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Non-invasive prenatal testing for fetal chromosomal abnormalities by low-coverage whole-genome sequencing of maternal plasma DNA: review of 1982 consecutive cases in a single center

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KEYWORDS: aneuploidy; chromosomal deletions; chromosomal duplications; fetal DNA; next-generation sequencing; NIPT; sex chromosomal abnormality; whole genome

ABSTRACT

Objective *To review the performance of non-invasive prenatal testing (NIPT) by low-coverage whole-genome sequencing of maternal plasma DNA at a single center.*

Methods *The NIPT result and pregnancy outcome of 1982 consecutive cases were reviewed. NIPT was based on low coverage (0.1×) whole-genome sequencing of maternal plasma DNA. All subjects were contacted for pregnancy and fetal outcome.*

Results *Of the 1982 NIPT tests, a repeat blood sample was required in 23 (1.16%). In one case, a conclusive report could not be issued, probably because of an abnormal vanished twin fetus. NIPT was positive for common trisomies in 29 cases (23 were trisomy 21, four were trisomy 18 and two were trisomy 13); all were confirmed by prenatal karyotyping (specificity = 100%). In addition, 11 cases were positive for sex-chromosomal abnormalities (SCA), and nine cases were positive for other aneuploidies or deletion/duplication. Fourteen of these 20 subjects agreed to undergo further investigations, and the abnormality was found to be of fetal origin in seven, confined placental mosaicism (CPM) in four, of maternal origin in two and not confirmed in one. Overall, 85.7% of the NIPT-suspected SCA were of fetal origin, and 66.7% of the other abnormalities were caused by CPM. Two of the six cases suspected or confirmed to have CPM were complicated by early-onset growth restriction requiring delivery before 34 weeks. Fetal outcome of the NIPT-negative cases was ascertained in 1645 (85.15%). Three chromosomal abnormalities were not detected by NIPT, including one case each of a balanced translocation, unbalanced translocation and triploidy. There were no known false negatives involving the common trisomies (sensitivity = 100%).*

Conclusions *Low-coverage whole-genome sequencing of maternal plasma DNA was highly accurate in detecting common trisomies. It also enabled the detection of other aneuploidies and structural chromosomal abnormalities with high positive predictive value.* *Copyright © 2013 ISUOG. Published by John Wiley & Sons Ltd.*

INTRODUCTION

Traditional fetal aneuploidy screening tests, based on sonography and maternal biochemistry, have a detection rate of 50–95% at a 5% false-positive rate[1]. The discovery of cell-free fetal DNA in maternal plasma[2] and the invention of massively parallel sequencing (MPS)[3] have made non-invasive prenatal testing (NIPT) for fetal trisomy 21 a clinical reality[4,5]. Review of published data suggests that NIPT is highly accurate in detecting fetal trisomy 21, with sensitivity and specificity both > 99% and a non-reportable (failure) rate of 0–4.9%[6]. Although initially less successful, the detection rates for trisomy 18 and 13 were reported to be 100% and 91.7%, respectively, with modification of methodologies, particularly the correction for GC

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bias in DNA sequencing[7,8]. The detection rate for sex-chromosome abnormalities has been reported to be 96.2%, with a false-positive rate of 0.3%[9]. However, most of the published data were based on experimental data or data collected as a well-designed study. Reports on NIPT performance based on real clinical data are still limited.

In most of the published MPS-based studies, the algorithm for the detection of fetal aneuploidy relied on calculating the genomic fraction of the target chromosome (e.g. chromosome 21) by some form of normalization against chromosomes assumed to be disomic or cases known to be disomic, and the deviation of this genomic fraction of the chromosome of interest from the expected value was measured and usually expressed as a *Z*-score[4,5]. The majority of commercial providers present their results as positive or negative, based on the *Z*-score exceeding a predefined threshold, using a one-hypothesis testing approach. Further statistical improvements have enabled the identification of cases with mosaicism and expanded aneuploidy detection to all 23 pairs of chromosomes[10]. An algorithm known as Fetal Copy Number Analysis through Maternal Plasma Sequencing (FCAPS) was further developed, making it possible to detect close to 100% of deletions/duplications that are ≥ 10 Mb without the need to increase the sequencing depth[11].

The objective of this study was to evaluate and report the clinical performance of NIPT based on low-level whole-genome sequencing in 1982 consecutive cases from a single center.

METHODS

Patients and sample collection

This was a prospective clinical audit of 1982 consecutive NIPT cases performed in a single prenatal diagnosis center, Paramount Medical Centre in Hong Kong, since the start of the service in August 2011 to the end of February 2013, including the first 567 cases reported previously[12]. The center accepted referral of any pregnant woman for NIPT, regardless of whether they had undergone any previous Down syndrome screening tests. The test protocol required that the pregnancy had to be of 12 weeks' gestation or beyond. All patients underwent pretest counseling and an informed consent process, as previously described, including analysis of other chromosomes. All subjects had a pretest ultrasound scan to ascertain the number of fetuses and gestational age, and to exclude major structural abnormalities.

Five milliliters of maternal peripheral blood was collected into a blood tube containing EDTA. The blood sample was stored immediately at 4°C before further processing. Plasma was prepared within 4 h after collection (subsequently extended to 8 h) using a two-step centrifugation protocol. The whole-blood sample was first centrifuged at 1600 ***g*** for 10 min at 4°C. The supernatant was transferred to sterile 2.0-mL Eppendorf (EP) tubes placed on ice, which were centrifuged again at 16 000 ***g*** for 10 min at 4°C. The final supernatant was transferred to new EP tubes, which were temporarily stored or transported in dry ice or at −20°C if DNA extraction was not performed immediately. Each plasma sample was frozen and thawed only once.

This cohort of NIPT samples included 23 internal control cases, in which the patient requested an invasive procedure for chromosome analysis for various reasons. Verbal consent was obtained from these patients to donate a 5-mL blood sample for NIPT before the invasive procedure to serve as internal control samples. These samples were sent to the NIPT laboratory and were processed as ordinary clinical samples. The NIPT laboratory staff were blinded to the clinical indications leading to invasive testing. The NIPT and chromosomal analysis from either chorionic villus sampling (CVS) or amniocentesis culture were performed independently by two different laboratories, each of which was blinded to the results of the other laboratory.

