zone. If either the A-value was >3 or the T-value was >3, the sample was in the warning zones. If the A-value was ≤ 3 and T-value ≤ 3, the sample was in the low-risk zone. If the sample result was in the 'low-risk zone', it was considered normal. If the sample result was in the 'high-risk zone', it was affected. If the sample fell within Warning Zone 1, the sample was considered affected by mosaicism or partial trisomy. Such cases were reported as high risk but were accompanied by appropriate comments. Samples in Warning Zone 2 were likely affected by inadequate fetal DNA concentrations. If clinically permitted, blood sampling and sequencing were repeated. Otherwise, a high-risk report was issued. This classification of twin pregnancies was the same as that used for singleton pregnancies in the laboratory. Of course, mosaicism cases with a high percentage of the acatastatic cell line might be in the high-risk zone, but those with a very low percentage of mosaicism might be regarded as low risk.

Karyotype Analysis and FISH Detection

Invasive diagnostic procedures for positive cases involved traditional amniotic fluid cell culture karyotype analysis. A karyotype analysis was performed using the conventional Giemsa banding (G-binding) method (Lanza et al., 1998). FISH was performed on postnatal placenta tissues. To prepare the placental tissues and probes, tissues (0.5 g) were collected from each of the four quadrants of the placenta. FISH was performed according to the previously established method (Klinger et al., 1992).

Results

Study Population

In the 92 patients, four positive reports were issued, and those patients underwent the prenatal diagnosis. The basic characteristics of the 92 parents are summarized in Table 2.Their average age was 30.54 years, ranging from 23 to 41 years. Twenty-one were 35 years old and older. The median gestational age at the time of MPS testing was 17.92 weeks. There were 53 dichorionic twin pregnancies and 39 monochorionic twin pregnancies. The method of conception included two types of pregnancy, of which 40 patients (including six who underwent ovulation induction) conceived naturally while 57.60% (53/92) conceived using ART. The four patients who received positive MPS results chose to undertake amniocentesis. The pregnancy outcomes included three terminations, 47 premature deliveries, and 42 term deliveries.

Karyotype Results and FISH

Fetal outcomes were ascertained either by prenatal karyotype or by clinical examination of the newborn after delivery. Four patients underwent karyotype analysis via amniocentesis. There were two cases of trisomy 21 and one case of trisomy 15 in the twin pregnancies. However, only one of the two fetuses was affected by trisomy 21 in all positive

https://doi.org/10.1017/thg.2017.23 Published online by Cambridge University Press

