RESEARCH ARTICLE

Maternal levels of free fetal DNA are elevated in pregnancies with growth restriction due to placental dysfunction

A preliminary study

Medhat S Alberry
Clinical Research Fellow, University of Bristol, St Michael's Hospital, Southwell Street, Bristol, UK/Ain-Shams University Maternity Hospital, Cairo, Egypt

Sebastian Illanis
Clinical Research Fellow, University of Bristol, St Michael's Hospital, Southwell Street, Bristol, UK

Deborah G Maddocks
Research Assistant, Centre for Research in Biomedicine, Faculty of Applied Sciences, University of the West of England, Bristol, UK

Sherif A Fattah
Consultant in Obstetrics and Gynaecology, University of Bristol, St Michael's Hospital, Southwell Street, Bristol, UK

Bernhard G Zimmermann
Postdoctoral Research Fellow, Dental Research Institute, Centre for Health Sciences, 10833 le Contre Ave, UCLA, Los Angeles, CA, USA

Neil D Avent
Director of Centre for Research in Biomedicine, Faculty of Applied Sciences, University of the West of England, Bristol, Coldharbour Lane, Frenchay, Bristol, UK

Peter W Soothill
Professor of Maternal and Fetal Medicine, Head of Obstetrics and Gynaecology, University of Bristol, St Michael's Hospital, Southwell Street, Bristol, UK

Objective. Fetal growth restriction (FGR) is associated with an increased risk of perinatal mortality and morbidity but can have many different causes. Non-cellular fetal DNA in maternal blood offers many opportunities for non-invasive prenatal diagnosis. It is likely that the source of the DNA is apoptosis or cell death in the placenta and that free fetal DNA (ffDNA) levels could theoretically be increased in placental dysfunction or infarction. We hypothesised that non-cellular fetal DNA levels would be increased only in the subset of FGR cases with placental dysfunction, which could explain previous contradictory reports.

Methods. We used plasma samples obtained during a previous study from pregnant women with singleton male pregnancies as controls and from women with small-for-gestational-age (SGA) infants that had been classified as having FGR due to placental dysfunction. A third group was defined as normal but small fetuses with placental function within the normal ranges indicated by Doppler studies. Twenty-two cases were identified in the third trimester of pregnancy (8 from the control group and 7 from each of the FGR and the normal small groups). DNA was extracted and the DYS14 gene of the Y chromosome was quantified by real-time quantitative polymerase chain reaction (PCR).

Results. ffDNA levels were higher in pregnancies with FGR due to placental dysfunction than in either normal pregnancies or those with SGA fetuses from causes other than placental dysfunction. There was no significant difference in the ffDNA levels between the fetuses of normal growth and those with FGR from other causes.

Conclusion. The level of ffDNA in maternal plasma is increased in pregnancies complicated by FGR secondary to placental dysfunction but not in those with small fetuses with normal placental function.

Fetal growth restriction (FGR) describes a decrease in the fetal growth rate that prevents the fetus from fulfilling its genetic growth potential. It is the second largest contributor to perinatal mortality in developed countries, affecting 3 - 5% of all deliveries. FGR may be secondary to placental dysfunction or the result of other causes.[1] It is the aim of modern perinatology to identify fetuses with FGR early enough to institute appropriate intervention and prevent further damage.[2-4] Doppler ultrasound, using measures of the resistance to flow in the placental circulation, has proved to be a useful technique for diagnosing the cause of a small fetus, making it possible to differentiate between small fetuses with normal placental transfer and cases in which the smallness is due to placental dysfunction.[5-7]

The discovery of cell-free fetal DNA in maternal plasma represents a promising non-invasive approach to prenatal diagnosis. Prenatal determination of fetal sex,[8,9] rhesus D blood genotyping[10] and detection of single-gene disorders[11,12] have all been achieved through the analysis of maternal plasma and are now being used in patient care.[13] There has been much speculation as to the source of the circulating fetal DNA, and three possible sources are fetal haematopoietic cells, the placenta, and direct transfer of DNA molecules.[14] There is considerable information supporting the view that the placenta is a major source of free fetal DNA, and if that proves correct, placental disease such as infarction might increase circulating levels. Placental disease can manifest either as FGR or pre-eclampsia, and there is some evidence that the fetal DNA concentration increases in the plasma of pregnant women with pre-eclampsia.[15-17]

