Correlation of maternal plasma total cell-free DNA and fetal DNA levels with short term outcome of first-trimester vaginal bleeding

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BACKGROUND: The current methods using sonographic parameters and/or maternal serum β-HCG levels to predict spontaneous abortion are not satisfactory. The aim of this study was to determine whether maternal plasma fetal DNA and total DNA levels could be used to predict spontaneous abortion. METHODS: We prospectively studied pregnant women who presented with vaginal bleeding in the first trimester of pregnancy, and those who had no vaginal bleeding (controls). DYS14 and the β-globin gene were used to measure the maternal plasma levels of fetal and total DNA, respectively, by real-time PCR. RESULTS: A total of 1114 women were studied. Both maternal plasma fetal and total DNA concentrations increased with gestation from 6 to 11.6 weeks in the controls. The multiple of medians (MoMs) of fetal and total DNA concentration in those who miscarried were significantly greater (P < 0.001) than in the normal controls by about 5- and 4-fold respectively. Using a cut-off value of 1.6 MoMs for total DNA to predict spontaneous abortion, the sensitivity was 98.2% and false positive rate was 47.6%. However, using a cut-off value of 1.8 MoMs for fetal DNA, the corresponding figures were 97% and 44.3%, respectively. CONCLUSIONS: Both maternal plasma fetal and total DNA concentrations increased throughout the first trimester. Significantly high levels of fetal and total DNA were found in those who miscarried.

Keywords: fetal DNA; maternal plasma; spontaneous abortion; total DNA

Introduction

In women presenting with vaginal bleeding in the first trimester and a live fetus demonstrated on pelvic scanning, the rate of subsequent fetal loss is ~15% (Falco et al., 1996). Due to the high prevalence of spontaneous abortion in these women, a test that can be used to predict the subsequent pregnancy outcome is useful in counselling. Sonographic parameters such as fetal heart rate (FHR) and crown rump length (CRL) and maternal serum β-HCG levels, either used alone or in combination, have been used for such prediction, but the results were inconsistent and not satisfactory (Benson and Doubilet, 1994; Qasim et al., 1997; Doubilet et al., 1999; Lipscomb et al., 2000; Reis et al., 2002; Florio et al., 2004). The presence of cell-free fetal DNA (cffDNA) in maternal plasma and serum and its potential for non-invasive prenatal diagnosis was first recognized in 1997 (Lo et al., 1997). The origin of the cff DNA has remained uncertain, although evidence suggests that the placenta or, more specifically, trophoblast degeneration is the most likely source (Bianchi, 2004). Fetomaternal transfusion of DNA is a potential indicator for placental health, and increased levels of fetal DNA in the maternal circulation is regarded as a breakdown of the placental barrier and linked to pregnancy-associated complications including aneuploidies (Zhong et al., 2000), pre-eclampsia (Leung et al., 2001), intrauterine growth restriction (Caramelli et al., 2003), preterm labor (Farina et al., 2005), placenta accreta (Jimbo et al., 2003), invasive placenta (Sekizawa et al., 2002), fetomaternal hemorrhage (Miura et al., 2006) and intrauterine fetal death (Zhong et al., 2006). In addition, recent studies showed β-globin (total DNA) levels were higher in those women who subsequently developed pre-eclampsia (Farina et al., 2004; Sekizawa et al., 2004) and in women carrying a fetus affected by trisomy 21 (Spencer et al., 2003).
cff DNA can be detected in the maternal circulation as early as 32 days of gestation (Birch et al., 2005). A recent study has shown that fetal DNA is present in the circulation of women with first trimester anembryonic spontaneous abortion (Skinner et al., 2001). We postulate that spontaneous abortion is associated with increased levels of fetal and total DNA in maternal circulation. In the present study, our objectives were to (i) determine normal values of maternal plasma fetal DNA and total DNA in the first trimester and (ii) determine whether maternal plasma fetal DNA and total DNA levels could be used to predict the subsequent outcome of women who had vaginal bleeding in the first trimester and a viable fetus on an ultrasound examination.