DNA sequencing and quality control

All subsequent molecular tests, including cell-free DNA isolation, library construction and sequencing, were performed in the ISO/IEC 17025-certified clinical laboratory of BGI-Shenzhen, as previously reported[10]. On average, the depth of sequencing of this NIPT method for each sample was about 0.1×.

A quality-control criteria system for each step from sampling to reporting was in place. A barcode tracking system was employed during the whole process. Blood samples with evidence of hemolysis, or those that were processed beyond 8 h after sample collection, were excluded. The quality parameters of acceptable samples were as follows: the peak size of a qualified DNA library was between 290 and 303 bp and the yield was more than 30 nM. The sequencing quality value (Q20) was over 90% for each base, and the GC content was around $40 \pm 1.5\%$. The minimal amount of unique sequencing reads was no less than 3.5 million after alignment. Only qualified sequencing data were used for subsequent analyses.

Computationally, the human reference genome (hg 18, NCBI build 36) was divided into 50-bp fragments and subsequently replaced with k-mers (k refers to the length of the sequencing reads) and then the k-mers were aligned back to the reference genome. All k-mers that could be uniquely mapped to a single position on the reference genome (i.e. the unique mapping reads) were named as the universal unique reads set. We selected the sequencing reads that could be mapped with 0-mismatch to the universal unique reads set (i.e. the tag) for the analysis. This was followed by computing the k-mer coverage with the corrected GC content for each chromosome[10].

Bioinformatics analysis

For autosomal aneuploidy detection, a binary (two) hypothesis *t*-test and logarithmic likelihood ratio (L-score) between the two *t*-tests were used to classify whether the

fetus had aneuploidy. The first (null) hypothesis was that the fetus was euploid, whereas the second (alternative) hypothesis was that the fetus was trisomic. The L-score measures whether the number of reads is closer to the distribution of reads of normal pregnancies or that of affected pregnancies. A case was classified as either high risk or low risk only when the results of both hypothesis testing agreed with each other. Cases with mosaicism or affected cases with a low fetal fraction usually would result in a situation in which the results of the two hypotheses testing were not conclusive. For example, in an affected pregnancy with a low fetal fraction, the *t*-scores for both hypotheses would be within the conventional cut-off in both tests and therefore both hypotheses could not be rejected. In such situations, a repeat blood sample would be requested if time allowed; otherwise, the result would be interpreted as high risk to minimize the possibility of a false-negative result[13].

Fetal gender classification and sex chromosomal aneuploidy detection were computed from the *t*-score for both X and Y chromosomes independently for interpretation, as previously reported[10].

Detection of chromosomal deletions and duplications using a specially designed FCAPS pipeline has been performed routinely in every sample since July 2012[11]: 759 cases in total. Before that, FCAPS was only performed for cases with a *t*-score of > 2.0, aiming to detect partial trisomy. All cases with significant findings on FCAPS identified during the entire study period were included in this report. With FCAPS, the human genome is divided into a total of 308 789 sliding, 99%-overlapping basic observation units, each with 84 000 expected unique reads. Potential breakpoints of deletions/duplications are localized by comparing the difference of the read numbers after GC correction. The detection power of FCAPS increases with increasing cell-free fetal DNA concentration and more sequencing reads. Under conditions of a concentration of 10% cell-free fetal DNA and a sequencing read number of 7M, the detection rate for deletions/duplications of > 10 Mb was close to 100%[11].

Results reporting

The median reporting time was 8 calendar days. The test report initially included a risk assessment for trisomy 21 and trisomy 18 only, but was extended to include trisomy 13 and Turner syndrome in early 2012. However, bioinformatics analysis actually included aneuploidy detection for all 23 pairs of chromosomes and analysis for deletions/duplications. In the event that there was suspicion of a chromosomal abnormality other than those included in the report, the clinician (T.K.L.) would be informed. All patients involved were informed of such an additional finding and were given appropriate counseling. Fetal sex was not reported, even on request, unless sex-chromosomal abnormalities were suspected.

Follow-up with confirmatory invasive testing was recommended for all positive results for trisomy 21, 18 and 13. For other suspected abnormalities, subsequent management depended on the result of individualized counseling. Whenever possible, additional tests were performed to exclude placental or maternal mosaicism if the invasive test was not performed or if the invasive test result was normal. Patients were contacted regularly for fetal outcome until either pregnancy termination or delivery.

Confirmatory invasive testing

Conventional G-banded cytogenetic studies, quantitative fluorescent polymerase chain reaction (QF-PCR) or array-CGH (aCGH) studies were performed at the laboratory of the Department of Obstetrics and Gynaecology, the Chinese University of Hong Kong, or the Department of Obstetrics and Gynaecology, Tsan Yuk Hospital, University of Hong Kong. The aCGH test offered by the Chinese University of Hong Kong is a custom-made panel for prenatal diagnosis targeting 100 of the common microdeletion and microduplication syndromes of relevance in prenatal diagnosis at high resolution, plus whole-genome coverage with a backbone resolution down to 100 kb[14]. The aCGH test offered by the University of Hong Kong was a NimbleGen CGX-135 K whole-genome oligonucleotide microarray with a resolution of 140 kb across the genome and ≤ 40 kb in regions of clinical relevance (> 245 genetic syndromes and > 980 gene regions of functional significance in human development).

Pregnancy outcome follow-up of NIPT-negative cases

The clinical details of all subjects were reviewed and summarized. Those with a negative NIPT result were contacted at least 3 months after the expected date of delivery (EDD) to reduce the chance of missing a case of trisomy 21, 18 and 13. The exception to this rule was for those who had NIPT in early 2013, and they were contacted in late October 2013. The latest EDD for this whole cohort was 9 September 2013, at least 1 month before being contacted for follow-up data. On the other hand, it is not possible to exclude other chromosomal abnormalities, in particular sex chromosomal abnormalities, by clinical examination at birth as they may be phenotypically normal at that age. Contact was made by telephone, or by e-mail if not contactable by telephone. A failure of contact was declared after three or more attempts to contact. When contacted, the following information was requested:

1. Whether fetal karyotyping was performed after NIPT, prenatal or postnatally. If yes, what was the indication and result of the confirmatory testing?
2. The status of the pregnancy outcome: live birth, spontaneous miscarriage, pregnancy termination, stillbirth or neonatal death
3. Date of delivery
4. Gestation at delivery
5. Fetal abnormalities detected before and/or at birth
6. Birth weight
7. Fetal sex

A negative NIPT was considered to be confirmed if (i) prenatal or postnatal karyotyping was normal or (ii) the neonate was phenotypically normal after birth.

