ORIGINAL ARTICLE

Validation of targeted sequencing of single-nucleotide polymorphisms for non-invasive prenatal detection of aneuploidy of chromosomes 13, 18, 21, X, and Y

K. H. Nicolaides[1,2]*, A. Syngelaki[1], M. Gil[1], V. Atanasova[1] and D. Markova[1]

[1]Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, London, UK
[2]Department of Fetal Medicine, University College London Hospital, London, UK
*Correspondence to: Kypros H. Nicolaides. E-mail: kypros@fetalmedicine.com

ABSTRACT

Objective To assess the performance of cell-free DNA (cfDNA) testing in maternal blood for detection of fetal aneuploidy of chromosomes 13, 18, 21, X, and Y using targeted sequencing of single-nucleotide polymorphisms.

Methods Prospective study in 242 singleton pregnancies undergoing chorionic villus sampling at 11 to 13 weeks. Maternal blood was collected before chorionic villus sampling and sent to Natera (San Carlos, CA, USA). cfDNA was isolated from maternal plasma, and targeted multiplex PCR amplification followed by sequencing of 19 488 polymorphic loci covering chromosomes 13, 18, 21, X, and Y was performed. Sequencing data were analyzed using the NATUS algorithm that determines the copy number and calculates a sample-specific accuracy for each of the five chromosomes tested. Laboratory personnel were blinded to fetal karyotype.

Results Results were provided for 229 (94.6%) of the 242 cases. Thirty-two cases were correctly identified as aneuploid, including trisomy 21 [$n = 25$; sensitivity = 100% (CI: 86.3–100%), specificity = 100% (CI: 98.2–100%)], trisomy 18 ($n = 3$), trisomy 13 ($n = 1$), Turner syndrome ($n = 2$), and triploidy ($n = 1$), with no false positive or false negative results. Median accuracy was 99.9% (range: 96.0–100%).

Conclusions cfDNA testing in maternal blood using targeted sequencing of polymorphic loci at chromosomes 13, 18, 21, X, and Y holds promise for accurate detection of fetal autosomal trisomies, sex chromosome aneuploidies, and triploidy. © 2013 John Wiley & Sons, Ltd.

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INTRODUCTION

Prenatal diagnosis of aneuploidies necessitates invasive testing by chorionic villus sampling or amniocentesis in women identified by non-invasive screening to be at increased risk for such aneuploidies. However, invasive testing carries a 1% risk of causing miscarriage. In the last 40 years, prenatal screening for aneuploidies has focused on trisomy 21, but a beneficial consequence of such screening has been the detection of many additional clinically significant aneuploidies in the screen positive group undergoing invasive testing and full karyotyping. The most effective method of screening for trisomy 21 is by a combination of maternal age, the sonographic measurement of fetal nuchal translucency thickness, and biochemical testing of maternal blood for free β-human chorionic gonadotropin (hCG) and PAPP-A at 11 to 13 weeks' gestation with a detection rate (DR) of about 90% and false positive rate (FPR) of 5%.[1] Use of additional biochemical and sonographic markers, including serum placenta growth factor and assessment of the nasal bone and blood flow across the tricuspid valve and ductus venosus, can increase the DR to more than 95% and decrease the FPR to less than 3%.[1]

Several recent studies have demonstrated that the most effective method of screening for trisomy 21, with DR of more than 99% and FPR of about 0.1%, is derived from examination of cell-free DNA (cfDNA) in maternal plasma.[2–11] There are two different approaches in analyzing the cfDNA: quantitative and single-nucleotide polymorphism (SNP)-based methods. In the first approach, maternal plasma cfDNA molecules are sequenced, and the chromosomal origin of each molecule is identified by comparing with the human genome. In trisomic pregnancies, the quantity of molecules derived from the trisomic chromosome, as compared with an assumed disomic reference chromosome, is higher than in euploid pregnancies.