Materials and Methods

Patients and study design

This study was approved by the Ethics Committee of Maternal and Children Hospital of Guangdong Province and informed consent was obtained from all subjects. This prospective study was performed between February 2005 and March 2006. During this period, all pregnant women who presented with vaginal bleeding in the preceding 24 h in the first trimester of pregnancy were invited to participate. A transvaginal ultrasound examination was performed in all women to determine the fetal viability and gestational age. Inclusion criteria were as follows: no significant past medical illness, a positive urinary pregnancy test result and a single intrauterine viable fetus of 6–11.6 weeks gestation on an ultrasound examination. Women who had no vaginal bleeding and sought antenatal care in our center were invited to participate as controls. Retrospective exclusion criteria for the control group were as follows: loss to follow-up; a complication of pregnancy in the second or third trimester including pre-eclampsia, gestational diabetes, intrauterine growth restriction, preterm delivery and spontaneous abortion after inclusion and detection of a fetal structural or chromosomal abnormality.

Preparation of samples

Ten milliliters of maternal blood was collected from all women who participated in the study on the first visit, and also shortly before surgery evacuation of the uterus in those women who were subsequently confirmed to have spontaneous abortion on scanning. Blood samples were collected into tubes containing EDTA, and centrifuged at 2,000g for 10 min. Plasma was carefully removed from the tubes without disturbing theuffy coat, transferred into the plain polypropylene tubes and recentrifuged at 16,000g for 10 min. The supernatants were collected into the fresh polypropylene tubes, and stored at −70°C until further processing. The samples were coded by an investigator not involved in the DNA extraction and amplification process for a subsequent blinded analysis.

Women with spontaneous abortion were scheduled to undergo surgical evacuation about 3 days following the ultrasound diagnosis. Products of conception (POCs) were collected into a container with normal saline solution and then frozen for subsequent determination of the fetal gender. For normal controls and those women with ongoing pregnancies, the fetal gender was ascertained after delivery or by the cytogenetic results of chorionic villus sampling (CVS), amniocentesis or cordocentesis.

DNA extraction and real-time PCR analysis

For all plasma samples, DNA was extracted from 800 μl maternal plasma samples by use of a QIAamp Blood Mini Kit (QIAGEN GmbH, Germany) according to the ‘blood and body fluid protocol’ recommended by the manufacturer (Lo et al., 1998). The exact amount used was documented to enable the calculation of target DNA concentration. For the gender determination in spontaneous abortion, we obtained 1 ml of saline solution in which the POC had been stored because some DNA diffused into the solution. This 1 ml solution was centrifuged at 11,500g for 10 min, with 800 μl of the supernatant used for DNA extraction, also by use of a QIAamp Blood Mini Kit. In all cases, the extracted DNA was eluted into a final volume of 50 μl distilled water.

PCR was performed with an ABI 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Extracted DNA was analysed for both DYS14 and the β-globin gene. The DYS14 sequence was used to measure the quantity of fetal DNA present in each sample from a woman carrying a male fetus, whereas the β-globin sequence was used to confirm the presence and determine the quality of DNA in each sample as well as to measure the quantity of total (maternal and fetal) DNA in each sample.

