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Cell-free fetal DNA levels in maternal plasma after elective first-trimester termination of pregnancy

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Objective: To determine if first-trimester elective termination of pregnancy affects cell-free fetal DNA (fDNA) levels in maternal plasma.

Design: Prospective cohort study.

Setting: Clinical and academic research centers.

Patient(s): One hundred thirty-four women who underwent first-trimester elective termination procedures.

Intervention(s): None.

Main Outcome Measure(s): Real-time polymerase chain reaction (PCR) amplification and measurement of *DYS1*, a Y-chromosome sequence, was used as a marker of fDNA.

Result(s): We detected fDNA in pretermination samples from 27 out of 71 patients in the surgical arm, and 29 out of 63 patients in the medical arm. Based on confirmation of male gender in placental tissue, the sensitivity of fDNA detection is 92.6%. We detected fDNA as early as 32 days of gestation, which increased 4.2 genome equivalents/mL/week. In the surgical arm, the mean level of posttermination fDNA, adjusted for the clearance of fDNA in maternal blood, was higher than projected based on an expected increase with gestational age. In the medical arm, six patients had increased fDNA levels up to 11 days following termination.

Conclusion(s): We found that fDNA can be reliably quantified in the early first trimester; fDNA elevation that occurs shortly after surgical termination may reflect fetomaternal hemorrhage or destruction of trophoblastic villi. Continued elevation of fDNA for several days may occur following medical termination. (Fertil Steril® 2004;81:638–44. ©2004 by American Society for Reproductive Medicine.)

Key Words: Cell-free fetal DNA, therapeutic abortion, fetomaternal hemorrhage

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Of the estimated 6 million pregnancies that occur annually in the United States, approximately 1.5 million, or 25%, are electively terminated. More than 90% of these terminations are performed in the first trimester (1). Termination of pregnancy (TOP) has been known to cause fetomaternal hemorrhage (FMH) (2). The accurate detection of FMH is of particular importance in RhD-negative pregnant women carrying RhD-positive fetuses, where prevention of maternal sensitization by RhD immune globulin is critical (3–5).

The Kleihauer-Betke (KB) test has traditionally been used to demonstrate FMH (6). This acid elution method measures the volume of FMH by determining the number of fetal hemoglobin (HbF) positive red blood cells in the maternal circulation (7). However, because adult cells are known to express HbF (8), the interpretation of this test is problematic, and its accuracy is further confounded by its subjective nature (9). Indeed, the incidence of FMH after first trimester TOP reported by the KB test varies from 0 to 11% (2, 10, 11).

Alternative methods to determine FMH are currently being developed. For example, maternal serum alpha-fetoprotein (MSAFP) is increased after the events that aggravate FMH (12). However, several factors affect the MSAFP level (13) and thus it may not be useful in validating FMH. Flow cytometry using specific fetal cell markers has also been

used to demonstrate FMH (14, 15), although this technique requires an expensive instrument and a well-trained operator, both of which are not always available. Using quantitative polymerase chain reaction (PCR) amplification of a Y-chromosome sequence, we previously showed that there is significant FMH of as many as 500,000 nucleated cells following TOP (16).

Since the discovery of the existence of cell-free fetal DNA (fDNA) in the maternal circulation (17), a number of clinical applications involving the use of this DNA, including prenatal diagnostic testing and screening, have been proposed (18). Lau et al. (19) demonstrated an increase of fDNA in maternal plasma from women undergoing near-term, external cephalic version. These investigators suggested that fDNA is a sensitive marker for the assessment of subclinical FMH.

During the first trimester, fDNA is detectable and quantifiable (20, 21), and may have an advantage over the KB test for the measurement of FMH because of its specificity as a fetal genetic marker. Indeed, use of the KB test has provided conflicting reports as to the occurrence of FMH following first-trimester TOP (2, 11, 22). The accuracy of fDNA in the quantification of FMH has not been demonstrated, as there is only a small amount of fDNA detectable in the maternal plasma during the first trimester when elective terminations are typically performed.