TABLE 1

Clinical Cases and the Sequencing Outcome

Case	Age	Gestational age (weeks)	Conception	Chorionicity (ultrasound scan)	NT (11–13) weeks	MPS testing result	Fetal fraction (%)	Karyotype	Pregnancy outcome	Fetal sex	Follow-up
1	28	17^{+1}	NP	DC	1.7/2.0	−ve	16	No	TD	M/F	N
2	28	17^{+2}	NP	DC	1.3/1.4	−ve	17	No	TD	M/F	N
3	41	14^{+5}	NP	DC	NA	High risk (T21)	14	T21/ N	TP	M/F	TP
4	26	17^{+3}	NP	DC	1.1/1.3	−ve	16	No	PD	M/F	N
5	32	14^{+6}	ART	DC	NA	High risk (T15)	16	T15/N	TP	F/M	TP
6	26	15^{+5}	NP	MC	2.9/2.1	High risk (T21)	14	T21/ N	TP	M/M	TP
7	33	15^{+4}	ART	MC	1.7/1.9	−ve	21	No	PD	M/M	N
8	24	16^{+2}	NP	DC	1.5/1.9	−ve	15	No	PD	M/F	N
9	33	18^{+2}	ART	DC	2.0/2.1	−ve	20	No	TD	M/F	N
10	30	18^{+3}	ART	DC	2.7/2.1	−ve	13	No	TD	M/F	N
11	34	16^{+2}	ART	DC	2.3/2.4	−ve	18	No	TD	M/F	N
12	38	16^{+3}	ART	DC	1.9/2.9	−ve	19	N/N	PD	M/F	N
13	34	17^{+1}	NP	DC	1.8/2.1	−ve	17	No	PD	M/F	N
14	38	21^{+1}	NP	DC	2.3/2.1	High risk (T13)	19	N/N	PD	F/M	N
15	26	17^{+5}	ART	DC	1.7/2.3	−ve	20	No	TD	M/F	N
16	25	19^{+1}	ART	DC	0.9/1.3	−ve	29	No	PD	M/F	N
17	36	20^{+2}	ART	DC	1.4/1.6	−ve	25	No	TD	M/F	N
18	29	17^{+2}	ART	DC	1.4/1.6	−ve	20	No	TD	F/M	N
19	28	18^{+4}	ART	DC	1.3/1.2	−ve	25	No	PD	M/F	N
20	29	17^{+2}	OI	DC	1.6/1.8	−ve	22	No	PD	M/F	N
21	35	17^{+3}	ART	MC	1.7/2.1	−ve	21	No	PD	F/F	N
22	24	16^{+3}	NP	DC	1.8/2.0	−ve	23	No	PD	M/F	N
23	25	18^{+2}	NP	MC	1.5/1.0	−ve	30	No	TD	M/M	N
24	23	16^{+2}	NP	MC	NA	−ve	29	No	TD	M/M	N
25	32	22^{+1}	NP	DC	NA	−ve	22	No	TD	F/M	N
26	33	17^{+2}	ART	MC	1.6/2.0	−ve	30	No	PD	M/M	N
27	25	20^{+1}	NP	MC	1.3/1.6	−ve	32	No	PD	F/F	N
28	30	17^{+5}	OI	MC	1.2/1.8	−ve	33	No	PD	F/F	N
29	30	19^{+4}	NP	MC	NA	−ve	35	No	TD	F/F	N
30	38	17^{+1}	ART	MC	1.5/1.6	−ve	36	No	TD	F/F	N
31	32	15^{+5}	ART	DC	1.6/1.9	−ve	21	No	PD	M/F	N
32	30	15^{+4}	ART	DC	1.7/2.3	−ve	20	No	PD	F/M	N
33	32	16^{+1}	ART	MC	1.8/2.0	−ve	21	No	PD	F/F	N
34	30	14^{+5}	NP	DC	2.2/1.9	−ve	15	No	TD	M/F	N
35	29	15^{+3}	NP	DC	1.4/1.6	−ve	20	No	PD	M/F	N
36	28	16^{+6}	ART	MC	1.5/1.8	−ve	30	No	PD	M/M	N
37	37	17^{+1}	ART	MC	1.5/1.7	−ve	37	No	TD	M/M	N
38	21	17^{+2}	NP	MC	1.5/1.6	−ve	20	No	PD	F/F	N
39	36	17^{+2}	ART	DC	1.8/1.9	−ve	21	No	SB(TP)	M/F	N
40	31	17^{+4}	ART	MC	2.0/1.9	−ve	18	No	TD	F/F	N
41	29	15^{+1}	OI	MC	2.0/2.1	−ve	14	No	PD	F/F	N
42	25	17^{+1}	ART	DC	2.2/2.1	−ve	18	No	SB(TP)	M/F	N
43	29	18	OI	MC	2.0/1.9	−ve	23	No	TD	F/F	N
44	37	19	ART	DC	1.8/1.9	−ve	26	No	PD	M/F	N
45	37	20	ART	DC	1.6/1.8	−ve	30	No	TD	F/M	N
46	31	20^{+1}	ART	MC	1.5/1.6	−ve	19	No	TD	F/F	N
47	23	20^{+2}	NP	MC	1.6/1.9	−ve	20	No	TD	F/F	N
48	28	19	NP	DC	1.7/1.8	−ve	21	No	TD	F/M	N
49	34	18	NP	DC	1.5/1.9	−ve	12	No	TD	F/M	N
50	24	18	ART	MC	1.5/1.9	−ve	18	No	TD	F/F	N
51	36	17^{+1}	ART	DC	1.0/1.1	−ve	20	No	PD	F/M	N
52	24	17^{+5}	ART	DC	1.2/1.1	−ve	19	No	TD	M/F	N
53	30	16^{+5}	ART	MC	1.0/1.2	−ve	16	No	PD	F/F	N
54	36	14^{+2}	NP	MC	1.2/1.5	−ve	13	No	TD	F/F	N
55	34	19^{+6}	NP	DC	2.0/2.1	−ve	17	No	TD	F/M	N
56	29	21^{+3}	ART	DC	1.9/2.0	−ve	16	No	TD	F/M	N
57	26	20	OI	DC	1.8/1.9	−ve	15	No	TD	F/M	N
58	34	18	NP	DC	0.9/1.2	−ve	21	No	PD	F/M	N
59	35	17	ART	MC	1.0/1.4	−ve	27	No	PD	M/M	N
60	32	16^{+5}	NP	MC	1.6/1.8	−ve	26	No	PD	M/M	N
61	31	17^{+4}	ART	MC	1.8/2.0	−ve	25	No	TD	F/F	N
62	32	15	NP	DC	1.5/1.8	−ve	23	No	PD	M/F	N
63	34	15^{+6}	ART	DC	1.9/1.8	−ve	21	No	TD	F/M	N
64	35	14^{+5}	NP	DC	1.5/2.0	−ve	22	No	TD	F/M	N
65	28	16^{+2}	ART	DC	1.0/1.4	−ve	18	No	PD	F/M	N
66	27	15^{+2}	ART	MC	1.5/1.6	−ve	16	No	PD	M/M	N
67	29	17	NP	DC	1.9/2.0	−ve	11	No	TD	M/F	N
68	25	15	ART	MC	1.8/1.6	−ve	10	No	TD	F/F	N
69	28	14^{+6}	OI	MC	1.7/1.9	−ve	23	No	TD	M/M	N
70	30	19	NP	DC	1.8/1.8	−ve	20	No	PD	M/F	N
71	37	20	ART	MC	1.9/2.0	−ve	29	No	PD	F/F	N
72	37	17	NP	DC	1.7/1.5	−ve	27	No	PD	M/F	N

https://doi.org/10.1017/thg.2017.23 Published online by Cambridge University Press