In this study, all women were in the first trimester, and thus the levels of maternal plasma fetal DNA could be very low, especially, at 6 weeks gestation. In order to minimize the false negative rates, we first used the DYS14 sequence, instead of SRY gene, to detect the fetal DNA, because the DYS14 sequence is a Y-specific multiplexed sequence and can therefore increase the sensitivity (Zimmermann et al., 2005). Then we used maximum volume extracted plasma DNA (12 μl) in the real-time PCR systems. The DYS14 TaqMan system consisted of the amplification primers: DYS14-F, 5′-GGG CCA ATG TTG TAT CCT TCT C-3′; DYS14-R, 5′-GCC CAT CCG TCA CTT ACA CTT C-3′; and a dual-labeled fluorescent TaqMan probe 5′-(FAM)TCT AGT GGA GAG TCT (TMRA)-3′ (Zimmermann et al., 2005). The β-globin TaqMan system consisted of the amplification primers β-globin-F, 5′-GGG CCA ATG TTG TAT CCT TCT C-3′; β-globin-R, 5′-GCC CAT CCG TCA CTT ACA CTT C-3′; and a dual-labeled fluorescent TaqMan probe β-globin -T, 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GTG GG(TMRA)-3′ (Lo et al., 1998). TaqMan probes were custom-synthesized by Applied Biosystems (Applied Biosystems). PCR primers were synthesized by Life Technologies (Shanghai, China). The PCR reactions were carried out in a final reaction volume of 25 μl, which consisted of 12 μl of eluted DNA, which corresponded to the amount of DNA recovered from 192 μl of maternal plasma, 400 nM each primer, 200 nM each probe, 1 μl of eluted DNA, which consisted of 12 μl of eluted DNA, which corresponded to the amount of DNA recovered from 192 μl of maternal plasma, 400 nM each primer, 200 nM each probe, 1 × TaqMan Universal PCR Master Mix (Applied Biosystems) and 0.5 U AmpErase uracil-N-glycosylase (Applied Biosystems). Identi cal thermal profiles were used for both the DYS14 and the β-globin TaqMan systems. PCR was carried out with an initial incubation at 50°C for 2 min to activate uracil-N-glycosylase, followed by incubation at 95°C for 10 min and 50 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was analysed in triplicate. To improve the specificity of DYS14 sequences, we applied a cut off at 1GE per PCR to distinguish between male and female (Zimmermann et al., 2005), and regarded a result as positive only if all the results of the triplicates were positive. The mean of each triplicate was used for further concentration calculations. A calibration curve was run in parallel and in triplicate with each analysis. To minimize the possibility of contamination, aerosol resistant tips were used throughout all the experimental procedures. Multiple negative controls were included in each analysis. Separate areas were used for the preparation of amplification reactions, the addition of DNA template and the carrying out of amplification reactions.

To determine the concentration of male fetal DNA present in the maternal plasma sample, a standard dilution curve using a known concentration of male genomic DNA (10 ng/μl, Applied Biosystems)
was used. The conversion factor of 6.6 pg of DNA per cell was used to express the results as genome-equivalents (Lo et al., 1998). One genome-equivalent was defined as the quantity of a particular DNA sequence present in one diploid male cell. The concentration was expressed as genome-equivalents per milliliter plasma (GE/ml).

Statistics
For statistical analysis, the women were classified into three groups: (i) spontaneous abortion, (ii) threatened spontaneous abortion but normal outcome and (iii) normal control. SPSS for Windows (SPSS Inc., Chicago, IL, USA) software package version 13 was used. The sonographic finding of CRL instead of last menstrual period was used to calculate the gestational age. Correlation of the maternal plasma fetal and total DNA concentration with gestation was assessed by Pearson correlation analysis. We checked that our data of plasma DNA were non-parametric. The median of fetal and total DNA in different gestational weeks were calculated and converted to MoMs using the group C (normal control) as a reference. Sensitivity, false positive value and ROC curve were used to assess the predictability of maternal plasma total DNA and fetal DNA in spontaneous abortion.

Results
Of 1187 women recruited into the study, 73 were subsequently excluded from analysis for various reasons (Table 1). Of a total of 1114 women being subsequently analysed, 164 were in group A (spontaneous abortion), 575 were in group B (threatened spontaneous abortion but with normal outcomes) and 375 were in group C (normal control). There were no statistically significant differences in maternal age and gestation age among the three groups (Table 1). We obtained first blood samples from all 1114 women and the second blood sample from 120 of 164 women in group A because 44 women refused to undergo another blood sampling. In total, we obtained a total of 1234 maternal plasma samples.

β-Globin analysis demonstrated the presence of amplifiable DNA in all the 1114 maternal first plasma samples. The DYS14 sequences were present in a total of 590 plasma samples, 39 samples obtained at 6 weeks gestation in groups B and C which were below 1GE/PCR but positive in all triplicates. However, all fetuses in the 39 cases were male according to the results at follow-up. Finally, male fetus was confirmed in 582 out of these 590 samples after the delivery, CVS, amniocentesis or surgical evacuation of uterus, but could not be confirmed in the remaining eight cases of positive DYS14 sequences because complete spontaneous abortion occurred outside our hospital and hence POCs could not be obtained for analysis. On the other hand, DYS14 sequences could not be detected in the plasma of four women with a male fetus, all of which were taken at 6 weeks gestation.

