Fragmentation of Fetal DNA in Maternal Plasma and Urine

Keiko Koide, Akihiko Sekizawa, Ryu Matsuoka and Takashi Okai

Abstract: The effect of freezing on fetal DNA fragmentation in maternal plasma, and maternal DNA fragmentation in plasma and urine, was investigated. Seven real-time PCR assays were designed to amplify different-sized amplicons targeting the SRY gene. Fragmentation of fetal DNA in maternal plasma was compared between new and four-year-old samples. With the exception of the smallest amplicon of 107 bp, which is more abundant in aged samples, the relative abundance of amplicons in new and old samples is essentially equivalent. To investigate differences in fragmentation of fetal DNA between plasma and urine from pregnant women, three different-sized amplicons were amplified and DNA fragmentation between plasma and urine was compared. Although fetal DNA in urine was not detected using the 107- and 137-bp amplicons of the SRY sequence, fetal DNA using a 63-bp amplicon was detectable in five of seven cases. In conclusion, cell-free fetal DNA in maternal plasma is stable under cryopreservation at -20°C for at least four years. Approximately 60% of fetal DNA in maternal plasma was fragmented to <100 bp and fetal DNA in urine was further fragmented. Maternal urine may be suitable for detection of fetal DNA, although a smaller target size is more important for PCR amplification of fetal DNA in urine than in the analysis of plasma from pregnant women.

Key words: fetal DNA, maternal plasma, maternal urine, DNA diagnosis

Introduction

Cell-free fetal DNA circulates in maternal plasma allowing prenatal determination of fetal gender, rhesus D blood type, and single-gene disorders via analysis of maternal plasma. A previous study of 302 pregnancies between weeks seven and 16 gestation obtained 97.2% sensitivity for fetal gender determination by analyzing maternal plasma for the Y-chromosome-specific gene DYS14. This clearly indicates that cell-free fetal DNA is detectable in plasma from pregnant women after seven weeks gestation. Evaluation of fetal DNA concentrations in various complications of pregnancy is performed by quantification of Y-chromosome specific sequences in women bearing a male fetus. Increased concentrations of fetal DNA have been reported in women with preeclampsia, invasive placenta, hyperemesis gravidarum and fetal aneuploidy.

Fetal DNA in maternal plasma is primarily derived from villous trophoblasts bordering the intervillous spaces, which are filled with maternal blood. As apoptosis of syncitio-
Table 1. Sequences of primers and probe for amplification of SRY gene

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Forward / reverse</th>
<th>Amplicon size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AAA GGC AAC GTC CAG GAT AGA G-3'</td>
<td>Forward</td>
<td></td>
</tr>
<tr>
<td>5'-ATC GCG AGA CCA CAC GAT GAA T-3'</td>
<td>Reverse</td>
<td>63</td>
</tr>
<tr>
<td>5'-TGA GTT TCG CAT TCT GGG ATT-3'</td>
<td>Reverse</td>
<td>107</td>
</tr>
<tr>
<td>5'-CCA CTG GTA TCC CAG CTG CT-3'</td>
<td>Reverse</td>
<td>137</td>
</tr>
<tr>
<td>5'-TGT AAT TTC TGT GCC TCC TGG A-3'</td>
<td>Reverse</td>
<td>193</td>
</tr>
<tr>
<td>5'-ACT TCG CTC CAG AGT ACC GAA-3'</td>
<td>Reverse</td>
<td>313</td>
</tr>
<tr>
<td>5'-ATG TTA CCC GAT TGT CCT ACA GC-3'</td>
<td>Reverse</td>
<td>524</td>
</tr>
<tr>
<td>5'- (FAM) AAG CGA CCC ATG AA (TAMRA) -3'</td>
<td>Probe</td>
<td></td>
</tr>
</tbody>
</table>

trophoblasts has been reported in placental tissue specimens\textsuperscript{15}, fragmented DNA might be released through apoptosis into the maternal circulation. In fact, Chan et al. noted that fetal DNA fragments are shorter than maternal-derived fragments\textsuperscript{16}. Trophoblast damage may be involved in the pathogenesis of preeclampsia, and fetal DNA might represent a molecular marker for evaluating trophoblast damage and monitoring the status of those pregnancies affected by preeclampsia\textsuperscript{6,17}. Fetal DNA in maternal plasma is thus a potentially valuable resource for prenatal DNA diagnosis, and for the evaluation of the occurrence or severity of complications associated with pregnancy.