Our previous studies have demonstrated that both fetal and maternal biochemical changes in inhibin and activin only occur in the group of FGR fetuses with abnormal Doppler studies.[14] We hypothesised that maternal free fetal DNA levels would be high in that group but not in small-for-gestational-age (SGA) fetuses with normal Doppler findings, which could explain differences in the results of previous studies in which anomalous results were obtained.[18]

Methods

After ethical committee approval and patient consent, we undertook serial maternal blood sampling and ultrasound assessment of normal pregnancies and those in which a fetus growing at less than the expected rate for gestational age had been identified. All the mothers were in the third trimester and carrying male fetuses. Serial growth scans with Doppler assessment were undertaken and documented on our Viewpoint Fetal Medicine Database. The small fetuses were classified as FGR due to placental dysfunction (umbilical artery Doppler, growth velocity pulsatility index > 2 SD for gestational age) or normal small (normal Doppler, growth velocity and amniotic fluid volume).[19] Twenty-

two pregnancies (8 controls, 7 SGA with normal Doppler and 7 FGR with abnormal Doppler) were identified. The samples were stored at –20oC for 7 years.

Genomic reference DNA was prepared as described previously.[20] Blood samples were collected into 10 ml collection tubes containing EDTA. The blood was centrifuged at 1 600 g for 10 minutes, after which the supernatant was transferred into polypropylene tubes and stored. After thawing, the samples were centrifuged and mixed thoroughly and plasma DNA was extracted from the supernatant with the high pure PCR template kit (Roche, Switzerland).

Real-time quantitative PCR amplification

To reduce inter-experimental variation, all DNA samples (total 48) were quantified in parallel on the same plate in single reactions. The quantification of free fetal DNA (ffDNA) was repeated three times and total DNA was also quantified once. The real-time quantitative polymerase chain reaction (PCR) measurement of male fetal DNA was performed in the ABI PRISM 7000 Sequence Detection System (Applied Bio Systems, ABI). The single-plex reactions in volumes of 15 μl were prepared contained 300 nM of each primer (HPLC purified), 200 nM of the MGB probe (ABI), and a 1 x concentration of the TaqMan Universal PCR master mix with Amperase UNG (ABI). Primer and probe sequences were as follows: DYS[14] Forwards: 5`GGG CCA ATG TTG TAT CCT TCT C, Reverse: 3`GCC CAT CGG TCA CTT ACA CTT C and Vic labelled MGB probe: TCT AGT GGA GAG GTG CTC.

PCR cycling conditions were 2 minutes at 50°C and 10 minutes at 95°C for initial denaturation of the DNA and polymerase activation, followed by 50 cycles of 1 minute at 60°C and 15 seconds at 95°C. Two microlitres of DNA was added per reaction. The analysis and quantification was performed using the ABI Prism 7000 SDS Software with automatic baseline setting, and we analysed all data at two thresholds with Rn = 0.1 and Rn = 0.2.

As quantitative standards fourfold dilutions of reference genomic DNA at 1 250, 312 and 78 genome equivalent (GE) per reaction were amplified in triplicate. The logarithm of the template copy number in a reaction is inversely proportional to the cycle threshold (CT). Thus, a linear regression can be used as a calibration curve when these two values are plotted against each other. The PCR efficiency was calculated from the slope of the curve using the following formula:

$$\text{Efficiency} = 10^{-(1/\text{slope})} - 1$$

For the determination of fetal sex by the SRY gene of the Y chromosome all three replicate amplifications of a sample had to be either positive or negative, otherwise the sample was undetermined. With the DYS14 gene protocol we applied a cut-off of 1 GE per

PCR to discriminate between true positives (male) and unspecific amplifications (female).

Results

Fetal DNA concentration (GE/ml) increased with gestational age in the control group and the normal small group (small fetuses with normal clinical assessment of placental function by Doppler). The relationship in these two groups was linear after $\log^{10}$ transformation (Fig. 1). Statistical modelling was used to assess whether these two normal groups had the same slopes and intercepts.
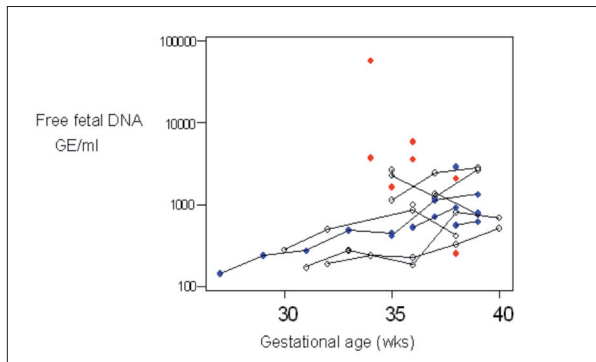

Fig. 1. Log^{10} of ffDNA from the three cohorts plotted against gestational age. Repeated results from the same individuals are linked. (Normal = open diamonds; 'small' normal = blue; FGR = red.)