Table 1: Demographic data of different groups of women at the time of first blood test

<table>
<thead>
<tr>
<th></th>
<th>Group A* (spontaneous abortion)</th>
<th>Group B* (threatened spontaneous abortion with normal outcomes)</th>
<th>Group C* (normal control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>164</td>
<td>575</td>
<td>375</td>
</tr>
<tr>
<td>Maternal age (years; mean ± SD)</td>
<td>30.0 ± 4.3</td>
<td>28.9 ± 3.6</td>
<td>28.7 ± 3.4</td>
</tr>
<tr>
<td>Gestation age (weeks; mean ± SD)</td>
<td>8 weeks and 4 days ± 1 week and 3 days</td>
<td>8 weeks and 4 days ± 1 week and 3 days</td>
<td>9 weeks and 2 days ± 1 week and 5 days</td>
</tr>
</tbody>
</table>

* A total of 776 patients with vaginal bleeding were recruited, and 22 of them defaulted follow-up. The remaining 754 patients were classified into two groups, non-spontaneous abortion (n = 590) and spontaneous abortion (n = 164), by the pregnancy outcome. In the non-spontaneous abortion group, 15 patients were excluded from analysis because of preterm delivery (n = 3), pre eclampsia (n = 4), fetal intrauterine growth restriction (n = 3), chromosome aberration (n = 3), and a fetal structural abnormality (n = 2).

A total of 411 women without vaginal bleeding were initially included in the control group. Thirty-six of them were subsequently excluded later because of preterm delivery (n = 3), pre eclampsia (n = 3), fetal intrauterine growth restriction (n = 4), trisome (n = 4), a fetal structural abnormality (n = 2) and loss to follow-up (n = 20), leaving a total of 375 patients who ultimately comprised the control group.

Concentration of maternal plasma cell-free total DNA and fetal DNA in normal controls at different gestational ages
In group C (normal control), the median concentration of maternal plasma fetal DNA and total DNA increased with gestational age from 6 to 11.6 weeks (Table 2). The correlation with gestational age was stronger with the maternal plasma total DNA concentration (r = 0.840, P < 0.001) than with the maternal plasma fetal DNA concentration (r = 0.546, P < 0.001). Whereas the increase in median concentration of plasma total DNA was gradual across the studied gestations, the increase in median concentration of maternal plasma fetal DNA varied with gestation. About 100% increase and relatively no changes were observed from 6 to 7 weeks gestation and from 10 to 11 weeks gestation, respectively. The concentration of maternal plasma fetal DNA was significantly correlated with that of total DNA (r = 0.536, P < 0.001).

Concentration of maternal plasma cell-free total DNA and fetal DNA in women with threatened spontaneous abortion
The medians of both maternal plasma fetal DNA and total DNA levels were significantly higher in group A (spontaneous abortion) than in group B (ongoing pregnancy with normal outcomes) and group C (normal control) (P < 0.001), but the differences between group B and group C were not significant.

To adjust for the effects of gestation, we converted the median values of maternal plasma fetal DNA and total DNA at different gestations in groups A and B into MoMs using the corresponding median values as a reference (Table 3). The MoMs of maternal plasma fetal and total DNA concentration in group A (spontaneous abortion) were significantly higher (P < 0.001) than that of group C (normal control) by about 5- and 4-folds, respectively (Table 3, Fig. 1a and b). However, there were no significant differences in the MoMs of maternal plasma fetal or total DNA concentration between...
the group B (threatened spontaneous abortion with normal outcomes) and group C (normal control).

