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Prenatal Detection of Fetal Triploidy from Cell-Free DNA Testing in Maternal Blood

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Non-invasive diagnosis · Prenatal diagnosis · Triploidy · Cell-free DNA

Abstract
Objective: To investigate potential performance of cell-free DNA (cfDNA) testing in maternal blood in detecting fetal triploidy. ***Methods:*** Plasma and buffy coat samples obtained at 11–13 weeks' gestation from singleton pregnancies with diandric triploidy (n = 4), digynic triploidy (n = 4), euploid fetuses (n = 48) were sent to Natera, Inc. (San Carlos, Calif., USA) for cfDNA testing. Multiplex polymerase chain reaction amplification of cfDNA followed by sequencing of single nucleotide polymorphic loci covering chromosomes 13, 18, 21, X, and Y was performed. Sequencing data were analyzed using the NATUS algorithm which identifies copy number for each of the five chromosomes. ***Results:*** cfDNA testing provided a result in 44 (91.7%) of the 48 euploid cases and correctly predicted the fetal sex and the presence of two copies each of chromosome 21, 18 and 13. In diandric triploidy, cfDNA testing identified multiple paternal haplotypes (indicating fetal trisomy 21, trisomy 18 and trisomy 13) suggesting the presence of either triploidy or dizygotic twins. In digynic triploidy the fetal fraction corrected for maternal weight and gestational age was below the 0.5th percentile. ***Conclusions:*** cfDNA testing by targeted sequencing and allelic ratio analysis of single nucleotide polymorphisms covering chromosomes 21, 18, 13, X, and Y can detect diandric triploidy and raise the suspicion of digynic triploidy.

Introduction

Triploidy, in which the fetus has three copies of all chromosomes, affects about 1% of recognized conceptions, but it is highly lethal and it is rarely observed in live births. The prevalence at 12 weeks' gestation is about 1 in 2,000 and this falls to 1 in 250,000 by 20 weeks [1, 2]. There are two phenotypes of triploidy, depending on whether the origin of the extra haploid set is paternal (diandric) or maternal (digynic) [3–6]. The digynic type is characterized by a small normal looking placenta, severely growth-restricted fetus with pronounced wasting of the body and sparing of the head, normal fetal nuchal translucency (NT) thickness and very low serum-free β-hCG and PAPP-A. In the diandric type, the placenta is enlarged and partially molar, the fetus is only mildly growth-restricted, the fetal NT tends to be high and maternal se-

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rum-free β-hCG is about 10 times higher than normal. Diandric triploidy can cause severe maternal complications, including severe early-onset preeclampsia and choriocarcinoma [7, 8].

First-trimester combined screening for trisomies 21, 18 and 13 by fetal NT and serum-free β-hCG and PAPP-A has the beneficial side effect of detecting about 85% of fetuses with triploidy [6, 9]. However, with the combined test there is a high false positive rate of about 5% and consequent need for unnecessary invasive diagnostic testing and related miscarriage. Recent evidence suggests that the most effective method of screening for fetal trisomies, with detection rates of more than 99% and false positive rate of less than 0.5%, is examination of cell-free DNA (cfDNA) in maternal blood [10–17]. The majority of clinically available methods for cfDNA testing employ a quantitative counting approach that relies on detecting higher relative amounts of fetal cfDNA from a trisomic chromosome when compared to disomic reference chromosomes [10–14]. The requirement for a reference chromosome with this method precludes triploidy detection. A second method of analyzing cfDNA, employed in this study, involves targeted amplification and analysis of single nucleotide polymorphisms (SNPs) on chromosomes 21, 18, 13, X, and Y in a single reaction and determination of chromosomal copy number by specific patterns in allelic measurements [15–17]. A bayesian-based statistical method (NATUS: Next-generation Aneuploidy Test Using SNPs) is then applied to determine the chromosomal count of these five chromosomes interrogated in each sample. Since 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.

A previous clinical validation study for detection of fetal aneuploidy from maternal plasma cfDNA by a SNP-based method and NATUS analysis reported the correct detection of a case of fetal triploidy [16]. The objective of this case-control study is to investigate the potential performance of the method in the detection of diandric and digynic triploidy.