TABLE 1
Continued

Case	Age	Gestational age (weeks)	Conception	Chorionicity (ultrasound scan)	NT (11–13) weeks	MPS testing result	Fetal fraction (%)	Karyotype	Pregnancy outcome	Fetal sex	Follow-up
73	31	15	ART	DC	1.6/1.8	−ve	26	No	PD	F/M	N
74	23	14^{+6}	NP	DC	1.6/1.8	−ve	22	No	PD	M/F	N
75	28	22	ART	DC	1.6/1.9	−ve	15	No	TD	M/F	N
76	30	20	ART	MC	1.8/1.9	−ve	17	No	TD	F/F	N
77	32	19	ART	MC	1.6/1.9	−ve	20	No	PD	M/M	N
78	33	17^{+4}	ART	MC	2.0/2.1	−ve	19	No	PD	M/M	N
79	34	16^{+3}	ART	DC	1.5/1.8	−ve	18	No	PD	M/F	N
80	35	17^{+6}	NP	DC	1.2/1.3	−ve	17	No	TD	M/F	N
81	25	16	NP	DC	2.0/1.6	−ve	15	No	PD	M/F	N
82	34	18^{+2}	NP	MC	1.6/1.9	−ve	40	No	PD	F/F	N
83	35	19	ART	DC	1.8/1.9	−ve	15	No	PD	M/F	N
84	38	18	ART	MC	1.9/2.2	−ve	16	No	PD	F/F	N
85	36	17	ART	MC	1.7/1.9	−ve	23	No	TD	M/M	N
86	35	16^{+5}	ART	MC	1.8/1.9	−ve	22	No	PD	M/M	N
87	30	15^{+4}	ART	MC	1.6/1.8	−ve	20	No	PD	M/M	N
88	34	15^{+5}	NP	DC	1.6/1.8	−ve	16	No	TD	F/M	N
89	24	15	ART	DC	1.5/1.8	−ve	10	No	PD	F/M	N
90	30	15^{+6}	NP	MC	1.0/1.4	−ve	12	No	PD	F/F	N
91	32	17	ART	MC	1.6/1.8	−ve	15	No	TD	F/F	N
92	28	16^{+1}	ART	DC	1.8/1.6	−ve	16	No	TD	M/F	N

Note: DC = dichorionic twin; MC = monochorionic twin; F = female; M = male; ART = assisted reproductive techniques; OI = ovulation induction; NP = natural pregnancy;NA = not available or not done; N = normal; No = not done; −ve = screened negative; PD = premature delivery; TD = term delivery; TP = terminate pregnancy.

TABLE 2
Basic Characteristics of the 92 Parents

Characteristics	
Maternal age	
Median (year)	30.54
Range (years)	23–41
Advanced maternal age (≥35years)	21 (21.74%)
18–24 years (*n*, %)	9 (9.78%)
25–29 years (*n*, %)	27 (29.35%)
30–34 years (*n*, %)	35 (38.04%)
35–39 years (*n*, %)	20 (21.74%)
≥40 years (*n*, %)	1 (1.09%)
Gestational age	
Median (week)	17.92
Range (week)	14–23
13–16^{+6} weeks (*n*, %)	36 (39.13%)
17–20^{+6}weeks (*n*, %)	52 (56.52%)
21–24^{+6} weeks (*n*, %)	4 (4.35%)
25–28^{+6} weeks (*n*, %)	0
≥29 week (*n*, %)	0
Chorionicity (*n*, %)	
MC	39 (42.39%)
DC	53 (57.61%)
Type of pregnancy (*n*, %)	
Natural pregnancy	40 (43.49%)
Assisted reproductive techniques	52 (56.52%)
Pregnancy outcome	
Premature delivery	47 (51.08%)
Term delivery	42 (45.65%)
Terminate pregnancy	3 (3.26%)

cases. A false-positive trisomy 13 result of MPS testing was correctly identified by karyotype analysis.

Identification of Fetal Trisomies 21, 18, and 13 with MPS Testing

Using the bioinformatics analysis approach, four cases were identified as positive. The cases of trisomy 21 ($n = 2$) and trisomy 15 ($n = 1$) identified with MPS testing were correctly identified with a full karyotype analysis of the amniocentesis sample. The trisomy 13 result identified with MPS testing was identified as a normal karyotype and correctly diagnosed as confined placental mosaicism. In this cohort, fetal trisomy 21, detected with MPS testing of cffDNA, was consistent with the karyotype analysis. The sensitivity and specificity of MPS testing for fetal trisomy 21 were 100%. Although the finding of fetal trisomy 13 with MPS testing of cffDNA was a false positive, it was correctly identified as confined placental mosaicism. However, no cases of trisomy 18 were detected (see Figure 1).