Using maternal plasma total DNA and fetal DNA to predict spontaneous abortion

ROC curves (Fig. 2) showed that maternal plasma total DNA was better than fetal DNA in the prediction of spontaneous abortion as the area under the ROC curve was greater (0.994 versus 0.978). Using maternal plasma total DNA 1.6 MoMs (just above the maximal level in group C) as a cut-off to predict spontaneous abortion, the sensitivity was 98.2% and false positive rate was 4.7%. However, using maternal plasma fetal DNA 1.8 MoMs (mean + 1SD in group C) as a cut-off to predict spontaneous abortion, the sensitivity was 97% but the false positive rate was 44.3%.

Changes of maternal plasma total DNA and fetal DNA in aborted pregnancies

In group A, of a total of 120 spontaneous abortions where a second blood sample could be obtained, 105 were silent spontaneous abortion and 15 women were incomplete spontaneous abortion. Since the number of the latter was small, we did not analyse the data according to different types of spontaneous abortion. There was a significant decrease in both the levels of maternal plasma total and fetal DNA from the time of presentation of vaginal bleeding to the diagnosis of spontaneous abortion \((P < 0.001)\) (Table 4).

Discussion

In the present study, we have shown that both plasma fetal and total DNA concentrations increase with gestation from 6 to 11.6 weeks gestation of normal pregnancy and there is a significant correlation between total DNA and fetal DNA. In previous studies, the results were inconsistent. Lo et al. (1998) first reported that both fetal and total DNA in maternal plasma increased with gestation. Other investigators reported a significant correlation between maternal plasma fetal DNA, but not total DNA, and gestational age in the first trimester (Stanghellini et al., 2006) and late pregnancy (Chan et al., 2003). Bauer et al. (2006) reported that total and fetal DNA levels were significantly correlated, but neither level was correlated with gestational age. However, two recent reports showed that maternal plasma fetal DNA levels in normal pregnancy increased by 4.2 genome equivalents/mL/week (Watagana, 2004) and by 21% per week in the first trimester (Tjoa et al., 2006).

There are several possible explanations for the differences in the results between different studies. One possible reason is the rather small sample size among the various studies in combination with the broad ranges in both cff and total DNA concentrations in the blood of mothers. Another possibility is the blood processing protocols that may have different efficacies in the generation of cell-free plasma and serum (Chiu et al., 2001). The third possibility is that cell-free DNA in serum samples is generally higher than that in plasma. This can be attributed to the release of cell-free DNA during the clotting process (Lo et al., 1998; Lee et al., 2002). There were several strengths in our study, first the sample size was much larger. Second, we

Table 2: Concentrations of maternal plasma fetal and total DNA in normal pregnancy

<table>
<thead>
<tr>
<th>Gestational weeks</th>
<th>Fetal DNA (GE/ml)</th>
<th>Total DNA (GE/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>Median (range)</td>
<td>Number of cases</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>7</td>
<td>23.0 (12.7, 21.7)</td>
<td>266.9 (206.9, 329.0)</td>
</tr>
<tr>
<td></td>
<td>1.62 (0.99, 2.11)</td>
<td>1.004 (0.38, 2.71)</td>
</tr>
<tr>
<td>8</td>
<td>27.5 (17.7, 27.5)</td>
<td>416.1 (383.2, 416.1)</td>
</tr>
<tr>
<td></td>
<td>1.00 (0.13, 1.9)</td>
<td>1.000 (0.42, 1.57)</td>
</tr>
<tr>
<td>9</td>
<td>61.5 (36.1, 61.5)</td>
<td>672.8 (537.2, 672.8)</td>
</tr>
<tr>
<td></td>
<td>1.34 (0.27, 2.8)</td>
<td>1.000 (0.42, 1.57)</td>
</tr>
<tr>
<td>10</td>
<td>69.1 (42.6, 69.1)</td>
<td>771.5 (692.3, 771.5)</td>
</tr>
<tr>
<td></td>
<td>2.33 (0.54, 4.1)</td>
<td>1.000 (0.42, 1.57)</td>
</tr>
<tr>
<td>11</td>
<td>57.9 (32.6, 57.9)</td>
<td>904.1 (804.1, 904.1)</td>
</tr>
<tr>
<td></td>
<td>1.16 (0.27, 4.1)</td>
<td>1.000 (0.42, 1.57)</td>
</tr>
<tr>
<td>12</td>
<td>69.1 (42.6, 69.1)</td>
<td>1041.7 (804.1, 1041.7)</td>
</tr>
<tr>
<td></td>
<td>2.33 (0.54, 4.1)</td>
<td>1.000 (0.42, 1.57)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>190</td>
</tr>
</tbody>
</table>