RESULTS

NIPT was performed as a clinical service on a total of 1959 patients, including 1929 singleton and 30 twin pregnancies, and as internal control samples in 23. As the demographics were similar for both singleton and twin pregnancies, they were combined and are presented in Tables 1 and 2. Over 90% of the patients were ethnic Chinese. The maternal age was significantly higher than that for the normal obstetric population in Hong Kong, with a mean of 36 (SD, 4.35; range, 20–46) years, and 63.85% of the patients were 35 years of age or older. The median gestational age at the time of NIPT was 14.5 weeks, and 56% of the NIPT studies were performed at 12 and 13 weeks of gestation. Sixteen patients had a previous pregnancy affected by trisomy 21, and 30 had a positive family history for Down syndrome. Approximately two thirds ($n = 1290$, 65.8%) of patients had a prior screening test before NIPT. The results of prior screens were available for 961 cases at the time of NIPT, 61.7% of which had a high-risk result.

Figure 1 shows the overall results and outcomes of the NIPT cases.

A repeat blood sample was required in 23 (1.16%) cases. A final test report was available in all cases except one. In this single case, the patient requested NIPT at 12 + 3 weeks of gestation. A vanished twin with a clearly visible fetal pole was detected. After counseling, the patient asked to continue with the test, understanding that there could be a higher chance of a 'false positive' if the demised fetus was abnormal. Repeat tests at 12 + 3, 13 + 2 and 15 + 4 weeks consistently showed a low level of Y-chromosome signals with a fractional concentration of 2.2–3.7% and a borderline *t*-score of 1.4–2.7 for chromosome 18, which did not satisfy the binary hypotheses requirement for classification as either positive or negative. As the presence of a vanished twin was known, a conclusive report was not issued, although the low-level abnormality was interpreted as probably a result of interference from the demised fetus. The patient ultimately had an amniocentesis which confirmed that the viable fetus had a normal karyotype (46,XX), consistent with the normal-appearing female fetus on ultrasound examination. Detailed examination of the placenta after delivery at term failed to identify any evidence of remnants of the vanished twin and therefore confirmation of the karyotype of the vanished twin was not possible.

Table 1 Basic patient characteristics of 1959 cases undergoing non-invasive prenatal testing (NIPT) as a clinical service

Characteristic	n *(%)*
Ethnicity	
Chinese	1781 (90.91)
Caucasian	102 (5.21)
Other	76 (3.88)
Maternal age	
20–24 years	23 (1.17)
25–29 years	140 (7.15)
30–34 years	545 (27.82)
35–39 years	873 (44.56)
40–44 years	355 (18.12)
≥ 45 years	23 (1.17)
Gestation at NIPT	
12 weeks to 13 + 6 weeks	1102 (56.25)
14 weeks to 15 + 6 weeks	449 (22.92)
16 weeks to 20 + 6 weeks	352 (17.97)
21 weeks and above	56 (2.86)
Previous trisomy 21 pregnancy	16 (0.82)
Previous trisomy 18 pregnancy	5 (0.26)
Previous trisomy 13 pregnancy	2 (0.10)
Family history of trisomy 21	30 (1.53)
Prior Down syndrome screening test	
None	669 (34.15)
Combined first-trimester NT + biochemistry	1018 (51.97)
First-trimester NT (± other ultrasound markers) only	86 (4.39)
First-trimester biochemistry only	16 (0.82)
Second-trimester biochemistry only	99 (5.05)
Other tests, or more than one test	71 (3.62)
Result of prior screening tests ($n = 1290$)	
High risk	593 (45.97)
Low risk	368 (28.53)
Result not available at time of NIPT	329 (25.5)

Table 2 Indication for invasive testing among the 23 internal control samples

	n *(%)*
Reason for referral	
NIPT	21 (91.3)
Second opinion for thickened NT	2 (8.7)
Prior Down screening	
No	2 (8.7)
Low risk	3 (13.0)
Increased NT	5 (21.7)
High risk	13 (56.5)
Reason for having invasive test instead of NIPT	
Congenital heart defects	6 (26.1)
Other structural anomalies	3 (13.0)
Multiple markers of chromosomal anomalies	7 (30.4)
Increased NT only	3 (13.0)
Maternal preference	3 (13.0)
Suspected triploidy	1 (4.4)

NIPT, non-invasive prenatal testing; NT, nuchal translucency.

Common autosomal aneuploidies

NIPT was high risk for trisomy 21 in 23 cases, for trisomy 18 in four cases and for trisomy 13 in two cases. All cases were confirmed by prenatal karyotyping. Detailed information for these cases is summarized in Table S1. Figure 2a shows the NIPT computed values (*t*-scores) according to MPS.

One case with trisomy 13 (Figure 2a, circled triangle) had a *t*-score below the standard cut-off of 3.0 because of a low fetal DNA concentration (estimated to be < 3.5%). The findings did not fulfill the binary hypotheses for a