In our study, we examined the level of fDNA by gestational age in women who underwent either surgical or medical TOP in the first trimester of pregnancy to evaluate the feasibility of using fDNA for FMH determination. The Y-chromosome–specific sequence *DYS1* was used to distinguish and quantify male DNA of presumed fetal origin in maternal plasma.

MATERIALS AND METHODS

This study was approved by the Institutional Review Boards at Tufts-New England Medical Center (Tufts-NEMC) and Boston University Medical Center (BUMC). Paired blood samples were obtained before and after elective first-trimester TOP from 134 patients of the Department of Obstetrics and Gynecology at BUMC. Gestational ages were established by ultrasonography and were expressed as menstrual age. If the gestational age was beyond 8 weeks, only surgical termination was offered. If the pregnancy was less than 8 weeks of gestation, the patient could opt for either medical or surgical termination after receiving counseling. The pretermination blood samples were drawn on the day the patients enrolled in the study. The samples were centrifuged at 800 $\times$ *g*, after which plasma was collected, labeled, and frozen at -80°C until analysis. The samples were coded by an investigator not involved in the DNA extraction and amplification process, and for a subsequent blinded analysis.

Surgical Termination Procedure

Patients obtaining a surgical termination were scheduled to undergo the procedure at BUMC approximately 1 week following recruitment. In all cases, the manual vacuum aspiration method was used. After the patient received adequate analgesia, her cervix was dilated using Pratt dilators as necessary. Sharp curettage was performed at the discretion of the operator. Posttermination blood samples were drawn shortly after the procedure was completed and processed identical to the pretermination samples. Times of suction initiation and blood drawing were recorded. Products of conception (POCs) were collected and placed in normal saline solution and frozen for subsequent fetal gender confirmation.

Medical Termination Procedure

Patients undergoing medical termination had 800 μg of misoprostol inserted in the posterior fornix. The patients were instructed to self-administer another dose of 800 μg at home if the conceptus was not expelled within 24 hours (day 2). They were then instructed to return to the hospital on the day following the second dose (day 3), regardless of whether the conceptus was expelled. Follow-up imaging of the uterine cavity by ultrasonography was performed in all cases. The second blood sample was then drawn on day 3. If the patient did not expel the conceptus by day 3, she was not included in the analysis. In some cases, the patient did not return to the clinic until several days after termination, and the second blood sample was drawn at that time. Because POCs could not be collected from patients undergoing medical termination, fetal gender could not be confirmed.