Table 3: Median and MoMs of maternal plasma total DNA and fetal DNA in different groups

<table>
<thead>
<tr>
<th></th>
<th>Group A (spontaneous abortion)</th>
<th>Group B (threatened spontaneous abortion with normal outcomes)</th>
<th>Group C (normal control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DNA</td>
<td>Number of cases</td>
<td>164</td>
<td>575</td>
</tr>
<tr>
<td></td>
<td>Median (range), (GE/ml)</td>
<td>1442.9 (354.2, 3041.4)</td>
<td>391.8 (153.1, 908.2)</td>
</tr>
<tr>
<td></td>
<td>MoM (mean ± SD)</td>
<td>3.804 ± 1.014</td>
<td>1.019 ± 0.227</td>
</tr>
<tr>
<td></td>
<td>MoM (median, range)</td>
<td>3.859 (0.99, 7.11)</td>
<td>1.004 (0.38, 2.71)</td>
</tr>
<tr>
<td>Fetal DNA</td>
<td>Number of cases</td>
<td>101</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>Median (range), (GE/ml)</td>
<td>58.3 (14.2, 166.9)</td>
<td>13.5 (1.7, 71.5)</td>
</tr>
<tr>
<td></td>
<td>MoM (mean ± SD)</td>
<td>5.634 ± 2.825</td>
<td>1.341 ± 0.846</td>
</tr>
<tr>
<td></td>
<td>MoM (median, range)</td>
<td>4.905 (1.04, 15.73)</td>
<td>1.161 (0.27, 6.29)</td>
</tr>
</tbody>
</table>
used the protocol of centrifugation at 16,000g to remove contaminating cells, which can be the origin of total cell-free DNA (Bischoff et al., 2005). Third, we used maternal plasma instead of serum to measure fetal and total DNA. The fetal DNA in maternal plasma increases with gestation because trophoblast degeneration occurs in all normal pregnancies as part of physiological villous remodeling (Tjoa et al., 2006). However, the source of increased β-globin is unclear. Poorly perfused placenta may release circulating factors into maternal circulation, which can result in the damage to maternal vascular endothelial cells and multi-system dysfunction (Farina et al., 2004). Other possible explanations include the increase in size of the fetomaternal interface as gestation progresses, and a possible reduction in DNA clearance associated with other physiologic changes during pregnancy (Lo et al., 1998).

Our results indicated that the MoMs of maternal plasma fetal and total DNA concentration in group A (spontaneous abortion) was significantly greater than that of group C (normal control) by about 5- and 4-folds, respectively. Just like other pregnancy-associated complications including aneuploidies, pre-eclampsia, intrauterine growth restriction, preterm labor, placenta accreta, invasive placenta, fetomaternal hemorrhage and intrauterine fetal death, spontaneous abortion is considered as a breakdown of the placental barrier resulting in increased levels of maternal plasma fetal DNA. Maternal plasma fetal DNA increased because of placenta separation which caused the release of fetal DNA into the maternal circulation when threatened spontaneous abortion happened (Farina et al., 2005).

When women presented with vaginal bleeding in the first trimester and a viable fetus, the use of maternal plasma total DNA was more informative than maternal plasma fetal DNA in predicting spontaneous abortion. Using a cut-off value of 1.6 MoMs for total DNA to predict spontaneous abortion, the sensitivity was 98.2% and false positive rate was 4.7%. On the other hand, using a cut-off value of 1.8 MoMs for fetal DNA to predict spontaneous abortion, the sensitivity was 97% but the false positive rate was as high as 44.3%, because there was a lot of overlap in the fetal DNA levels between the

Figure 1: Multiple of medians of (A) maternal plasma total DNA and (B) fetal DNA in group A (spontaneous abortion), group B (threatened spontaneous abortion but with normal outcome) and group C (normal control).