Follow-Up Investigations

All cases were followed-up after birth. Table 1 summarizes the outcomes of these subjects, including the karyotype analyses of amniocentesis samples, sonographic anomalies, and the results of follow-up. However, it is regrettable that in one case, the parents decided to terminate both twins because one of the fetuses had trisomy 21. Although the MPS result of one fetus showed trisomy 13, the full karyotype analysis of the amniocentesis showed a normal karyotype. Placental tissue was collected and confined placental mosaicism was diagnosed. The remaining 87 cases were classified as negative for trisomies 21, 18, and 13. Among the 87 patients with reported pregnancy outcomes, 87 delivered normal fetuses (Table 2). The delivery of twin fetuses was reported for 87 participants, 47 had premature delivery, and 45.98% (40/87) were delivered at term (see Table 2).

Discussion

In twin pregnancies, invasive diagnostic methods are more complex than in singleton pregnancies because of the

https://doi.org/10.1017/thg.2017.23 Published online by Cambridge University Press

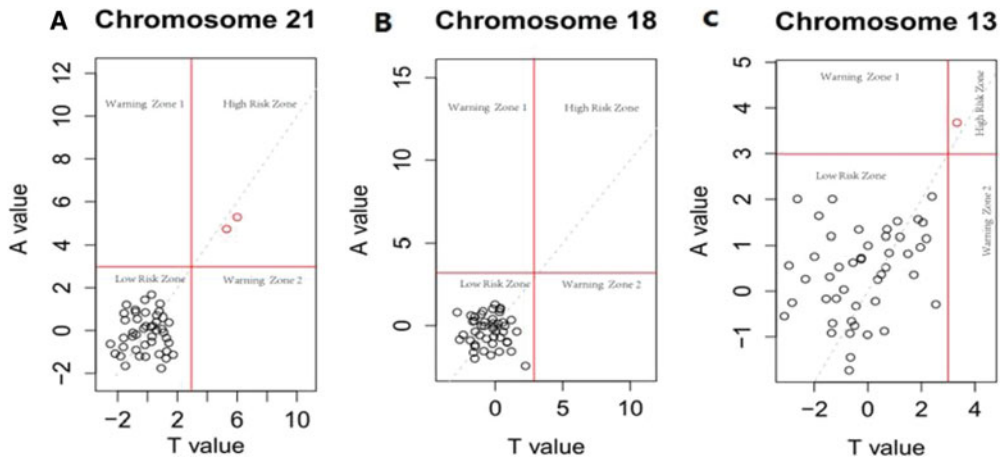

FIGURE 1

(Colour online) Identification of fetal trisomies 21, 18, and 13. The risk of fetal aneupoloidy is described by the *T*-value (*x*-axis) and *A*-value (*y*-axis). Red circles represent the positive results and open circles represent the negative results. The high-risk zone is defined by a *T*-value >3 and *A*-value >3.

higher possible incidence of procedure-related miscarriage and stillbirth, the possibility of sampling errors, and the increase in technical difficulty (Lau et al., 2013). Non-invasive prenatal diagnosis is as formidable as invasive prenatal diagnosis. Many studies have confirmed the accuracy of MPS in singleton pregnancies for the prenatal testing of fetal trisomies 21, 18, and 13. However, there are only five published studies with a small amount of data for twin pregnancies (Canick et al., 2012; Gromminger et al., 2014; Huang et al., 2014; Lau et al., 2013; Leung et al., 2013). One study detected two pregnancies with trisomy 18, one of which was a false-positive result (Huang et al., 2014). Another previously published study correctly detected one fetus with trisomy 13 and seven twin pregnancies with trisomy 21 out of 25 twin pregnancies. The sensitivity and specificity of MPS for fetal trisomies 21 and 13 were 100% (Canick et al., 2012). Five studies used MPS testing of cffDNA to identify trisomy 21 in twin pregnancies, with a sensitivity of 1.000 (95% CI [0.846, 1.000]) and a specificity of 0.999 (95% CI [0.999, 0.999]). A meta-analysis of twin pregnancies tested with cffDNA has been published; the diagnostic rate for trisomy 21 was 93.7% (95% CI [83.6, 99.2]), and the FPR was 0.23% (95% CI [0.00, 0.92]; Gil et al., 2015). There were also nine trisomy 18 pregnancies and two trisomy 13 pregnancies that were classified correctly (Canick et al., 2012; del Mar Gil et al., 2014; Huang et al., 2014). It is feasible to use MPS of cffDNA in twin pregnancies. The fraction of cffDNA in maternal plasma in twin gravidas is about 5% ∼ 30%, and increases with increasing gestational age (del Mar Gil et al., 2014). There is evidence that each fetus in dizygotic twin pairs can contribute different amounts of cffDNA to the maternal circulation, and the difference can vary by nearly twofold. Twin pregnancies are more complex than singleton pregnancies because monozygotic twins are genetically identical and dizygotic twins are genetically different, in which case it is possible that only one fetus may have aneuploidy (Leung et al., 2013; Qu et al., 2013). However, monozygotic twins discordant for chromosomes 13, 18, and 21 are reported (Choi et al., 2013; Ramsey et al., 2012; Reuss et al., 2011). There are several potential mechanisms that may lead to discordant karyotypes in monozygotic twins. Monochorionic–diamniotic twins may be discordant for karyotypes, for which anaphase lagging, chromosomal non-disjunction, and trisomy rescue may be the underlying reasons (Machin, 2009). In twin pregnancies undergoing first-trimester screening for trisomies using cffDNA testing, the fetal fraction is lower and the failure rate is higher than those of singletons (Sarno et al., 2016).Therefore, our study was carried out during the second trimester, screening for trisomies using cffDNA testing. It is feasible to use MPS testing of cffDNA in dizygotic twin pregnancies that are discordant for aneuploidy; however, doing so could obtain an erroneous result regarding the risk of aneuploidy because a high contribution from the disomic cotwin could lead to a satisfactory total fetal fraction. To avoid this potential risk, researchers have proposed that for non-invasive prenatal diagnosis in twin pregnancies, the lower fetal fraction of the two fetuses, rather than the total fetal fraction, be used to assess the risk of aneuploidies (del Mar Gil et al., 2014). However, an inevitable result of such a policy is that no-result findings may be more common in twin pregnancies than in singleton pregnancies (Bevilacqua et al., 2015). Although there are five studies that have reported the successful use of MPS in twin pregnancies, direct evidence is very limited.