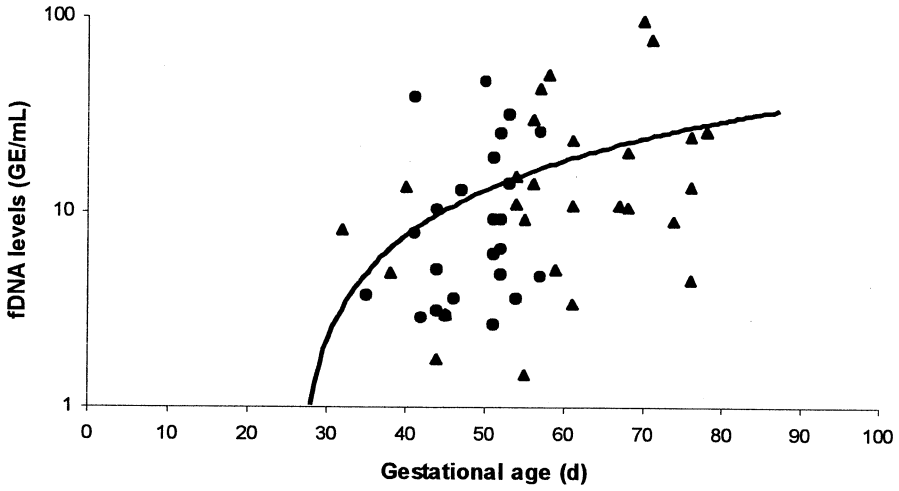
DNA Extraction and Real-Time PCR

We centrifuged 900 μL from each plasma sample at 11,500 $\times$ *g* for 10 minutes to remove any residual cells. Then 800 μL of the supernatant was used for DNA extraction via the QIAamp blood kit (QIAGEN, Inc, Chatsworth, CA) using the Blood and Body Fluid Spin Protocol as described by the manufacturer. For gender determination, we obtained 500 μL of the saline solution in which the POC had been stored, as some of the DNA diffuses into the solution. This was centrifuged at 11,500 $\times$ *g* for 10 minutes, with 400 μL of the supernatant used for DNA extraction. In all cases, the extracted DNA was eluted into a final volume of 50 μL.

The concentration of fDNA in maternal plasma and the POC solution was determined by real-time PCR amplification using a Perkin-Elmer Applied Biosystems 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The *DYS1* sequence on the Y chromosome was used in this study to detect and quantify male DNA. The *DYS1* primers (forward: 5′-TCCTGCTTATCCAAATTCACCAT-3′, reverse: 5′-ACTTCCCTCTGACATTACCTGATAATTG-3′) are derived from the Y-chromosome–specific sequence p49a (23). The fluorogenic *DYS1* probe (5′-FAM-AAGTCGCCACTG-GATATCAGTTCCCTTGT-TAMRA-3′) combines both a

FIGURE 1

Maternal plasma fDNA levels. The solid trendline represents the increase in fDNA with gestation based on pretermination levels of fDNA, which is linear in arithmetic scale (4.2 genome equivalents [GE]/mL/week) but presented in logarithmic scale for clarity of data presentation. ▲ = Pretermination, surgical arm. ● = Pretermination, medical arm.



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fluorescent reporter dye (6-carboxyfluorescein, FAM) with a second fluorescent quencher dye (6-carboxytetramethylrhodamine, TAMRA).

During PCR extension by *Taq* polymerase, the reporter dye is cleaved from the probe and quencher dye, thus incrementally increasing fluorescent emission at 518 nm. The fluorescent emission was continuously measured during the DNA amplification process and recorded by computer using Applied Biosystems Sequence Detection System software, version 1.7. The PCR amplification proceeded in a 50 μL volume using 5 μL of DNA extracted from plasma. Reaction components included 100 nmol of each *DYS1* primer, 50 nmol of *DYS1* probe, 20 μL of Universal MasterMix (Applied Biosystems) containing 200 μmol each of dATP, dCTP, and dGTP, 400 μmol dUTP, 5.0 mmol $MgCl_2$, 8% glycerol, and 1X Taqman Buffer A. Thermal cycling was initiated with a 2-minute incubation at 50°C, followed by an initial denaturation phase at 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Each sample was analyzed in triplicate with the mean value used for further calculations. A standard curve of titrated, purified male DNA was run simultaneously with the samples. For all samples, the β-globin gene was amplified to estimate the total amount of DNA in the sample as previously described (24).

RESULTS

Seventy-one and 63 paired blood samples were obtained from the surgical and medical termination arms of this study, respectively. At the time of enrollment, mean gestational ages of the patients in these two arms were 58 and 45 days, respectively. Male DNA was detected in maternal plasma as early as 32 days of gestation, and was consistently detected after 52 days of gestation. The linear incremental slope of the pretermination fDNA was 4.2 GE/mL per week (0.6 GE/mL per day) with an X axis intercept at 29 days of gestation, as demonstrated in Figure 1.