Methods

Maternal venous blood samples were obtained at 11–13 weeks' gestation from singleton pregnancies immediately before chorionic villus sampling for fetal karyotyping. The women had undergone prior screening for trisomies 21, 18 and 13 by the combined test and found to be at increased risk for these aneuploidies [18]. All patients gave written informed consent to participate in the study which was approved by the UK NHS Research Ethics Committee.

Blood samples were collected in ethylenediaminetetraacetic acid BD Vacutainer™ tubes (Becton Dickinson UK Ltd, Oxford, UK) and within 15 min of collection they were centrifuged at 2,000 *g* for 10 min to separate plasma from packed cells and buffy coat and subsequently at 16,000 *g* for 10 min to further separate cell debris. Plasma samples were divided into 0.5-ml aliquots in separate Eppendorf tubes which were labeled with a unique patient identifier and stored at –80°C until subsequent analysis.

For the present study we searched our database of stored samples and identified 8 cases of triploidy and 48 of euploid fetuses (24 male and 24 female). In 4 of the triploid cases there was increased maternal serum-free β-hCG ranging from 5.7 to 13.4 multiples of the normal median (MoM) and these cases were considered to be diandric [6, 18]. In the other 4 cases, serum-free β-hCG ranged from 0.02 to 0.26 MoM and these cases were classified as digynic.

Plasma samples (4 ml) and buffy coat samples (1 ml) were shipped overnight, frozen on dry ice, from London to the USA (Natera, Inc. Laboratory, San Carlos, Calif., USA) for cfDNA testing. The laboratory was informed that the study included triploid and euploid fetuses but not the karyotype of individual samples. The information given for each case was: patient unique identifier, gestational age, maternal age, maternal weight and date of blood collection.

Natera, Inc. confirmed sufficient volume and adequate labeling. Cell-free and genomic DNA were isolated from the maternal plasma and buffy coat samples, respectively, subjected to massively multiplexed polymerase chain reaction amplification, underwent sequencing and the results were analyzed using the NATUS algorithm as previously described [15–17]. Briefly, the NATUS algorithm considers parental genotypes, crossover frequency data, linkage disequilibrium, and possible fetal chromosome copy number to generate different probability distributions for each of the two possible alleles at 19,488 loci. It then compares the predicted allele distributions to the actual allelic distributions as measured in the cfDNA sample, calculates the likelihoods of each ploidy state hypothesis (monosomy, disomy or trisomy) based on the sequencing data, and calls the hypothesis with the maximum likelihood as the ploidy state. To identify triploidy the algorithm looks for high-confidence trisomy copy number calls at chromosomes 21, 18 and 13. Determination of the ploidy state of the fetus was not made directly if the fetal fraction in the sample was less than 4%. However, samples with a fetal fraction that fell below the 0.5th percentile of the value after adjustment for gestational age and maternal weight (derived from the study of more than 10,000 commercial samples) were considered as abnormally low, a characteristic raising the possibility of digynic triploidy.

Results

Maternal and pregnancy characteristics of the study population are summarized in table 1 and the results from cfDNA testing are given in table 2. In the euploid group, cfDNA testing provided a result in 44 (91.7%) of the 48

Table 1. Maternal and pregnancy characteristics of the study population

Characteristics	Euploid (n = 48)	Diandric triploid (n = 4)	Digynic triploid (n = 4)
Maternal age, years	34.7 (22.7–46.0)	29.3 (25.3–35.2)	33.4 (29.7–36.2)
Maternal weight, g	65.9 (52.3–118.0)	57.2 (55.9–68.0)	64.0 (60.0–69.6)
Racial origin			
Caucasian	38 (79.2)	4 (100)	4 (100)
Afro-Caribbean	6 (12.5)		
Asian	4 (8.3)		
Spontaneous conception	44 (91.7)	4 (100)	4 (100)
Gestational age, weeks	12.9 (11.5–13.9)	12.3 (11.5–13.8)	11.7 (11.3–12.2)
Crown-rump length, mm	65.7 (48.3–83.9)	58.4 (47.6–79.7)	50.5 (45.3–57.0)
Nuchal translucency, mm	2.6 (1.2–7.3)	2.7 (1.1–3.5)	1.6 (1.5–2.6)
Serum-free β-hCG in MoM	1.582 (0.270–6.380)	9.279 (5.655–13.365)	0.075 (0.019–0.262)
Serum PAPP-A in MoM	0.619 (0.097–2.655)	0.802 (0.068–1.998)	0.034 (0.022–0.043)
Fetal fraction	10.1 (3.5–18.1)	23.4 (14.3–40.8)	2.8 (1.4–3.5)

Median (range) or n (%) values are shown.