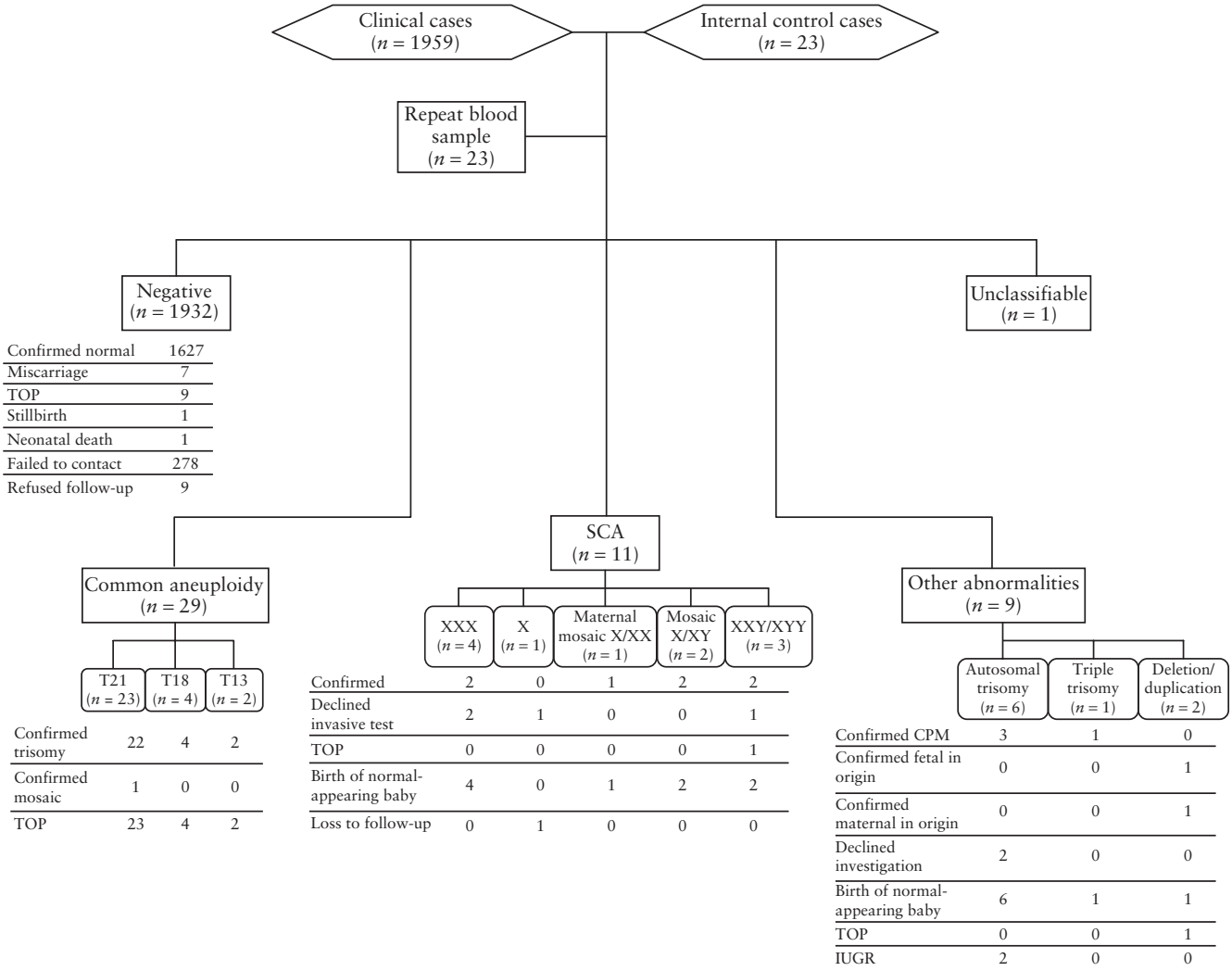

Figure 1 Diagram summarizing study cohort and test results. All values are *n*. CPM, confined placental mosaicism; IUGR, intrauterine growth restriction; SCA, sex-chromosomal abnormalities; T13, trisomy 13; T18, trisomy 18; T21, trisomy 21; TOP, termination of pregnancy.

low-risk result. The standard procedure of requesting a repeat blood sample was not possible because this was an internal control sample. Therefore, according to the standard procedure, the case was classified as high-risk.

All but eight of the NIPT-positive cases had other screening tests performed before NIPT, indicating a high risk, with the exception of Case 27, which was considered to be low risk based on first-trimester combined screening. However, in this case, ultrasound evaluation performed at the time of NIPT revealed an absent nasal bone and left superior vena cava, consistent with the diagnosis of trisomy 21.

Although all patients were fully aware that NIPT has a false-positive rate of < 1%, most challenged the need for karyotyping when they were informed of the positive result. After careful counseling, all patients agreed to an invasive test. In all cases the chromosomal abnormality was confirmed. Of note, mosaicism was detected in Case 16, with trisomy 21 observed in 42% of the cultured amniocytes.

Sex chromosomal abnormalities

Eleven cases were positive for sex chromosomal abnormalities (SCA) according to NIPT. Details of these cases are summarized in Table S2. Of these 11 cases, six were high risk on prior screening or had sonographic markers, three had not had any previous screening tests and two were low risk on prior screening. The NIPT computed *t*-scores for sex chromosomes, according to MPS, are plotted in Figures 2b and c.

There were four cases with a computed *t*-score above (triple X) 3.0 in a 'female' fetus, as predicted by MPS (Figure 2b). In all four cases of suspected triple X, all agreed to continue with the pregnancy. Two declined any further investigations, even after birth, but the babies were reported to be normal at the time of the follow-up survey. One case was confirmed to be 47,XXX on placental biopsy at birth, and the fourth was confirmed by cord blood sample to be 47,XXX/45,X mosaic at birth.

There were four cases with a computed *t*-score below (monosomy X) −3.0 in a 'female' fetus, as predicted by