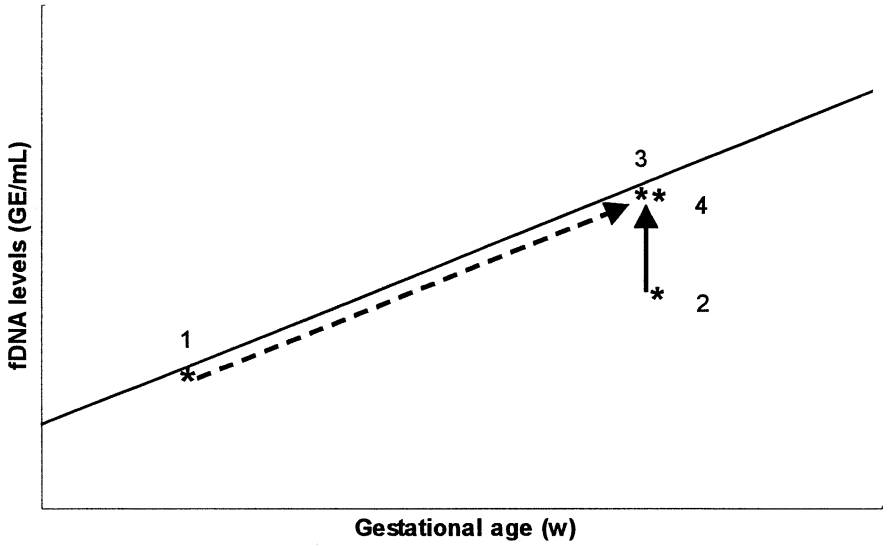
In the surgical arm, 29 fetuses were identified as male by analysis of POCs; 27 of these patients had detectable male DNA in their plasma, resulting in a 92.6% sensitivity of male fetus identification. One false-positive result was found in a pretermination blood sample; the analysis of POCs showed the fetus was female and the posttermination sample from this patient had no Y signal detected. The direct comparison of pretermination and posttermination fDNA levels showed that 18 of 27 patients (66.7%) had increased fDNA after the procedure.

Due to the approximately 1-week time lapse between the enrollment and the surgical termination procedure, we created a statistical model to correct for this confounder, as demonstrated in Figure 2. Projected levels of posttermination fDNA in the surgical arm were calculated based on the pretermination levels and the estimated linear increase in fDNA with gestational age.

In addition, owing to the variation in time lapse between vacuum aspiration and subsequent posttermination blood draw (16 to 79 minutes) and the rapid clearance of fDNA in maternal circulation, adjusted levels of posttermination

FIGURE 2

Schematic diagram to illustrate calculations of posttermination fDNA in plasma samples from surgical termination patients. The continuous line shows the linear increase in fDNA with gestation based on pretermination levels of fDNA (4.2 genome equivalents [GE]/mL/week). For one woman, 1 represents the pretermination level of fDNA, 2 represents the observed posttermination level, 3 represents the projected posttermination level based on the expected increase with gestation (*dashed arrow*), and 4 represents the adjusted posttermination level based on the clearance of DNA in maternal plasma (16.3 minute half-life) (*solid arrow*).



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fDNA were calculated based on the measured posttermination levels and the mean half-life of fDNA in maternal blood (16.3 minutes), as reported by Lo et al. (25). We then used a series of paired *t* tests to assess for statistically significant differences between the projected and adjusted levels of posttermination fDNA. We also used the *t* test to assess for differences in fDNA between women who did and did not have sharp curettage.

After adjustment for the expected half-life of fDNA in maternal plasma, 28 of 29 patients (96.6%) had a higher mean posttermination fDNA level compared with the projected mean fDNA level ($P=.0017$). Twelve patients had sharp uterine curettage after vacuum aspiration, which did not appear to alter their levels of fDNA as compared with those who did not have sharp curettage ($P=.8020$). Figure 3 depicts the adjusted posttermination fDNA levels compared with the trendline of the pretermination fDNA at the comparable gestational age.

For the medical arm, the posttermination blood samples were drawn at least 2 days and up to 11 days after the pregnancy was terminated. Because of the short half-life of fDNA and the long delay between the expulsion of the conceptus and the posttermination blood draw, we did not calculate the projected fDNA or adjusted posttermination fDNA for this arm. Instead, we compared the pretermination and observed posttermination fDNA levels in this arm directly, and determined the proportion of patients who had increased levels of fDNA after pregnancy termination. We then used the paired *t* test to assess for statistically significant differences between the pretermination and posttermination levels of fDNA.