In contrast, SNP-based methods determine chromosomal copy number by looking for specific patterns in allelic measurements. The first reports of successful detection of trisomic pregnancies utilized massively parallel shotgun sequencing (MPSS), where several millions of DNA fragments from all chromosomes are identified and quantified.[12,13] Subsequently, digital analysis of selected regions (DANSR) was introduced, where selective sequencing of loci from chromosomes under investigation is undertaken, thereby increasing throughput and reducing cost.[14] Clinical studies demonstrated that both MPSS and DANSR can be successfully applied in highly effective screening for trisomies 21 and 18 and less so for trisomy 13, in high-risk women.[4–11,15–17] There is also one report of sex chromosome aneuploidy detection by MPSS.[10] An alternative to MPSS and DANSR is an SNP-based method involving targeted amplification and analysis of 19 488 SNPs on chromosomes 21, 18, 13, X, and Y in a single reaction.[18] A Bayesian-based maximum likelihood statistical method is then applied to determine the chromosomal count of the five chromosomes interrogated in each sample. Because this method analyzes allele distributions and does not require the use of a disomic reference chromosome, it is uniquely expected to be capable of detecting triploidy.

The aim of this study was to assess the ability of the SNP-based approach, coupled with an algorithm called NATUS (Next-generation Aneuploidy Test Using SNPs), to detect trisomies 21, 18, and 13, sex chromosome aneuploidies, and triploidy from maternal blood samples.

PATIENTS AND METHODS

Study population

The data for this study were derived from 242 singleton pregnancies undergoing fetal karyotyping by chorionic villus sampling at 11 to 13 weeks' gestation in our fetal medicine center because first trimester combined screening had demonstrated that the risk for trisomy 21, 18, or 13 was more than 1 in 300 ($n = 227$), or there was a previous aneuploid pregnancy ($n = 6$) and advanced maternal age ($n = 5$), or the women were undergoing invasive testing for sickle cell disease and wanted fetal karyotyping as well ($n = 4$). On the day of chorionic villous sampling, gestational age was determined from the measurement of the fetal crown-rump length,[19] and the risk for trisomies 21, 18 and 13 was derived from the measurements of fetal translucency thickness and serum free β-hCG and PAPP-A.[1,20,21]

Maternal venous blood samples (20 mL in Streck cell-free DNA BCT™ tubes) were obtained before chorionic villus sampling from the 242 singleton pregnancies. The patients gave written informed consent to provide samples for research into early prediction of pregnancy complications, which was approved by the NHS Research Ethics Committee (REC reference number 03-04-070).

Laboratory analysis

Maternal blood samples were sent overnight from London to the laboratory of Natera Inc. in San Carlos, CA. The information provided to the laboratory for each case was the following: patient unique identifier, maternal age, gestational age, racial origin, and date of blood collection, but not fetal karyotype. Natera Inc. confirmed sufficient volume and adequate labeling, and no samples were excluded from analysis.

Sample preparation (including cfDNA and maternal genomic DNA isolation from the buffy coat) and measurement (including PCR amplification, sequencing, and informatics analysis of the sequencing results) were performed as previously described,[18] with two modifications as follows: firstly, the individual PCR assays included in a single reaction increased from roughly 11 000 to 19 488, and secondly, sequencing results were analyzed using an advanced version of the algorithm with the expanded capability to analyze data generated from 19 488 assays (NATUS). NATUS analyzes a number of quality control metrics to identify lab or sequencing failure, estimate the amount of total starting DNA, determine the fetal fraction, and calculate the extent to which the measured cfDNA data fit expected case-specific distributions. In this study, a determination of the ploidy state of the fetus was not made if the sample had less than 3.5% fetal fraction, if the amount of input DNA was below 1500 genome equivalents, or if contamination was greater than 0.2%. Maternal genotypic information was incorporated into the analysis as previously described.[18]

Results were provided in the form of a copy number of the five chromosomes interrogated in each sample, along with a sample-specific calculated accuracy for each chromosome, to one of the authors A. S. who then determined the correlation between the assay results with the fetal karyotype.

RESULTS

The median maternal age of the study population was 35.7 (range: 18.5–46.5) years, and the median gestational age at sampling was 13.1 (range: 11.3–13.9) weeks. The median estimated risk for trisomy 21, 18, or 13 by the combined test was 1:75 (range 1:2–1:12 433).