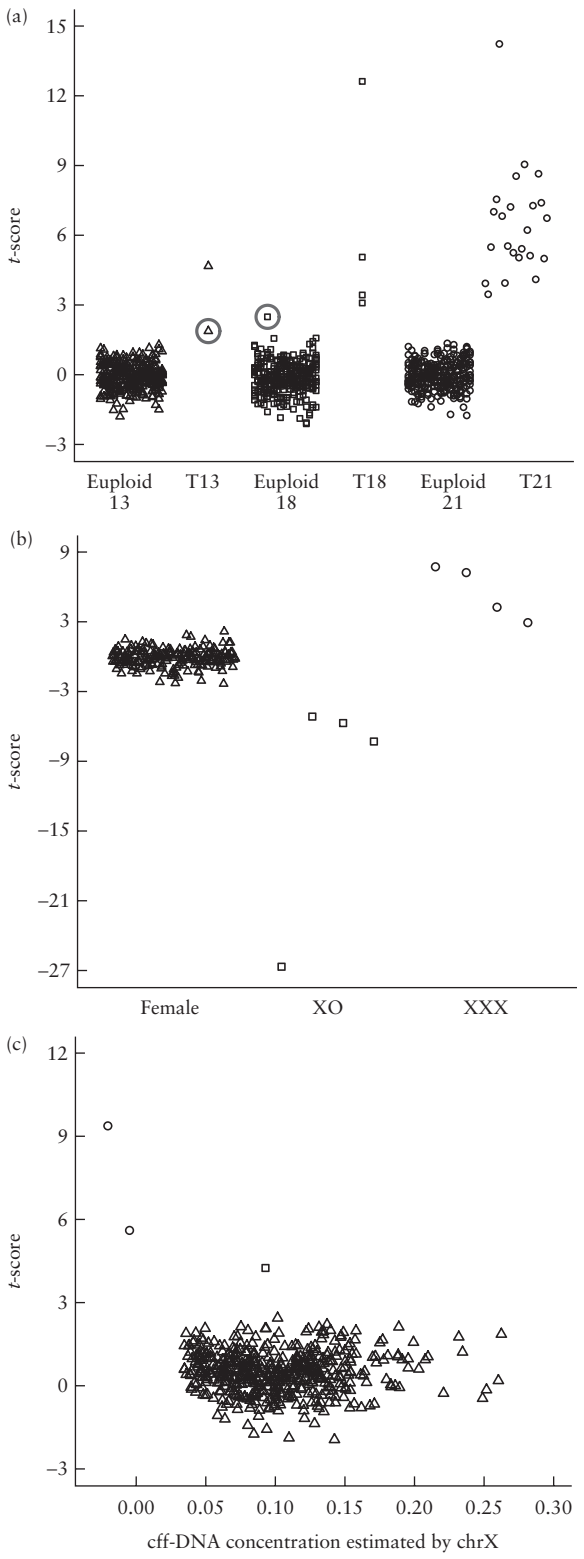

Figure 2 Summary of results of bioinformatic analysis of study cases. (a) Standard deviation (*t*-score) of each sample for the corresponding chromosome according to their karyotyping status for the common trisomies. The circled trisomy (T) 18 case had an 'unclassifiable result'. The circled T13 case was classified by the binary hypotheses as probably trisomy with a low fetal fraction. (b) *t*-score for X-chromosome, according to karyotyping result. (c) *t*-score for Y-chromosome plotted against cell-free fetal DNA (cff-DNA) concentration, estimated by chromosome (chr) X for normal males (△) and for sex chromosome aneuploidies XXY (○) and XYY (□).

MPS (Figure 2b). In one case, the cause was suspected to be maternal mosaicism, and was confirmed[15]. In the other case, the NIPT result was suggestive of fetal Turner syndrome. The couple, after counseling, agreed to continue with the pregnancy. However, this case was lost to follow-up and no further investigation could be performed. In the remaining two cases, low-level Y-chromosome signals were present in the MPS data, below the cut-off for classification as a male fetus. However, ultrasound examination in both cases confirmed male gender. Therefore, XO/XY mosaicism was suspected in both cases, and both were confirmed by amniocentesis. In one case, there was low-level (6%) mosaicism of monosomy X cells in a male fetus. Data collected from fluorescence *in-situ* hybridization (FISH) analysis and PCR studies are depicted in Figure 3. In the other case, about 50% of the cells were monosomy X, whilst the remaining cells had an isodicentric chromosome of the short arm of Y up to q11.2. Detailed FISH analysis for this case is shown in Figure 4. This special chromosomal structural rearrangement explained (i) why the overall Y-chromosome dosage was low because of the loss of most of the long arm and (ii) the overall normal dosage for the short arm of the Y chromosome, resulting in normal fetal sexual development. After counseling by the clinician and a geneticist, both couples opted for continuation of pregnancy, and at birth both babies were clinically normal.

There were two cases suspected to be XXY (Figure 2c), confirmed by either cord blood at delivery or amniocentesis. In the last case of suspected XYY, the couple continued with the pregnancy but declined any further investigation.

Overall, of the 11 cases of suspected SCA, four declined any further investigations, and a biological cause could be confirmed in all seven cases in whom investigations were performed (fetal in origin in six, and maternal in origin in one).

Other positive findings

Nine cases screened by NIPT were positive for other chromosome abnormalities, as detailed in Table S3. A biological cause was confirmed in six cases. Two patients declined follow-up studies and in one patient a cause could not be identified.

Six of the nine cases were positive for whole-chromosome aneuploidy involving one autosome. One case had amniocentesis for fetal short limbs, showing a normal fetal karyotype and normal genetic studies for dwarfism. Postnatal placental karyotyping confirmed confined placental mosaicism (CPM). CPM was suspected in the remaining five cases because no structural abnormalities could be identified by prenatal ultrasound examination. One patient requested prenatal karyotyping, which was normal. All six pregnancies resulted in live births, although two had early-onset growth restriction requiring preterm delivery. Postdelivery placental karyotyping was declined by two patients, was confirmed CPM in another two and was negative in one.