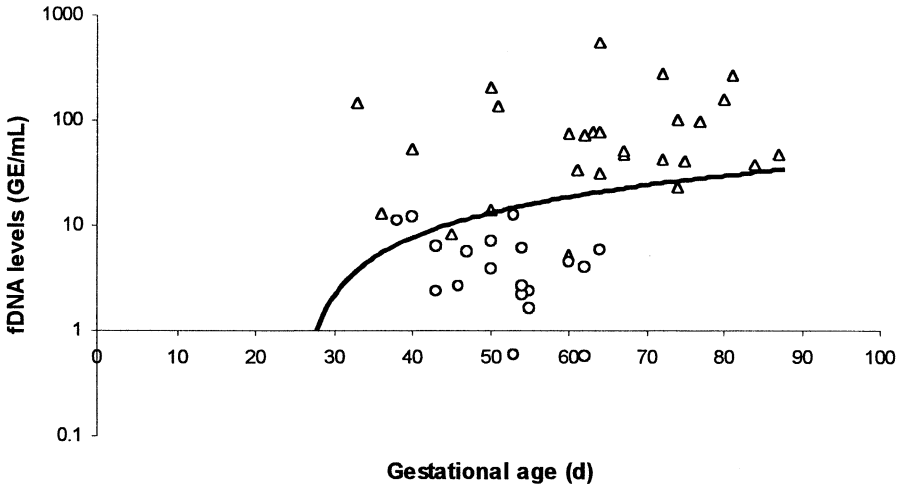
In the medical arm, 29 patients had male DNA detected in their plasma before termination and 23 of those had decreased fDNA after 2 days posttermination. In these patients, the mean posttermination level of fDNA was statistically significantly lower than the mean pretermination level ($P = .0012$), as shown in Figure 3. Six patients had elevated posttermination fDNA compared with their pretermination levels: four at 2 days and one each at 6 and 11 days posttermination, as shown in Figure 4. Two samples had pretermination fDNA levels of 0; therefore, the percentage change cannot be calculated. In all patients who underwent medical termination, none had remarkably prolonged vaginal bleeding or other clinical evidence of retained POCs, and intrauterine gestational sacs were not observed by ultrasonographic studies.

DISCUSSION

In this study, fDNA in maternal plasma was detected as early as 32 days of gestation and increased by 4.2 GE/mL per

FIGURE 3

Maternal plasma fDNA levels. The open triangles represent adjusted posttermination fDNA levels from the surgical arm based on the clearance of DNA in maternal plasma (16.3-minute half-life); the majority of the points are above the trendline representing the pretermination fDNA levels, shown in the logarithmic scale for clarity of presentation. The open circles represent posttermination fDNA levels from the medical arm; the majority of these points are below the pretermination trendline. △ = Adjusted posttermination, surgical arm. ○ = Postermination, medical arm.



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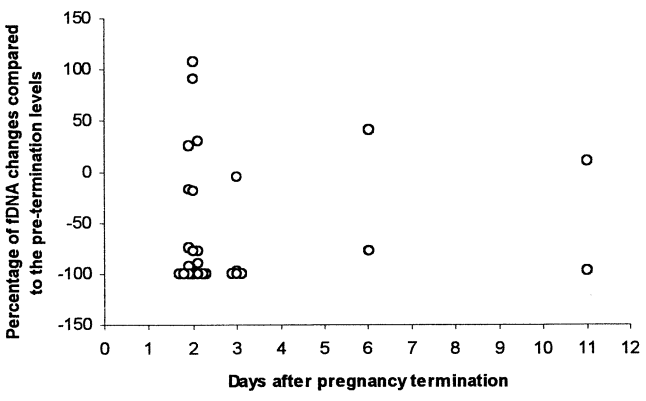
week. By extrapolation, fDNA is projected to first appear in the maternal circulation as early as 29 days of gestation. The sensitivity of first-trimester fetal gender identification in this study was 92.6%, which is comparable with previous reports (20, 21). These investigators have also shown that as gestational age advances, the sensitivity of gender detection also increases.