A 'random coefficient' model was used to take into account the correlation between repeated results on the same pregnancy, and the model allowed for inter-pregnancy-variation in both the slopes and the intercepts. SAS 'Proc MIXED' was used for the fitting.[21] There was no significant difference between the mean slopes of the two groups (p = 0.283) or their mean intercepts (p = 0.266), suggesting that the two normal groups might be combined. For the combined group there was negligible variation between individual slopes (data not shown), but there was some variation between the individual intercepts; the slope estimate was 0.05834 (SE 0.01055), with mean intercept 0.7492 (SE 0.3884). The variance between the individual intercepts was estimated to be 0.06319 and the residual variance 0.02420.

Approximate 95% prediction intervals were calculated and plotted for a series of gestational ages (Fig. 2). The majority of the FGR cases (small fetuses with evidence of placental dysfunction by abnormal Doppler studies) had free fetal DNA levels outside the estimated 95% prediction interval, while almost all of the ffDNA levels of the normal and SGA groups lay within the 95% prediction interval as shown in Fig. 2. Although initially this may appear to indicate a fall with gestational age, it is likely to result from the fact that more severe FGR is detected earlier in pregnancy.
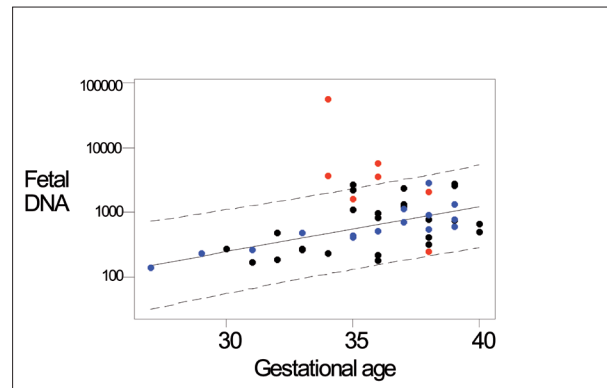

Fig. 2. 95% prediction intervals for the FGR group (red diamonds), normal (black) and normal small (blue).

Discussion

This study showed high levels of ffDNA in 4 out of 7 cases of FGR caused by utero-placental insufficiency due to either placental infarction or utero-placental blood flow reduction. An important observation is that the FGR cases with higher ffDNA were at an earlier gestational age. Early cases of FGR are likely to be more severe than those reaching term, and this might explain the presence of ffDNA within the normal range in the cases reaching term. More longitudinal prospective studies may be able to establish the role of ffDNA as an indicator of severity of FGR and subsequently assist in deciding the optimal timing of delivery.[22,23] The increased ffDNA in FGR cases associated with placental dysfunction supports the hypothesis that most fetal DNA in maternal plasma originates from trophoblasts. Nevertheless, the ability to extract and detect DNA in these stored samples after such a long period indicates stability of DNA when stored at –20°C, which may be useful for future studies.[24]

There was no statistical evidence of any difference between the controls and the SGA cases, and after log transformation the results demonstrated a linear increase with gestational age. Although the data need extending, this assessment of a normal range could be useful for future studies of ffDNA quantification. Moreover, the data from both groups showed similar medians, trends and variance, which supports the hypothesis that pregnancies with SGA fetuses do not have any of the fetal or maternal changes associated with placental dysfunction.[15,18] Conversely, the data from the FGR group provide a different modality from the normal and normal small groups. Previous protein-based studies have also shown that when the umbilical artery Doppler is abnormal the placental tissue is affected, probably by increased apoptosis or infarction.