Our results suggest that three positive results of the 92 twin pregnancies (two cases with discordant fetal trisomy 21 and one case with discordant fetal trisomy 15) were correctly identified with high precision. One case that had been classified as having trisomy 13 by MPS testing was

https://doi.org/10.1017/thg.2017.23 Published online by Cambridge University Press

determined to be a false positive when the karyotype analysis of amniotic fluid showed a normal karyotype. The fetus was found to have a normal karyotype, but the placenta had an acatastatic karyotype because of confined placental mosaicism. Mosaicism is a condition in which an individual has two or more genetically distinct cell lines that develop from a single zygote. Mosaic trisomies do occur with varying degrees of trisomic cells present in different tissues. Affected individuals have both trisomic and euploid cell lines in the case of trisomy 21 mosaicism and Down syndrome. However, the differing percentages of trisomic and euploid cells in individuals with mosaicism for trisomy 21 is related to the varying proportion and/or tissue distribution of the trisomic cells (Papavassiliou et al., 2009). Everyone may carry some cells with an extra mosaicism for trisomy 21 in some tissues (Hultén et al., 2013). Discordance between MPS testing and karyotype analysis may reflect the limitations of the positive predictive value of MPS testing. Confined placental mosaicism is the presence of an acatastatic cell line in the placenta of a fetus with a normal karyotype. It is diagnosed when both normal and acatastatic cell lines are detected in placenta samples, while only the normal cell line is detected in fetal blood samples and by amniocentesis (Kalousek & Vekemans, 2000). It occurs in approximately 1–3% of viable pregnancies, according to chorionic villus sampling (CVS). A large proportion of pregnancies with confined placental mosaicism proceed uneventfully, resulting in normal liveborn infants. Confined placental mosaicisms have been classified as two different types: mitotic and meiotic. In mitotic confined placental mosaicism, the conception is diploid, and the acatastatic division takes place in the progenitors of specific placental cell lineages. In contrast, in meiotic confined placental mosaicism, the zygote has an initial acatastatic chromosomal complement (most often a trisomy), and a subsequent mitotic 'error' causes the loss of the acatastatic chromosome in the true embryonic cell lineage, leaving the acatastatic cell line confined to the placenta, a process known as trisomic zygote rescue (Baffero et al., 2012; Chan Wong et al., 2012; Kalousek, 2000). Amniotic fluid-derived stem cells are derived from the amniotic fluid of developing fetus and give rise to three germ layers: the endoderm, mesoderm, and ectoderm. However, the origin of the cffDNA is uncertain. Evidence suggests that cffDNA mostly derives from placental trophoblast cells (Alberry et al., 2007; Bianchi, 2004; Flori et al., 2004; Gahan & Swaminathan, 2008; Rosner & Hengstschläger, 2013). Therefore, the karyotype of amniotic fluid is truer than cffDNA in identifying the fetus karyotype. MPS testing is very precise, but MPS testing of cffDNA might yield a low false-positive result (Chen et al., 2011; Mao et al., 2014). Although MPS testing can detect an affected fetus in twin pregnancies, it cannot accurately note which of the two fetuses is the affected one. Nonetheless, the high precision of MPS testing can correctly detect the vast majority of pregnancies with normal fetuses, thereby reducing the need for invasive prenatal diagnosis. With the limited evidence, it is still premature to offer MPS testing of cffDNA for trisomies 21, 18, and 13 as a routine clinical service for twin pregnancies. However, all reported evidence suggests that MPS testing of cffDNA for trisomies 21, 18, and 13 in twin pregnancies is likely to be as effective as such testing for singleton pregnancies. Therefore, MPS testing of cffDNA for trisomies 21, 18, and 13 should be an option for women with twin pregnancies at increased risk, in lieu of amniocentesis, especially for those who have been shown to be at high risk by conventional testing, after extensive counseling from specialists with experience in this specialized field. Our study suggested that MPS testing of cffDNA for trisomies 21, 18, and 13 in twin pregnancies is available. Moreover, other aneuploidies can correctly be detected. If the result of MPS testing of cffDNA is positive, genetic counseling may facilitate patient-focused decision making. Although MPS testing of cffDNA could enhance the diagnostic spectrum of non-invasive prenatal testing and decrease the invasive prenatal diagnosis of higher precision, the parents should consider the questions about pregnancy loss following first-trimester CVS and mid-trimester genetic amniocentesis in twins. In both amniocentesis and CVS, fetal loss rate does not affect significantly the outcomes evaluated (Agarwal & Alfirevic, 2012; Simonazzi et al., 2010). The evaluation of fetus, placenta, umbilical cord, and amniotic cavity by ultrasound scan is beneficial to amniocentesis.