FIGURE 4

Percentage change of posttermination levels of fDNA compared with pretermination levels in plasma from medical termination patients. Overlapping data points at days 2 and 3 on the X axis are offset for clarity of presentation. Note that an increase is seen in six patients, and a decrease is seen for all others.



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In our study, we demonstrated an increase of fDNA in maternal plasma over pretermination levels after first-trimester termination of pregnancy by vacuum aspiration, which may reflect FMH or villus destruction resulting from the procedure. In addition, fDNA was found to be elevated days after medical termination, which may be explained by low-level retention of trophoblastic villi that shed fetal genetic material into the maternal circulation.

We demonstrated that the unadjusted fDNA levels in maternal plasma increased after surgical termination in 66.7% of the patients, which is comparable with that reported by Lau et al. (19), who measured fDNA in maternal plasma after external cephalic version near term. After the adjustment of the posttermination fDNA measurements based on the expected clearance of fDNA in maternal plasma (25), the occurrence was even more striking (96.6% of patients). Due to the short half-life of fDNA in maternal plasma and the brief period between surgical termination and blood draw, the adjustment for this clearance in the calculation of fDNA levels is simple and may be important for the accurate determination of the posttermination level of fDNA. In addition, this rapid clearance means it is unlikely that fDNA from previous pregnancies will be present.

Note that an average half-life of 16.3 minutes was used in the adjustment calculations as per Lo et al. (25). A recent

study from this same group suggests that the clearance of fDNA in postpartum control women may be as long as 28 minutes (26). This longer clearance time would affect the magnitude of increase in fDNA levels after surgical termination, but the reported variation in half-life suggests that additional investigation is required to apply this factor more reliably to studies of fDNA persistence.

Most patients in the medical arm of our study had decreased levels of fDNA in their plasma 2 or more days after the POCs were expelled. In some patients, however, fDNA was found to be elevated days after medical termination, although the clinical importance of this is unclear. These patients had no evidence of gross retained POCs either from their clinical history or ultrasonographic study. This elevation could be explained by subclinical retention of trophoblastic villi that continued to shed fetal genetic material into the maternal circulation. This suggests that fDNA may be an additional indicator to confirm the completeness of pregnancy termination, particularly in patients who have inconclusive clinical or ultrasonographic findings.

During pregnancy, FMH is relatively common, although massive FMH associated with catastrophic events occurs very rarely (3, 4). In normal pregnancies, apoptosis of trophoblastic cells is thought to be a major source of fDNA in maternal circulation. The source of fDNA after surgical termination of pregnancy may be from direct fetal hemorrhage as a result of disturbance of the fetomaternal interface during the procedure. Procedure-related placental injury could cause trophoblastic cells to shed DNA into the circulation; this might also contribute to the postprocedural elevation of fDNA. However, we found that patients who underwent sharp curettage after vacuum aspiration did not have a higher mean level of fDNA than those who did not, suggesting this component of surgical termination may not increase FMH.

The quantification of fDNA is currently performed by the detection of Y-chromosome sequences, which is applicable only to pregnant women carrying male fetuses. There is no evidence that the trafficking of female fetal DNA is different from that of male fetuses. However, it may be possible to accurately measure female fDNA by exploiting differences between maternal and fetal DNA such as differential DNA methylation, an epigenetic marker currently being studied (27).

Our study showed that fDNA can reliably be quantified in early first trimester, and that fDNA is elevated shortly after surgical TOP, which may reflect either FMH or destruction of trophoblastic villi. The recent demonstration of placental-specific messenger RNA in the circulation of pregnant women (28) may eventually allow us to more definitively determine the tissue source of the fetal nucleic acids in the circulation of pregnant women. In addition, our findings suggest that fDNA quantification may be more sensitive than the KB test for the measurement of FMH. Future studies should include the comparison of fDNA analysis to the KB test for the detection and accurate quantification of FMH, as the rapid, reliable, and objective method of measuring fDNA presented here may complement the more subjective method currently in clinical use.

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