For clinical purposes, it is very important to be able to evaluate the stability of maternal plasma DNA. The first part of the present study (Study 1) assessed the effect of freezing on fragmentation of fetal DNA in maternal plasma. Fetal DNA is reportedly cleared from maternal blood with a half-life of 16.3 min\textsuperscript{18}. Since fetal DNA might be excreted in urine, such a source could be used for noninvasive prenatal DNA diagnosis. Therefore, the second part of the present study (Study 2) assessed differences in DNA fragmentation between plasma and urine from pregnant women.

Materials and Methods

Study 1

Plasma samples were compared between Group A, in which blood was analyzed within one month of collection, and Group B, in which blood collected in the year 2000 had been preserved at \(-20^\circ\text{C}\) for at least four years. Each group was comprised of ten plasma samples from normal pregnant women bearing a male fetus. The median gestational week at the time of blood collection was 33 weeks (range, 29–37 weeks) in Group A and 32 weeks (range, 25–40 weeks) in Group B. None of the women experienced vaginal bleeding or labor pain. In all cases, gestational age was confirmed at early gestation by crown-rump length measurement.

To evaluate fragmentation of fetal DNA in maternal plasma during freezing, six real-time PCR assays were prepared to amplify different-sized amplicons targeting the SRY gene\textsuperscript{16}. The sequences of the primers and probe are listed in Table 1. To obtain each of the amplicons, the same forward primer was used in each PCR, while the reverse primer was different for each amplicon. The primer sequences and resulting amplicon sizes are given in Table 1.
Study 2

The subject group was comprised of seven pregnant women bearing a male fetus at 25-40 weeks gestation (median, 33 weeks). Samples of peripheral blood and urine were collected from all pregnant women. After extracting fetal DNA from both plasma and urine samples, three different-sized SRY gene amplicons (63, 107 and 137 bp) were amplified, and the fragmentation of fetal DNA in plasma and urine from pregnant women was evaluated. Fragmentation was then compared between urine DNA and plasma DNA.

DNA preparation

Maternal blood samples (7 ml) were collected into tubes containing ethylene diamine tetraacetic acid (EDTA), and plasma was separated by centrifugation at 3000 rpm for 10 minutes within 3 h of collection. Plasma was then transferred into plain polypropylene tubes and centrifuged repeatedly. Supernatants were collected into fresh tubes and stored at −20°C until needed for DNA extraction. Urine samples were collected in plain cups and transferred into EDTA tubes, then centrifuged at 3000 rpm within 1 h of collection. The urine supernatant was transferred into fresh tubes. In study 2, plasma and urine samples were not frozen and the analysis was performed on the day of sampling. DNA was extracted from 1.5 ml of plasma and 30 ml of urine using a QIAamp Blood mini Kit (Qiagen, Hilden, Germany), in accordance with the instructions of the manufacturer, with minor modifications. Total DNA was eluted from the columns with 50 µl of water.

Quantification of fetal DNA

Real-time PCR analysis was performed using a 7900 T Sequence Detection System (PE Applied Biosystem, Foster City, CA). For PCR analysis, 25 µl reaction volumes were used, each containing 400 nM of each amplification primer, 200 nM of dual-labeled probe (FAM, TAMRA), and 12.5 µl of QuantiTeck probe PCR master mix (Qiagen). For amplification, 5 µl of extracted plasma DNA was used. Thermal profiles were performed using an initial denaturation step at 95°C for 15 min, followed by 40 cycles of 15 s at 94°C and 1 min at 60°C. The number of copies of the SRY sequences and of male DNA present in the plasma samples was determined by comparison with a calibration curve of serially diluted, male genomic DNA. A conversion factor of 6.6 pg DNA per cell was used for the expression of the results as copy numbers.

Each sample was analyzed in duplicate, and the mean was used for further concentration calculations. In each case, fetal DNA concentrations from the 107-, 137-, 193-, 313- and 524-bp amplicons were converted to a relative concentration compared to the concentration of the 63-bp amplicon. To confirm the efficiency of PCR amplification in each primer set, genomic male DNA was also amplified.