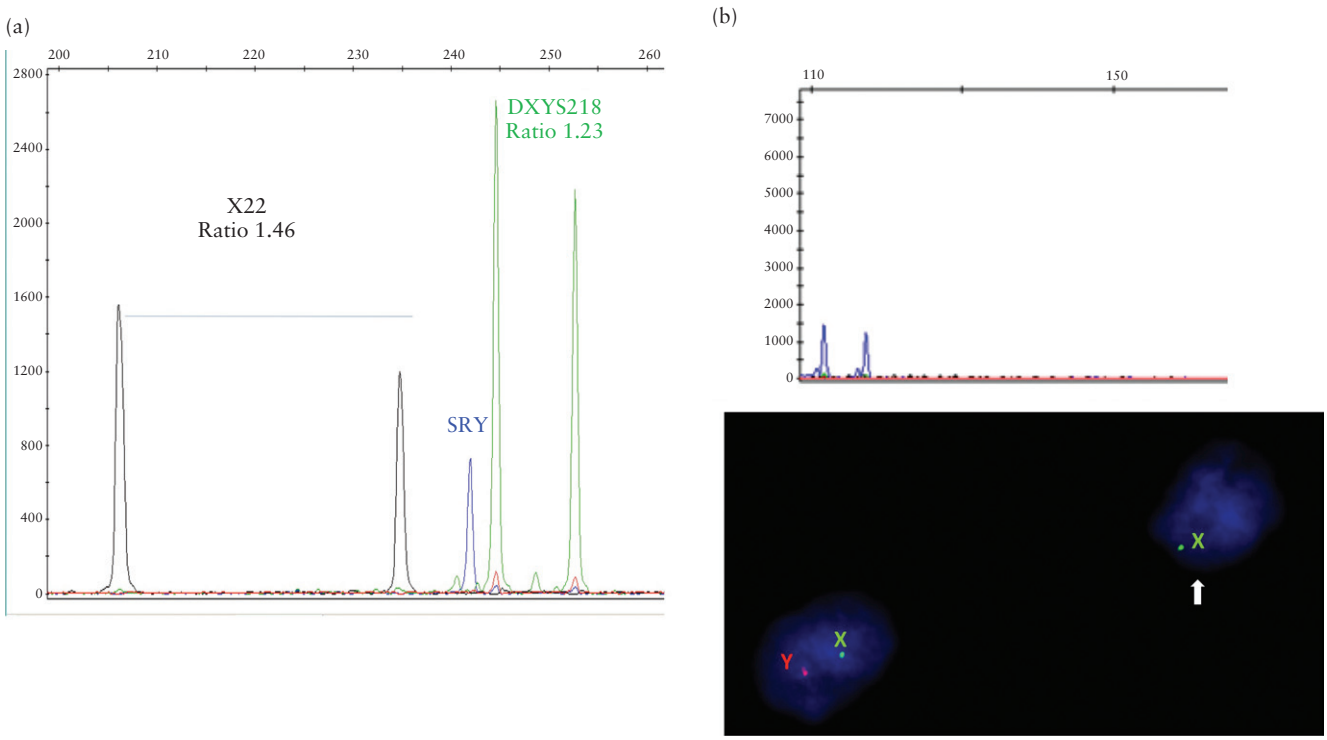

Figure 3 (a) Quantitative fluorescent polymerase chain reaction analysis showing an abnormally high ratio of STR markers on chromosomes X and Y, with an AMEL ratio of 1.23, a DXYS218 ratio of 1.23 and an X22 ratio of 1.46, indicating presence of one SRY allele. (b) Fluorescence *in-situ* hybridization (FISH) analysis of chromosome X (CEP X (green)) and chromosome Y (CEP Y (red)) on amniocytes isolated from amniotic fluid (1-day-old culture). FISH analysis demonstrates one signal for the X chromosome (arrow) in 6.0% of cells, and one signal each for both X and Y chromosomes in 94.03% of cells from a total of 486 cells counted.

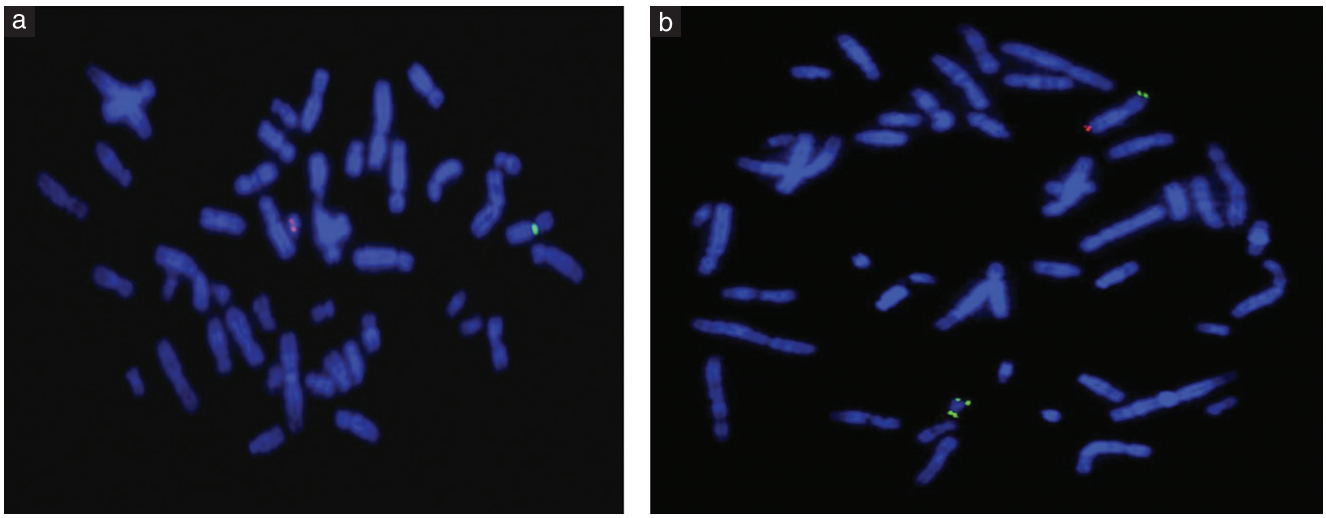

Figure 4 (a) Fluorescence *in-situ* hybridization (FISH) with chromosome X (CEP X (green)) and chromosome Y (CEP Y (red)) probes showing that the marker chromosome has dicentric Y signals. (b) FISH with subtelomeric XpYp (green) and XqYq (red) probes showing Yp signals at termini of marker chromosome.

The three cases with fetal triple trisomy, fetal partial trisomy 18p and partial monosomy 18q of maternal origin, have been reported previously[15].

Internal control samples

Twenty-three cases were included for internal control purposes in which the NIPT was performed immediately before invasive testing. Twenty-one were initially referred for NIPT, but these patients decided to have an invasive procedure for various reasons, mostly because of abnormalities detected by ultrasound evaluation before NIPT or maternal preference for a diagnostic test (Table 2). The remaining two were initially referred for a second opinion because of thickened nuchal translucency. Eleven of the internal control cases were

Table 3 Summary of pregnancy outcome of non-invasive prenatal testing (NIPT)-negative cases ($n = 1932$)

Outcome variable	n *or* n *(%)*
Failed to contact	278/1932 (14.39)
Declined to provide information	9/1932 (0.47)
Fetal outcome available	1645/1932 (85.15)
Confirmed normal (at birth or by prenatal karyotyping)	1627/1645 (98.91)
Spontaneous miscarriage (none had karyotyping of abortus)	7/1645 (0.43)
Termination of pregnancy	9/1645 (0.55)
Major fetal anomalies, normal karyotype	5
Major fetal anomalies, unbalanced translocation	1
Triploidy	1
Confirmed single-gene disorder	1
Personal reason	1
Stillbirth	1/1645 (0.06)
Neonatal death	1/1645 (0.06)
Fetal karyotyping performed	26/1645 (1.58)
Internal control cases	12
Unresolved anxiety despite negative NIPT	10
Absence of nasal bone at 20-week scan	1
Suspected fetal anomalies	3