Results from analysis of cfDNA in maternal blood were provided for 229 (94.6%) of the 242 cases. In 13 cases (5.4%), including two cases of trisomy 21 and 11 of euploid fetuses, no result was given because samples failed internal quality controls for one or more of the following reasons: insufficient total DNA after sample preparation (<1500 input genomic DNA units), insufficient fetal cfDNA fraction (<3.5%), or high noise levels, which may include high contamination levels or low model fit parameter due to, for example, bottlenecking (where bottlenecking refers to the oversampling of too few original DNA fragments). The lowest fetal fraction on a case that returned a result was 3.95%. In the group with no result, compared with those with result, the mean serum PAPP-A was significantly lower (0.520 vs 0.892 MoM, Mann–Whitney *U*-test $p = 0.016$), but mean maternal weight was not significantly different (77.2 vs 68.5 kg, $p = 0.141$).

The fetal karyotype obtained from chorionic villus sampling in the 229 cases with results and the predicted copy number for chromosomes X, Y, 21, 18, and 13 are summarized in Table 1. Cell-free DNA testing using SNPs correctly identified all cases of autosomal trisomy and Turner syndrome with no false positives, and correctly determined fetal sex in all cases. For the one triploidy case, cfDNA testing identified multiple maternal haplotypes, indicating either twins or triploidy; as ultrasound confirmed a single gestation, the sample was

Table 1 Fetal karyotype obtained from chorionic villus sampling in the 229 cases with results from cell-free DNA testing and the predicted copy number for chromosomes X, Y, 21, 18, and 13

Fetal karyotype	Predicted copy number for chromosomes:				
	X	Y	21	18	13
47,XY,+21 (n = 10)	1	1	3	2	2
47,XX,+21 (n = 15)	2	0	3	2	2
47,XX,+18 (n = 3)	2	0	2	3	2
47,XY,+13 (n = 1)	1	1	2	2	3
45,X (n = 2)	1	0	2	2	2
69,XXX (n = 1)	3	0	3	3	3
47,XY,+22 (n = 1)	1	1	2	2	2
46,XY,del(2)(q37.1) (n = 1)	1	1	2	2	2
46,XX,+der(9)t(9;15)(q33.2;q11.2)pat, −15 (n = 1)	2	0	2	2	2
46,XY,t(8;10)(p23.1;q24.1)pat (n = 1)	1	1	2	2	2
45,XX,der(14;21)(q10;q10)mat (n = 1)	2	0	2	2	2
46,XY (n = 103).	1	1	2	2	2
46,XX (n = 89)	2	0	2	2	2

identified as triploid (69,XXX). The estimated risk for aneuploidies by the combined test is compared with the result of cfDNA testing in Figure 1. In all cases of trisomy 21, the copy number of chromosome 21 was three with a sample-specific accuracy of 99.9% (sensitivity: 100%; CI: 86.3–100%; specificity: 100%; CI: 98.2–100%). In all cases of trisomy 18, the copy number of chromosome 18 was three with accuracy of 99.9%, in the case of trisomy 13, the copy number of chromosome 13 was three with accuracy of 99.9%; in the cases of Turner syndrome, the copy number of chromosome X was one, and the copy number

of chromosome Y was zero, both with sample-specific accuracy of 99.9%. For the triploidy case, the copy number of chromosomes 13, 18, 21, and X was three, and the copy number of chromosome Y was zero. Because of the small number of trisomy 13, trisomy 18, monosomy X, and triploidy samples, sensitivities and specificities are not reported. In the 116 male fetuses, there was one copy number for chromosome X with accuracy of 99.9% in all cases, and one for chromosome Y with a median accuracy of 99.9% (range: 96.0–99.9%), and in the 109 female fetuses, excluding the two Turner and one triploidy cases, the copy number of chromosome X was two, and chromosome Y was zero with a median sample-specific accuracy of 99.9% (range: 96.0–100%).

DISCUSSION

Principal findings of this study

This externally blinded validation study has demonstrated that SNP-based analysis of cfDNA in maternal blood obtained at 11 to 13 weeks' gestation from high-risk singleton pregnancies correctly identified all cases of trisomies 21, 18, and 13, Turner syndrome, and triploidy with no false positives and correctly determined the fetal sex in all cases. The test did not provide results in 5.4% of cases, for which a redraw and reanalysis would be recommended.