This paper presents evidence supporting the fundamental importance of classifying FGR and SGA by using Doppler ultrasound for clinical management.
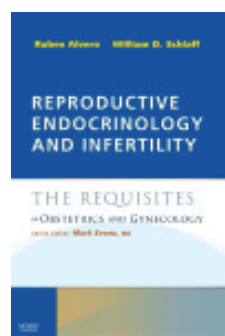
1. Wolfe HM, Gross TL. Increased risk to the growth retarded fetus. In: Gross TM, Soko RJ, eds. *Intrauterine Growth Retardation*. Chicago: Year Book Medical Publishers, 1989.
2. Baschat AA, Hecher K. Fetal growth restriction due to placental disease. *Semin Perinatol* 2004; 28: 67-80.
3. Lubchenco LO, Hansman C, Boyd E. Intrauterine growth as estimated from live born birth-weight data at 24 - 42 weeks of gestation. *Paediatrics* 1963; 32: 793.
4. Bernstein IM, Horbar JD, Badger GJ, *et al.* Morbidity and mortality among very-low-birth-weight neonates with intrauterine growth restriction. The Vermont Oxford Network. *Am J Obstet Gynecol* 2000; 182: 198-206.
5. Bobrow CS, Soothill PW. Fetal growth velocity: a cautionary tale. *Lancet* 1999; 353: 1460.
6. Ott WJ. Diagnosis of intrauterine growth restriction: comparison of ultrasound parameters. *Am J Perinatol* 2002; 19: 133-137.
7. Kingdom JC, Burrell SJ, Kaufmann P. Pathology and clinical implications of abnormal umbilical artery Doppler waveforms. *Ultrasound Obstet Gynecol* 1997; 9: 271-286.
8. Lo YM, Tein MS, Lau TK, *et al.* Quantitative analysis of fetal DNA in maternal plasma and serum: implications for non-invasive prenatal diagnosis. *Am J Hum Genet* 1998; 62: 768-775.
9. Sekizawa A, Kondo T, Iwasaki M, *et al.* Accuracy of fetal gender determination by analysis of DNA in maternal plasma. *Clin Chem* 2001; 47: 1856-1858.
10. Lo YM, Hjelm NM, Fidler C, *et al.* Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998; 339: 1734-1738.
11. Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma [letter]. *Lancet* 2000; 356: 1170.
12. Lo YM, Lau TK, Zhang J, *et al.* Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* 1999; 45: 1747-1751.
13. Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* 2002; 42(8): 1079-1085.
14. Bianchi DW. Circulating fetal DNA: Its origin and diagnostic potential – a review. *Placenta* 2004; 25: Supplement A, Trophoblast Research, Vol. 18, S93-S101.
15. Lo YM, Leung TN, Tein MS, *et al.* Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem* 1999; 45: 184-188.
16. Bianchi DW. Fetal DNA in maternal plasma: The plot thickens and the placental barrier thins. *Am J Hum Genet* 1998; 62: 763-764.
17. Sekizawa A, Yokokawa K, Sugito Y. Evaluation of bidirectional transfer of plasma DNA through placenta. *Hum Genet* 2003; 113: 307-310.
18. Bobrow CS, Holmes RP, Muttukrishna S, *et al.* Maternal serum activin A, inhibin A, and follistatin in pregnancies with appropriately grown and small-for-gestational-age fetuses classified by umbilical artery Doppler ultrasound. *Am J Obstet Gynecol* 2002; 186(2): 283-287.
19. Holmes RP, Holly JM, Soothill PW. A prospective study of maternal serum insulin-like growth factor-I in pregnancies with appropriately grown or growth restricted fetuses. *Br J Obstet Gynaecol* 1999; 106: 1232-1233.
20. Zimmermann B, El-Sheikhah A, Nicolaides K, Holzgreve W, Hahn S. Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma. *Clin Chem* 2005; 51: 1598-1604. Epub 14 July 2005.
21. *SAS Version 8.2.* Cary, NC: SAS Inst. Inc., 1999-2001.
22. Illanis S, Avent N, Soothill PW. Cell-free fetal DNA in maternal plasma an important advance to link fetal genetics to obstetric ultrasound. *Ultrasound Obstet Gynecol* 2005; 25: 317-322.
23. Alberry M, Soothill PW. Management of FGR. *Arch Dis Child* 2007; 92: 62-67.
24. Koide K, Sekizawa A, Iwasaki M, *et al.* Fragmentation of cell-free fetal DNA in plasma and urine of pregnant women. *Prenat Diagn* 2005; 25: 604-607.