If one fetus in a twin pregnancy is discordant for structural and chromosomal anomalies, genetic counseling may facilitate patient-focused decision making. Selective fetal reduction is performed for a variety of indications in multiple pregnancies. The reduction of multiple pregnancies using potassium chloride (KCl) is a usual method for reduction in cases of diamniotic twins, but this method is complicated in cases of monochorionic multiple pregnancies, in which KCl cannot be used. For monochorionic twins, vascular ablative techniques are used for selective reduction. Such techniques include bipolar cord occlusion (BCO), interstitial laser and, more recently, radiofrequency ablation (RFA; Nobili et al., 2013). In this scenario, the usual fetal reduction procedure of monochorionic twins could endanger both fetuses, which share a placenta; thus, normal saline was used to create a cardiac tamponade and realize cardiac asystole, which is a novel way of reducing the acatastatic fetus in monochorionic twins without an adverse effect on the co-twin and or a maternal spillage effect of the commonly used drug (Gupta et al., 2016).

Conclusion

In summary, MPS testing of cffDNA for trisomies 21, 18, and 13 is precise and effective for successfully detecting discordant structural and chromosomal anomalies and reduces the need for invasive prenatal diagnosis. In the future,

https://doi.org/10.1017/thg.2017.23 Published online by Cambridge University Press

massive parallel sequencing tests will be gradually used for non-invasive screening for fetal trisomies 21, 18, and 13 in twin pregnancies. When twin fetuses are classed as discordant for structural and chromosomal anomalies, selective fetal reduction is performed to reduce the birth defects after genetic counseling by specialists.

Acknowledgments

This work was supported by Department of Obstetrics and Gynecology, Zhongnan Hospital of Wuhan University, Department of Obstetrics and Gynecology, Renmin Hospital, Hubei University of Medical, Shiyan, China. This work was also supported by National Natural Science Foundation of China (81370707) to W.-H. Z, Science and technology support program of Hubei Province (2015BCA310) to W.-H.Z. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted

References

Agarwal, K., & Alfirevic, Z. (2012). Pregnancy loss after chorionic villus sampling and genetic amniocentesis in twin pregnancies: A systematic review. *Ultrasound in Obstetrics and Gynecology*, *40*, 128–134.

Alberry, M., Maddocks, D., Jones, M., Abdel Hadi, M., Abdel-Fattah, S., Avent, N., & Soothill, P. W. (2007). Free fetal DNA in maternal plasma in embryonic pregnancies: Confirmation that the origin is the trophoblast. *Prenatal Diagnosis*, *27*, 415–418.

Baffero, G. M., Somigliana, E., Crovetto, F., Paffoni, A., Persico, N., Guerneri, S., … Fedele, L. (2012). Confined placental mosaicism at chorionic villous sampling: Risk factors and pregnancy outcome., *32*, 1102–1108.

Bevilacqua, E., Gil, M. M., Nicolaides, K. H., Ordoñez, E., Cirigliano, V., Dierickx, H., … Jani, J. C. (2015). Performance of screening for aneuploidies by cell-free DNA analysis of maternal blood in twin pregnancies. *Ultrasound in Obstetrics and Gynecology, 45*, 61–66.

Bianchi, D. W. (2004). Circulating fetal DNA: Its origin and diagnostic potential: A review. *Placenta, 25*, S93–S101.