The pregnancies examined had undergone screening for trisomies 21, 18, and 13 by a combination of maternal age, ultrasound examination, and maternal serum biochemical testing, most were identified as being at high risk for these aneuploidies and the parents chose to have chorionic villus sampling for fetal karyotyping. In this respect, the study population reflects current clinical practice in the UK and many other countries where screening and diagnosis of aneuploidies is based on the first trimester combined test and chorionic villus sampling, respectively.
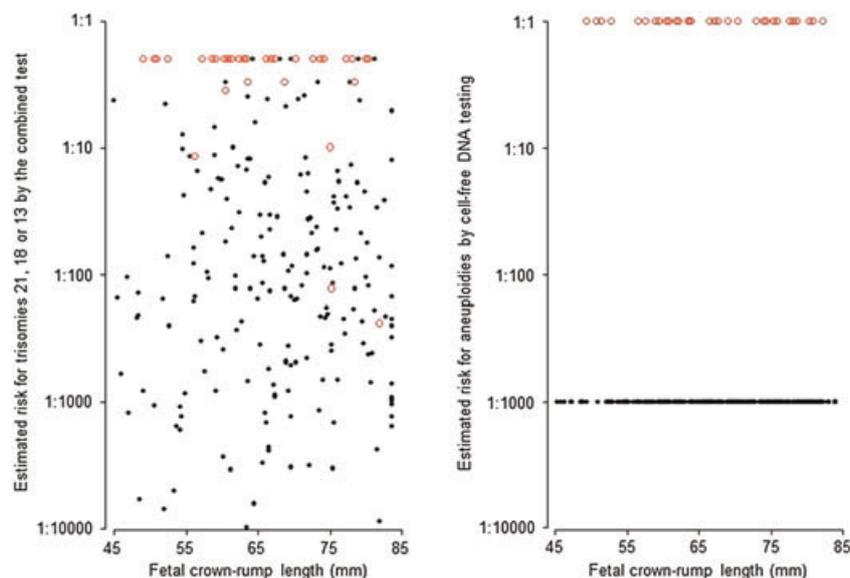

Figure 1 Distribution of risk for aneuploidies by the combined test (left) and cfDNA testing (right) in euploid (black) and aneuploid (red) pregnancies plotted on the fetal crown-rump length

Comparison of results and method of analysis of cfDNA to that of prevous studies

There is extensive evidence from the results of previous externally blinded validation studies with a combined total of 594 cases of trisomy 21, 193 of trisomy 18, and 38 of trisomy 13, that testing of cfDNA in maternal blood can identify more than 99%, 97%, and 79% of cases, respectively, with respective FPR of 0.1%, 0.1%, and 0.4% for each aneuploidy.[4–11,13,14,16,17] In our study, we correctly identified all three trisomies with no false positives but acknowledge that the number of cases of trisomies 18 and 13 was too small for reliable estimation of test performance.

The previous studies used MPSS or DANSR to analyze the cfDNA in maternal blood. Both methods determine fetal ploidy state by measuring the ratio of the number of reads mapping to the chromosome of interest to those mapping to diploid reference chromosomes. Because MPSS is not selective in the chromosomal origin of the sequenced DNA fragments and chromosome 21 represents only about 1.5% of the human genome, it is necessary to sequence many millions of fragments to ensure sufficient chromosome 21 counts for high-confidence results. Although this method has been shown to detect structural rearrangements, to accomplish this, it is necessary to obtain a very high number of reads.[22] In the DANSR approach, there is selective sequencing of loci from only chromosomes 21, 18, and 13 with consequent tenfold decrease in the required DNA sequencing compared with MPSS approaches, leading to an improvement in efficiency.

The approach in this study selectively amplifies and sequences polymorphic loci, targeting 19 488 SNPs covering chromosomes 21, 18, 13, X, and Y, and copy number is identified by NATUS analysis of the sequencing results. This method differs from previous ones in that it uses maternal genotypic information and recombination frequencies to construct, *in silico*, billions of possible fetal genotypes. It then compares each hypothesis with the actual plasma measurements and calculates a relative likelihood for each hypothesis. The method only considers possible fetal genotypes and does not require the use of a disomic reference chromosome with two potentially beneficial consequences. Firstly, it is the only currently available method that could detect triploidy. Secondly, it avoids the lower detection rates for chromosomes that are prone to high levels of amplification variation, such as chromosomes 13 and X. As demonstrated in this study, it was possible to correctly identify the case of triploidy, but it would be necessary to validate this finding by examining a considerably higher number of affected pregnancies. The test correctly determined the fetal sex in all cases and detected the two cases of Turner syndrome in the study population. The exact performance of the test in detecting trisomy 13, trisomy 18, and sex chromosome aneuploidies requires further investigation.