Table 2. Fetal karyotype obtained from chorionic villus sampling and results from cell-free DNA testing with predicted copy number for chromosomes X, Y, 21, 18 and 13

Fetal karyotype	No result		Result	Predicted copy number for chromosomes				
	assay failure	fetal fraction <4%		X	Y	21	18	13
Diandric triploidy								
69,XXY (n = 1)			1	2	1	3	3	3
69,XXX (n = 3)			3	3	0	3	3	3
Digynic triploidy								
69,XXY (n = 1)		1						
69,XXX (n = 3)		3						
Euploid								
46,XY (n = 24)	1		23	1	1	2	2	2
46,XX (n = 24)	1	2	21	2	0	2	2	2

cases and correctly predicted the fetal sex (XX or XY) and that there were two copies each of chromosomes 21, 18 and 13 and two total copies of the sex chromosomes. In 4 cases no result was provided; 2 because of low fetal fraction and 2 due to assay failure.

In the 4 cases of diandric triploidy, cfDNA testing identified high fetal fraction and multiple paternal haplotypes (indicating fetal trisomy 21, trisomy 18 and trisomy 13) suggesting the presence of either triploidy or dizygotic twins.

In the 4 cases of digynic triploidy, cfDNA testing demonstrated very low fetal fraction. Indeed, in 3 of the cases the laboratory reported that the fetal fraction was abnormally low after correction for maternal weight and gestational age (below the 0.5th percentile) and were therefore suspected digynic triploidy cases. The fourth case was above the 0.5th percentile, but below the threshold required for making a call.

Figure 1 illustrates the relationship between maternal weight and unadjusted fetal fraction in pregnancies with euploid and triploid fetuses. In the euploid group the distribution of fetal fraction was gaussian (Kolmogorov-Smirnov test, p = 0.20), the median, 99.5th and 0.5th percentiles were 10.1, 18.24 and 3.51%, respectively. There was no significant association between fetal fraction and maternal weight (r = 0.120, p = 0.415), serum-

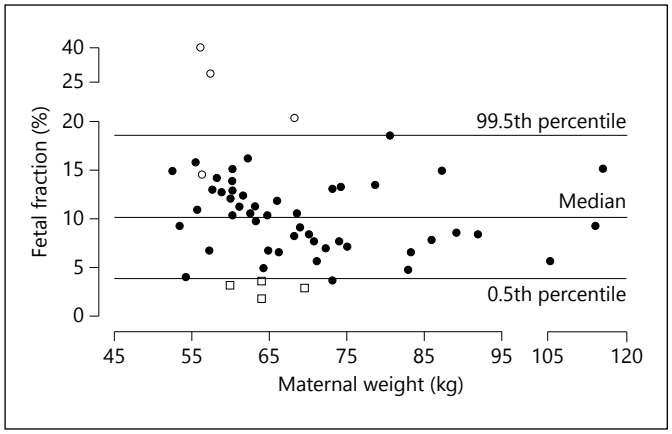

Fig. 1. Fetal fraction in pregnancies with euploid (closed circles), diandric triploidy (open circles) and digynic triploidy (open squares) in relation to maternal weight.

free β-hCG MoM (r = 0.156, p = 0.290) or PAPP-A MoM (r = 0.098, p = 0.508). In 3 of the 4 cases of diandric triploidy the unadjusted fetal fraction was above the 99.5th percentile of the euploid group and in all 4 digynic triploidies the unadjusted fetal fraction was below the 0.5th percentile.

Discussion

This study demonstrates the feasibility of prenatal diagnosis of fetal triploidy from cfDNA testing in maternal blood. All 4 cases of diandric triploidy were correctly detected and in cases of digynic triploidy the diagnosis was suspected from the inappropriately low fetal fraction after correction for maternal weight and gestational age.

Diagnosis of triploidy was made possible because the SNP-based approach used for the study examines the relative distributions of alleles at polymorphic loci and does not require a reference chromosome for comparison. Other clinically available methods for cfDNA testing detect aneuploidy by comparing the amount of DNA from the chromosome-of-interest to one or more reference chromosomes and since in triploid fetuses all chromosomes are trisomic the ratio is identical to that of euploid fetuses. A third method of cfDNA testing has been described which uses a targeted approach involving amplification and sequencing of SNPs [19]. However, this method is also unable to distinguish triploid from euploid fetuses because it too requires the use of reference chromosomes.