Figure 2: ROC curves: using maternal plasma total DNA and fetal DNA to predict spontaneous abortion.
spontaneous abortion and non-spontaneous abortion groups. In addition, the correlation with gestational age was stronger with the maternal plasma total DNA concentration than with the maternal plasma fetal DNA concentration. Whereas the increase in median concentration of plasma total DNA was gradual across the studied gestations, the increase in median concentration of maternal plasma fetal DNA varied with gestation. Furthermore, fetal DNA can predict the outcome of women carrying a male fetus only because DYS14 sequence is a Y-specific multi-copy sequence, but total DNA is gender-independent and can predict the outcome of all pregnant women with threatened spontaneous abortion. Similarly, other studies also showed the use of total DNA in plasma or serum of pregnant women was more informative than fetal DNA in detecting pre-eclampsia (Farina et al., 2004; Sekizawa et al., 2003) and trisomy 21 (Spencer et al., 2004). This hint that we can serially detect fetal DNA in the near future. However, when ultrasound examination shows a viable fetus, the measurement of maternal plasma total DNA can be used to predict spontaneous abortion which may occur a few weeks later. Such a prediction provides an opportunity for potential interventions. Women with recurrent spontaneous abortions have been treated with progesterone alone (Nardo and Sallam, 2006) or in combination with prednisone, aspirin and folate (Temper et al., 2006) with various degrees of success. Whether women with a high level of maternal plasma total DNA would benefit from the use of progesterone and other medications is unknown.

### Table 4: Maternal plasma total and fetal DNA in the first sample (taken when women presented with first trimester vaginal bleeding and a viable fetus) and the second sample (taken after the diagnosis of spontaneous abortion but before the suction evacuation of uterus) versus the time interval between the first and second sample (interval). *a*

<table>
<thead>
<tr>
<th></th>
<th>First sample</th>
<th></th>
<th></th>
<th>Second sample</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Interval (weeks)</td>
<td>n</td>
<td>Median (GE/ml)</td>
<td>Range</td>
<td>n</td>
<td>Median (GE/ml)</td>
</tr>
<tr>
<td>Total DNA</td>
<td>1</td>
<td>47</td>
<td>2224.9</td>
<td>1147.6, 3337.1</td>
<td>39</td>
<td>668.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>73</td>
<td>1205.4</td>
<td>354.2, 2389.5</td>
<td>54</td>
<td>566.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>43</td>
<td>1243.0</td>
<td>787.7, 2581.4</td>
<td>26</td>
<td>617.3</td>
</tr>
<tr>
<td>Fetal DNA</td>
<td>1</td>
<td>29</td>
<td>75.3</td>
<td>27.1, 166.9</td>
<td>24</td>
<td>53.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37</td>
<td>58.3</td>
<td>19.3, 121.2</td>
<td>28</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>34</td>
<td>46.4</td>
<td>14.2, 102.7</td>
<td>20</td>
<td>15.1</td>
</tr>
</tbody>
</table>

*aIn one case, the interval between the first and second sample was 4 weeks. We did not include this case in the above analysis.
When ultrasound examination does not show a viable fetus, we envisage that serial measurements of maternal plasma cfDNA and total DNA may be potentially useful to differentiate between spontaneous abortion, ectopic pregnancy and early viable pregnancy. A decrease in level of maternal plasma fetal and total DNA may indicate spontaneous abortion (Stanghellini et al., 2006), whereas a higher than normal level of cfDNA may suggest ectopic pregnancy (Lazar et al., 2006).

In conclusion, we have found that both maternal plasma fetal and total DNA concentrations increased throughout the first trimester. Significantly high levels of fetal and total DNA were found in those who miscarried. When women presented with vaginal bleeding in the first trimester and a viable fetus, the use of maternal plasma total DNA was more informative than maternal plasma fetal DNA in predicting spontaneous abortion. Although it is unlikely that DNA measurements will replace ultrasound in the near future, such a prediction provides an opportunity for potential interventions. We believe that these results are helpful in understanding the mechanisms of early pregnancy loss and open new avenues for fundamental research in this field.

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