NIPT positive and concordant with karyotyping including seven cases of trisomy 21, two cases of trisomy 18, one case of trisomy 13 and one case of triple X (all detailed in Tables S1 and S3). The remaining 12 cases were NIPT negative and all but two had a normal karyotype. There was one case of a *de-novo* balanced translocation 46,XX,t(3;20),(p25;q11.2), confirmed by a normal aCGH study. The couple decided to continue with the pregnancy. As this is a balanced translocation, it will not be counted as false negative in this series. In the last case, the patient was originally referred for NIPT, but ultrasound examination before the test detected early intrauterine growth restriction (IUGR), small placenta, cardiac defect and abnormal hands, which were consistent with triploidy. Owing to methodological limitations, triploidy cannot be detected by the current method of whole-genome shotgun sequencing and therefore CVS was suggested. Karyotype confirmed triploidy.

Pregnancy outcome

NIPT was negative in 1932 cases, including 12 internal control cases. Follow-up data were successfully obtained in 1645 (85.15%) subjects (Table 3).

Prenatal karyotyping was performed in 26 cases, including 12 in the internal control group. The reasons for karyotyping in the remaining 14, after a negative NIPT, were: absence of nasal bone at the 20-week scan; suspected fetal anomalies at the 20-week scan; and unresolved anxiety either in the patient (eight cases) or in their attending obstetrician (two cases). All cases in the last group occurred in patients who had the NIPT before June 2012, within the first year of the implementation of NIPT. It might reflect the initial uncertainty of patients/obstetrician about a new technology.

Table 4 Screening performance for common aneuploidies in the current cohort

Chr	*Sensitivity*	*Specificity*
21	23/23 (100 (85.7–100))	1959/1959 (100 (99.8–100))
18	4/4 (100 (51.0–100))	1978/1978 (100 (99.8–100))
13	2/2 (100 (34.2–100))	1980/1980 (100 (99.8–100))

Values are given as no. positive/total no. (% (95% CI)).
Chr, chromosome.

Only one chromosomal abnormality (unbalanced translocation) was detected in the 14 additional karyotypes. Routine FCAPS analysis had not started when that case had NIPT. FCAPS was requested and performed at the time of amniocentesis and correctly identified the 16-Mb duplication[15] before karyotyping or aCGH results were available.

Normality of the fetus was confirmed in 1627 (98.91%) cases. In a total of seven cases there was spontaneous miscarriage without karyotypic confirmation. Nine cases had termination of pregnancy: five for major fetal anomalies with normal karyotype; one for major fetal abnormalities with an unbalanced translocation (see above); one for triploidy (see above); one for confirmed single-gene disorder; and one for personal reasons.

There was one case of stillbirth and one case of neonatal death because of prematurity (born at 24 weeks). Karyotyping was not performed in either case.

DISCUSSION

The current study provides a true reflection of what NIPT could achieve in a real clinical setting. In this study, a low-level whole-genome sequencing approach was adopted. Overall, the screen-positive rate, excluding the internal control cases, was 38/1959 (1.94%, one in 52); or 19/1959 (0.97%, one in 103) for the three common trisomies. The need for a repeat blood sample, or the so-called unclassifiable[16] or failure[6] rate, was only 1.16%, one of the lowest compared with previous studies[6]. There was only one 'false-positive' case, of trisomy 9, for which no biological reason could be identified.

In this cohort, sensitivity and specificity for the three common trisomies (trisomy13, trisomy 18 and trisomy 21) were all 100% (Table 4), comparable with other published data[7,16–19]. The wide confidence intervals were caused by the small sample size. The concordance rate of NIPT with fetal karyotype was 100% and there were no false positives for the common aneuploidies, indicating that NIPT is highly reliable, even in real clinical practice, under a strict protocol and quality control.

Among the 29 cases with common trisomies, all, except three, had been classified as high risk by a previous screening test or by the presence of abnormal markers on the pre-NIPT ultrasound scan. If NIPT had not been available, these three cases would have been missed. On the other hand, NIPT substantially reduced the number of invasive tests because most of the 593 women who had

high-risk screen results according to conventional testing before NIPT would probably have had an invasive test if NIPT had not been available.

We believe that the excellent results achieved in this cohort were due to two main factors. First, we insisted that the original protocol used in previous experimental studies should be followed strictly, starting from the pre-analytical stage, including case selection, sample handling, immediate refrigeration, gentle handing during transportation and early separation of plasma. We decided to accept samples from 12 weeks' gestation and onwards, only because in published papers at that time, and even now, data on cases before 12 weeks of gestation were very limited[6]. Second, the use of binary hypotheses in bioinformatics analysis enabled a much more robust estimation of the fetal status. Of course, bioinformatics alone can help to minimize, but cannot totally prevent, problems associated with low fetal DNA fraction.

We believe that NIPT should not be a standalone blood test but part of a full risk assessment. Performing the test at 12 weeks or beyond provides an excellent opportunity for a detailed sonographic examination of the fetus, enabling the early detection of both structural and chromosomal abnormalities for more appropriate investigation other than NIPT (as reflected by data from the internal control group), and screening for other obstetric complications. A similar viewpoint was recently expressed in an Opinion article by Yagel[20].

The whole-genome approach enabled the detection of other chromosomal abnormalities. The additional analysis for other chromosomal abnormalities increased the screen-positive rate by about 1%. About half of these additional abnormalities were SCA. Unlike previous studies[21], the algorithm presented here for detection of SCA appears to be highly sensitive and specific. A biological reason was identified in all NIPT-positive SCA cases, with a high positive predictive value. Although a true estimation of the detection rate was not possible because routine karyotyping was not performed in NIPT-negative cases, the incidence of SCA in this cohort, even if counting only the seven confirmed fetal cases, was six (0.3%) of 1983, which agrees very well with previous reports that SCA occur in approximately 0.3% of all live births[22]. One of the challenges in the detection and interpretation of SCA is mosaicism, which has been reported in NIPT[18,21], and is as high as 60% in postnatal series[23]. In this cohort, fetal mosaicism was detected in three (42.9%) of the seven cases by confirmation studies, although all were originally thought to be simple monosomy or trisomy. The suspicion of X/XY mosaicism (Case 36 and Case 37) would have been missed if the fetuses were phenotypically female. Therefore, caution should be taken when counseling women with SCA-positive NIPT results, which should include the possibility of mosaicism, and confirmatory tests such as CVS and amniocentesis should be discussed.