Canick, J. A., Kloza, E. M., Lambert-Messerlian, G. M., Haddow, J. E., Ehrich, M., van den Boom, D., … Palomaki, G. E. (2012). DNA sequencing of maternal plasma to identify Down syndrome and other trisomy in multiple gestations. *Prenatal Diagnosis*, *32*, 730–734.

Chan Wong, E., Hatakeyama, C., Minor, A., & Ma, S. (2012). Investigation of confined placental mosaicism by CGH in IVF and ICSI pregnancies. *Placenta, 33*, 202–206.

Chen, E. Z., Chiu, R. W., Sun, H., Akolekar, R., Chan, K. C., Leung, T. Y., … Lo, Y. M. (2011). Noninvasive prenatal diagnosis of fetal trisomy 18 and trisomy 13 by maternal plasma DNA sequencing. *PLoS One, 6*, e21791.

Chiu, R. W., Akolekar, R., Zheng, Y. W., Leung, T. Y., Sun, H., Chan, K. C., … Lo, Y. M. (2011). Noninvasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: Large scale validity study. *BMJ, 342*, c7401.

Choi, S. A., Ko, J. M., Shin, C. H., Yang, S. W., Choi, J. S., & Oh, S. K. (2013). Monozygotic twin discordant for Down syndrome: mos 47, XX, + 21/46, XX and 46, XX. *European Journal of Pediatrics, 172*, 1117–1120.

Cleary-Goldman, J., & Berkowitz, R. L. (2005). First trimester screening for Down syndrome in multiple pregnancy. *Seminars in Perinatology, 29*, 395–400.

Dan, S., Wang, W., Ren, J., Li, Y., Hu, H., Xu, Z., … Zhang, X. (2012). Clinical application of massively parallel sequencing-based prenatal noninvasive fetal trisomy test for trisomies 21 and 18 in 11,105 pregnancies with mixed risk factors. *Prenatal Diagnosis, 32*, 1225–1232.

del Mar Gil, M., Quezada, M. S., Bregant, B., Syngelaki, A., & Nicolaides, K. H. (2014). Cell-free DNA analysis for trisomy risk assessment in first-trimester twin pregnancies. *Fetal Diagnosis and Therapy, 35*, 204–211.

Flori, E., Doray, B., Gautier, E., Kohler, M., Ernault, P., Flori, J., & Costa, J. M. (2004). Circulating cell-free fetal DNA in maternal serum appears to originate from cyto- and syncytiotrophoblastic cells. Case report. *Human Reproduction, 19*, 723–724.

Gahan, P. B., & Swaminathan, R. (2008). Circulating nucleic acids in plasma and serum. Recent developments. *Annals of the New York Academy of Sciences, 1137*, 1–6.

Gil, M. M., Quezada, M. S., & Revello, R. (2015). Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: Updated meta-analysis. *Ultrasound in Obstetrics and Gynecology, 45*, 249–266.

Gregg, A. R., Gross, S. J., Best, R. G., Monaghan, K. G., Baja, j K., Skotko, B. G., … Watson, M. S. (2013). ACMG statement on noninvasive prenatal screening for fetal aneuploidy. *Genetics in Medicine, 15*, 395–398.

Gromminger, S., Yagmur, E., Erkan, S., Nagy, S., Schöck, U., Bonnet, J., … Stumm, M. (2014). Fetal aneuploidy detection by cell-free DNA sequencing for multiple pregnancies and quality issues with vanishing twins. *Journal of Clinical Medicine, 3*, 679–692.

Gupta, A., Vaid, A., & Arora, R. (2016). Diachorionic triamniotic triplets — Saline cardiac tamponade for fetal reduction: A novel approach. *Journal of Fetal Medicine, 3*, 167–170.

Hansen, M., Kurinczuk, J. J., Milne, E., de Klerk, N., & Bower, C. (2013). Assisted reproductive technology and birth defects: A systematic review and meta-analysis. *Human Reproduction Update, 19*, 330–353.

Huang, X., Zheng, J., Chen, M., Zhao, Y., Zhang, C., Liu, L., … Wang, W. (2014). Noninvasive prenatal testing of trisomies 21 and 18 by massively parallel sequencing of maternal plasma DNA in twin pregnancies. *Prenatal Diagnosis, 34*, 335–340.

Hultén, M. A., Jonasson, J., Iwarsson, E., Uppal, P., Vorsanova, S. G., Yurov, Y. B., & Iourov, I. Y. (2013). Trisomy 21 mosaicism: We may all have a touch of Down syndrome. *Cytogenetic and Genome Research, 139*, 189–192.

Kalousek, D. K. (2000). Pathogenesis of chromosomal mosaicism and its effect on early human development. *American Journal of Medical Genetics, 91*, 39–45.

https://doi.org/10.1017/thg.2017.23 Published online by Cambridge University Press

Kalousek, D. K., & Vekemans, M. (2000). Confined placental mosaicism and genomic imprinting. *Journal of Medical Genetics, 14*, 723–730.