The SNP method used in this study is able to identify diandric triploid samples based on the presence of a second paternal haplotype. However, there are only minor differences in allele distributions between diandric triploidy and dizygotic twins and it is therefore difficult to distinguish between these two conditions by cfDNA testing in maternal blood. For example, for alleles where the maternal genotype is AA (where the dimorphic alleles are arbitrarily labeled as A and B), the expected fetal genotypes include AAA, AAB, and ABB. This results in the contribution of zero, one, or two B alleles. In the case of dizygotic twins, for alleles where the mother is AA, the expected fetal genotypes include AA/AA, AA/AB and AB/AB. This results in identical B allele contributions and very similar A allele contributions. At this stage, the NATUS algorithm is unable to reliably distinguish between these A allele distributions in the case of diandric triploidy or dizygotic twins. Ongoing studies aim to distinguish between the two conditions based on the presence (in the case of twins) or absence (in the case of triploidy) of an extra fetal haplotype. In the case of vanishing twins, the placenta from the dead embryo may continue to release cfDNA into the maternal circulation and with the SNP allele ratio method these samples are identified as triploidy or twins.

In digynic triploidy the fetal fraction was inappropriately low for maternal weight and gestational age, and this is likely to be the consequence of the associated small placental mass. Although in our euploid group there was no significant association between fetal fraction and either maternal weight or serum-free β-hCG and PAPP-A, previous studies examining a higher number of pregnancies demonstrated a significant inverse association between fetal fraction and maternal weight and linear association between fetal fraction and both free β-hCG and PAPP-A [10, 20–22]. The maternal blood level of fetal cfDNA decreases with increasing maternal weight due to a dilutional effect. This is also true for other fetoplacental products, including free β-hCG and PAPP-A. A contributing factor to the decrease in fetal fraction with increasing maternal weight is the increase in maternal cfDNA levels because with increased weight there is active remodeling of adipose tissue with accelerated turnover of adipocytes [22, 23]. In terms of the association of fetal fraction with serum-free β-hCG and PAPP-A, these metabolites are produced by the placenta and inevitably their maternal serum concentration provides an indirect measure of placental mass. Similarly, the likely source of fetal cfDNA in maternal plasma is dying cells in the placenta [24] and consequently the number

of apoptotic cells would be proportional to the placental mass.

The first-trimester combined test screens for trisomies 21, 18 and 13 with a detection rate of about 90% at a FPR of 5% [18]. This method also identifies about 85% of fetuses with triploidy; this is because diandric triploidy, with the associated very high level of serum-free β-hCG, can present as high risk for trisomy 21 [6]. Similarly, digynic triploidy, with very low serum-free β-hCG and PAPP-A, can present as high risk for trisomies 18 and 13 [6]. Several studies have demonstrated that the performance of screening for trisomies with maternal blood cfDNA testing is superior to that of the combined test [10–17]. There are essentially two options in the introduction of cfDNA testing: the first option is to carry cfDNA testing together with serum biochemistry at 10 weeks' gestation and an ultrasound scan at 12 weeks in all women, and the second option is to perform cfDNA testing contingent on the results of the combined test at 12 weeks' gestation [25, 26]. These retain the advantages of the combined test, specifically that diagnosis of aneuploidies within the first trimester allows for earlier and safer termination of pregnancy, and that early detection of major defects and prediction of a wide range of pregnancy complications allows for earlier therapeutic intervention and better pregnancy management [27].

Irrespective of the strategy for cfDNA testing if the results suggest diandric triploidy or dizygotic twins, the two can be easily distinguished by ultrasound and if the pregnancy is singleton, invasive testing should be recommended to confirm the suspected diagnosis. In pregnancies where cfDNA testing raises the possibility of digynic triploidy because the fetal fraction corrected for maternal weight and gestational age is very low, management is more difficult because the positive predictive value is still undefined. Consequently, until there is more data addressing abnormally low fetal cfDNA fractions, invasive diagnostic testing could be reserved only for cases where serum levels of free β-hCG and PAPP-A are very low and there is ultrasound evidence of severe asymmetric fetal growth restriction.

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