Limitations of the test

The test did not provide results in about 5% of cases, and one of the reasons for this was low fetal fraction. In the group with no result, compared with those with results, the median serum PAPP-A was significantly lower, and maternal weight was higher, but the difference was not significant. This is compatible with our previous report that the fetal fraction increases with serum PAPP-A, which provides an indirect measure of the mass of the placenta and source of fetal cfDNA in maternal plasma, and decreases with maternal weight, which could be attributed to a dilutional effect.[23]

Although in the UK and most other countries screening for aneuploidies essentially focuses on screening for trisomy 21, invasive testing in the screen positive group leads to the detection of many additional clinically significant aneuploidies. As shown in our study population, there was one case each of trisomy 22, deletion in chromosome 2 and deletion in chromosome 15. Consequently, a potential criticism of replacing the traditional methods of screening for trisomy 21 by maternal cfDNA testing is that many clinically significant cytogenetic abnormalities that are currently detectable would be missed. However, the biomarker profile for many of the rare aneuploidies is not clearly defined, and it is uncertain whether their incidence in the screen positive group for trisomy 21 is higher than in the screen negative group. Nevertheless, SNP-based methods are expected to detect such clinically significant aneuploidies, including microdeletions and microduplications, but this would require specifically selecting anomalies to target.

Implications for practice

The data of this study provide further evidence that the performance of non-invasive prenatal aneuploidy tests based on analysis of cfDNA in detecting trisomies 21, 18, and 13 is far superior to all currently available methods that rely on maternal age, ultrasound examination of the fetus, and maternal serum biochemistry. This is well illustrated in Figure 1, which compares the estimated risk for aneuploidies by the combined test with that from cfDNA testing. The data also demonstrate the potential of expanding the scope of screening to include sex chromosome aneuploidies and triploidy.

This study, as most previous ones, has investigated high-risk pregnancies. However, the ability to detect aneuploidy with cfDNA is dependent upon assay precision and fetal DNA percentage in the sample rather than the prevalence of the disease in the study population, and we have previously shown that screening by cfDNA testing in a routine population is as effective as in high-risk groups.[11] Consequently, the extent to which cfDNA testing could be applied as a universal screening tool for aneuploidies in all pregnant women would depend on whether access to this technique becomes comparable with that of current methods of sonographic and biochemical testing. In the meantime, cfDNA testing would be useful as a secondary test contingent on the results of a more universally applicable primary method of screening.

As shown in our study, the majority of pregnancies identified by the combined test as being at high-risk for trisomy 21, 18, or 13 are euploid, and in such cases, the use of cfDNA testing would considerably reduce the number of unnecessary invasive tests and eliminate their associated risk of causing miscarriage. Another population that may benefit from screening by maternal plasma cfDNA is the one identified by the combined test as being at intermediate risk for autosomal trisomies, because in these cases, their risk will be revised to

either very high or very low, thereby making their decision in favor or against invasive testing easier.[24,25]

CONCLUSION

This blinded validation study has demonstrated that cfDNA testing in maternal blood using targeted sequencing of SNPs at chromosomes 13, 18, 21, X, and Y and use of the NATUS algorithm holds promise as an accurate method for detecting fetal autosomal aneuploidies, sex chromosome aneuploidies, and triploidy in the first trimester of pregnancy.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- A novel, effective method of screening for trisomies 21, 18, and 13 is derived from examination of cell-free DNA in maternal plasma.

WHAT DOES THIS STUDY ADD?

- Targeted sequencing of single-nucleotide polymorphisms at chromosomes 13, 18, 21, X, and Y holds promise for accurate detection of fetal autosomal aneuploidies, sex chromosome aneuploidies, and triploidy.

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