Klinger, K., Landes, G., Shook, D., Harvey, R., Lopez, L., Locke, P., … Dackowski, W. (1992). Rapid detection of chromosome aneuploidies in uncultured amniocytes by using fluorescence in situ hybridization (FISH). *American Journal of Human Genetics, 51*, 55–65.

Lanza, F., Castoldi, G., Castagnari, B., Todd, R. F. 3rd., Moretti, S., Spisani, S., … Traniello, S. (1998). Expression and functional role of urokinase-type plasminogen activator receptor in normal and acute leukaemic cells. *British Journal of Haematology, 103*, 110–123.

Lau, T. K., Jiang, F., Chan, M. K., Zhang, H., Lo, P. S., & Wang, W. (2013). Non-invasive prenatal screening of fetal Down syndrome by maternal plasma DNA sequencing in twin pregnancies. *Journal of Maternal-Fetal & Neonatal Medicine, 26*, 434–437.

Leung, T. Y., Qu, J. Z., Liao, G. J., Jiang, P., Cheng, Y. K., Chan, K. C., … Lo, Y. M. (2013). Noninvasive twin zygosity assessment and aneuploidy detection by maternal plasma DNA sequencing. *Prenatal Diagnosis, 33*, 675–681.

Machin, G., (2009). Non-identical monozygotic twins, intermediate twin types, zygosity testing, and the non-random nature of monozygotic twinning: A review. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*, *151*, 110–127.

Mao, J., Wang, T., Wang, B. J., Liu, Y. H., Li, H, Zhang, J., … Chen, Y. (2014). Confined placental origin of the circulating cell free fetal DNA revealed by a discordant non-invasive prenatal test result in a trisomy 18 pregnancy. *Clinica Chimica Acta, 10*, 190–193.

Nobili, E., Paramasivam, G., & Kumar, S. (2013). Outcome following selective fetal reduction in monochorionic and dichorionic twin pregnancies discordant for structural chromosomaland genetic disorders. *Australian and New Zealand Journal of Obstetrics and Gynaecology, 53*, 114–118.

Odibo, A. O., Lawrence-Cleary, K., & Macones, G. A. (2003). Screening for aneuploidy in twins and higher-order multiples: Is first-trimester nuchal translucency the solution? *Obstetrical & Gynecological Survey, 58*, 609–614.

Papavassiliou, P., York, T. P., Gursoy, N., Hill, G., Nicely, L. V., Sundaram, U., & Jackson-Cook, C. (2009). The phenotype of persons having mosaicism for trisomy 21/Down syndrome reflects the percentage of trisomic cells present in different tissues. *American Journal of Medical Genetics Part A, 149*, 573–583.

Qu, J. Z., Leung, T. Y., Jiang, P., Liao, G. J., Cheng, Y. K., Sun, H., … Lo, Y. M. (2013). Noninvasive prenatal determination of twin zygosity by maternal plasma DNA analysis. *Clinical Chemistry, 59*, 427–435.

Ramsey, K. W., Slavin, T. P., Graham, G., Hirata, G. I., Balaraman, V., & Seaver, L. H. (2012). Monozygotic twins discordant for trisomy 13. *Journal of Perinatology, 32*, 306–308.

Reuss, A., Gerlach, H., Bedow, W., Landt, S., Kuhn, U., Stein, A., … Eiben, B. (2011). Monozygotic twins discordant for trisomy 18. *Ultrasound in Obstetrics & Gynecology, 38*, 727–728.

Rosner, M., & Hengstschläger, M. (2013). Amniotic fluid stem cells and fetal cell microchimerism. *Trends in Molecular Medicine, 19*, 271–272.

Sarno, L., Revello, R., Hanson, E., Akolekar, R., & Nicolaides, K. H. (2016). Prospective first- trimester screening for trisomies by cell-free DNA testing of maternal blood in twin pregnancies. *Ultrasound in Obstetrics & Gynecology*, *47*, 705–711.

Simonazzi, G., Curti, A., Farina, A., Pilu, G., Bovicelli, L., & Rizzo, N. (2010). Amniocentesis and chorionic villus sampling in twin gestations: Which is the best sampling technique? *American Journal of Obstetrics and Gynecology, 202*, 365.e1–5.

Spencer, K. (2000). Screening for trisomy 21 in twin pregnancies in the first trimester using free beta-hCG and PAPP-A, combined with fetal nuchal translucency thickness. *Prenatal Diagnosis, 20*, 91–95.

Vink, J., Wapner, R., & D'Alton, M. E. (2012). Prenatal diagnosis in twin gestations. *Seminars in Perinatology, 36*, 169–174.

Wright, D., Kagan, K. O., Molina, F. S., Gazzoni, A., & Nicolaides, K. H. (2008). A mixture model of nuchal translucency thickness in screening for chromosomal defects. *Ultrasound in Obstetrics & Gynecology*, *31*, 376–383.

https://doi.org/10.1017/thg.2017.23 Published online by Cambridge University Press