Strict precautions were taken against contamination and multiple negative controls (water) were included in every analysis. All patients in the study were enrolled at the Department of Obstetrics and Gynecology, Showa University Hospital, Tokyo, Japan. All subjects provided written informed consent to the use of biological specimens for research purposes. Study protocols were approved by the Ethics Committee of the Showa University School of Medicine.
Results

Study 1

Analysis of male genomic DNA revealed that the efficiency of PCR was almost identical among the seven primer sets (Fig. 1). In the analysis of maternal plasma, fetal DNA was detected in all plasma samples. When fetal DNA concentrations were determined using PCR amplification of the 63-bp amplicon, median concentrations in Groups A and B were 166 copies/ml (range, 59–930 copies/ml) and 175 copies/ml (range, 57–896 copies/ml), respectively (not significant, Mann-Whitney U-test). Relative concentrations of fetal DNA compared to the 63-bp amplicon in Group A samples were 53.1% (median; range, 23.3–140%), 42.0% (range, 22.0–71.4%), 9.2% (range, 2.3–578%), 2.0% (range, 0–11.8%) and 0.0% for PCR amplicons of 107 bp, 137 bp, 193 bp, 313 bp and 524 bp, respectively. In Group B, relative concentrations were 70.4% (range, 31.4–122%), 40.9% (range, 22.7–112%), 11.9% (range, 0–20.7%), 2.3% (range, 0–79%) and 1.9% (range, 0–2.5%), respectively (Fig. 1). Almost 60% of circulating fetal DNA was fragmented to <107 bp. Comparison between new and four-year-old samples revealed no significant difference in each amplicon size (Mann-Whitney U-test).

Study 2

Fetal DNA in maternal plasma was detectable for all amplicon sizes, and relative concentrations for 107- and 137-bp amplicons compared to 63 bp were 71.9% (range, 22.7–107%) and 33.3% (range, 4.3–127%), respectively. Although fetal DNA in urine was not detected for the 107- and 137-bp amplicons of the SRY sequence (Fig. 2), fetal DNA in urine was detectable using a 63-bp amplicon in five of seven cases, although concentrations were extremely low (mean: 18.3 copies/l).

Discussion

Circulating fetal DNA in maternal plasma can be used for fetal genetic testing and evaluation of complications of pregnancy, and therefore the stability of fetal DNA is very
important for these clinical applications. Angert et al have reported that fetal cell-free plasma DNA concentrations in maternal blood are stable 24 h after collection. However, plasma DNA concentrations are thought to gradually decrease under long-term cryopreservation, and therefore plasma DNA would be expected to become increasingly fragmented in storage. To confirm this hypothesis, the long-term effect of cryopreservation on fragment size of fetal DNA in maternal plasma was assessed. The present study found no differences in the fragmentation of plasma fetal DNA between time intervals of less than one month and <4 years. This result indicates that plasma DNA is stable at -20°C for more than 4 years. The majority of fetal DNA in plasma from pregnant women appears to come from apoptosis of villous trophoblasts. During apoptosis, DNA is thought to fragment to the size of nucleosomal DNA. Fragmented DNA is thus released into maternal blood and may further fragment in the maternal circulation. The present study shows that detection of longer fetal DNA fragments in maternal plasma is more difficult since approximately 60% of fetal DNA is fragmented to <107 bp. Again, the results indicate that a smaller target size is better for amplifying fetal sequences in maternal plasma. Lastly, the study reveals that cryopreservation does not affect fragmentation of plasma DNA.

Compared to plasma DNA, fetal DNA in urine was much shorter. Although the mechanism of fetal DNA excretion into urine is unclear, DNA fragmented to a small size in maternal blood may facilitate excretion. Li et al analyzed fetal DNA in 800 μL of maternal urine using 133-bp amplicons of the SRY gene, and concluded that urinary DNA represents an inadequate source for noninvasive prenatal DNA diagnosis. However, the present study extracted DNA from a larger volume of maternal urine and targeted smaller amplicon-sized DNA for detecting fetal SRY sequence. This allowed detection of fetal DNA in 70% of urine samples. Smaller target size is thus more important for detecting fetal DNA in urine than in maternal plasma.

In summary, this is the first report to find that cell-free fetal DNA in plasma from pregnant women is stable under cryopreservation at -20°C for at least 4 years. Plasma DNA appears stable to freezing and concentrations appear suitable for the development of noninvasive screening methods against complications of pregnancy. Furthermore, the present...
results suggest that prenatal diagnosis from maternal urine may be possible if smaller sized targets are used for PCR amplification.

References


22) Sekizawa A, Yokokawa K, Sugito Y, Iwasaki M, Yukimoto Y, Ichizuka K, Saito H and Okai T: Evaluation of


[Received January 28, 2005 : Accepted February 4, 2005]