In this cohort, a very low incidence of prenatal karyotyping for SCA-positive NIPT results was performed. This agreed very well with a previous survey which showed that 98.5% of pregnant women wanted to be informed if NIPT suspected a SCA, although only one-third would consider amniocentesis[24]. Reasons stated for wanting to be informed was so that they could make informed choices and for preparation. The low incidence of prenatal karyotyping in this cohort was mostly because of the very high proportion of couples who were willing to continue the pregnancy regardless of the fetal chromosomal status after counseling, which has been found to be significantly affected by the counseling process and the genetic experience of the healthcare provider[25].

CPM is another formidable challenge for interpretation of NIPT results. About half of the additional chromosomal abnormalities in this cohort were caused by uncommon fetal autosomal abnormalities, including seven cases of trisomies and two cases of deletions and duplications. When uncommon autosomal trisomies were suspected by NIPT, CPM was confirmed or suspected in the vast majority (six out of seven). This was not surprising because most fetuses with uncommon trisomies do not survive beyond the first trimester, and cell-free fetal DNA in maternal plasma is of placental, rather than fetal, origin[26]. Therefore, amniocentesis is indicated in some cases to confirm CPM and to rule out fetal mosaicism. IUGR and uniparental disomy (UPD) resulting from trisomic rescue are well known potential complications of CPM[27]. Indeed, early-onset IUGR requiring preterm delivery occurred in two of the seven suspected cases of CPM in this cohort. Therefore, the likelihood of fetal mosaicism, CPM and UPD should be discussed when counseling patients with positive NIPT results for uncommon aneuploidies. Confirmatory testing using CVS or amniocentesis, and serial ultrasound examinations to monitor fetal growth for IUGR, are warranted in such cases.

An unexpected diagnostic dilemma encountered was the discovery of maternal chromosomal abnormalities. A 'false-positive' NIPT result can occur in patients with copy number imbalances and mosaicism, especially for conditions associated with normal or mild phenotypes, such as SCA[28]. Therefore, interpretation of NIPT results should take this possibility into consideration. In this cohort, two maternal abnormalities were detected, including one case of maternal 45,X/46,XX mosaicism and a case of an 18q microdeletion. In both cases, NIPT predicted correctly the maternal origin of the abnormalities, based either on a very high *t*-score, which is uncommon if the abnormality is of fetal origin (mosaic case), or if the copy number ratio of the affected segment was about 0.5 (microdeletion case), equivalent to the loss of one haploid dosage, which is not possible to be of fetal origin unless the fetal fraction was 100%. The ability to predict the maternal origin of the suspected abnormality by NIPT significantly facilitated the counseling process.

Our cohort suggests that detection of chromosomal deletions and duplications are feasible in routine NIPT, even with low-level coverage sequencing. At the current sequencing depth, it is likely that structural chromosomal abnormalities of 15–20 Mb or more would be detectable[11]. Although the resolution is not yet as

good as routine karyotyping, such findings come without additional cost or risk of invasive tests. It is possible to detect smaller deletions by increasing the sequencing depth[29], but adoption of deep sequencing as a routine clinical practice is unlikely at present because of the additional cost.

There was only one case in which a report could not be issued after repeat blood sampling. The most likely cause in that case was due to a vanishing twin. The inconsistencies between the fetal ultrasound findings and NIPT results, in addition to the low fetal DNA concentration, led to cautious result reporting in this case. At present, it is still uncertain for how long after fetal death the DNA of a demised fetus will still be present at a significant level in the maternal plasma. Our service protocol suggests at least a 6-week window after the demise of a vanished twin before the NIPT, unless patients are willing to accept a potentially higher chance of a false-positive result as a result of a demised abnormal fetus. If NIPT is going to be performed in earlier pregnancy, such as at 9 or 10 weeks, the chance of encountering a vanished twin will be higher and potentially will cause more unclassifiable or false-positive results.

There were only two identified cases of 'false negatives', neither of which involved the common trisomies. The case of triploidy was expected not to be identified by the MPS method, but could be detected by ultrasound scan. In the other case with an unbalanced translocation, the abnormality was missed only because the FCAPS analysis was not routinely performed at the time when the case was evaluated.

As NIPT is a new technology, careful monitoring of its performance in a real clinical setting is essential[30]. Although all steps were carefully monitored, we decided to implement the internal control system using clinical samples. Patients undergoing an invasive test were invited to donate a blood sample, which was processed as a real clinical sample. The NIPT laboratory was blinded to the karyotype result. Such information provided continuous assessment and reassurance that the NIPT was reliable. This is similar to interlaboratory quality assurance programs in place for different laboratory tests, such as the United Kingdom National External Quality Assessment Service (UK-NEQAS) for the Down Screening in the First Trimester.

In conclusion, NIPT utilizing a low coverage ($0.1\times$) whole-genome sequencing approach provides a unique possibility to screen for a wider spectrum of fetal chromosomal abnormalities beyond common aneuploidies at an affordable cost. Challenges encountered in this study, such as a vanishing twin, fetal and placental mosaicism and maternal abnormalities, are not unique to NIPT and have been, to a certain extent, complications for traditional cytogenetic techniques for decades. We suggest that all NIPT screen-positive subjects should be carefully counseled for the possibility of contribution from the fetal, placental or maternal compartment. Additionally, all screen-positive cases must be confirmed by fetal karyotyping before pregnancy termination is performed.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Table S1 Details of 29 cases screened by NIPT to be positive for common autosomal aneuploidies

Table S2 Details of 11 cases screened by NIPT to be positive for sex chromosomal abnormalities

Table S3 Details of nine cases screened by NIPT to be positive for other chromosomal abnormalities