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Circulating cell-free DNA levels increase variably following chorionic villus sampling

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Abstract

Objective—Cell-free fetal DNA (cffDNA) in maternal plasma results from degradation of fetal and/or placental cells. Our objective was to determine if chorionic villus sampling (CVS) causes increased release of fetal and/or maternal DNA.

Methods—Fifty-two pregnant women were recruited prior to CVS, performed for clinical indications, at 10 5/7 to 13 2/7 weeks. Maternal blood was collected before and within 15 minutes after CVS. cffDNA was extracted from plasma. Real-time polymerase chain reaction (PCR) amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and the Y chromosome sequence *DYS1* were used as measures of total and fetal DNA, respectively. All samples were analyzed in triplicate without knowledge of fetal gender.

Results—Sensitivity of *DYS1* detection in male fetuses was 100% (n=30); specificity in female fetuses was 100% (n=22). While a majority of women had >50% post-procedure increases in both fetal and total DNA, some showed post-procedure decreases. However, overall median proportional increases were not statistically significant. Gestational age (GA), placental location, and individual CVS operator did not correlate with changes in DNA levels.

Conclusions—While there were no statistically significant overall changes in DNA levels after CVS, as-yet undiscovered variables may influence the extent of post-procedure release of cell-free DNA in the circulation of pregnant women.

Keywords

CVS; cell-free DNA; prenatal diagnosis

Introduction

Current methods of invasive prenatal diagnosis include chorionic villus sampling (CVS) and amniocentesis. However, these methods are expensive, require specific expertise, and incur a small (0.5 to 1%) but devastating possibility of pregnancy loss. Because of these limitations,

noninvasive diagnostic methods are actively being pursued, such as the measurement of cell-free fetal (cff) DNA in maternal circulation (Lo *et al.*, 1997; Bianchi *et al.*, 1997; Lo *et al.*, 1998; Guibert *et al.*, 2003; Chiu *et al.*, 2004).

One potential clinical application for the measurement of cell-free fetal DNA is the quantification of fetomaternal hemorrhage after amniocentesis or CVS. Currently, the Kleihauer Betke (KB) test is the most commonly used test to quantify fetomaternal hemorrhage (Lachman *et al.*, 1977; Lenke *et al.*, 1985). This acid elution test identifies fetal cells in the maternal circulation by the relative resistance of cells containing hemoglobin F to acid treatment. While the KB test is sensitive, its accuracy is low because of multiple sources of error. These include variations in the thickness of blood films, the number of red blood cells (RBCs) in a low power microscope field, the fact that some fetal cells do not stain, and the difficulty of classifying cells of intermediate staining. In addition, the KB test is unable to differentiate between fetal RBCs and adult cells that contain hemoglobin F that may be present in women with thalassemias or sickle cell disease (Johnson *et al.*, 1995).

The KB test has reduced accuracy when fetomaternal hemorrhage is greater than 4 mL (Johnson *et al.*, 1995). After amniocentesis, up to 20% of patients have evidence of fetomaternal hemorrhage by the KB test or increases in alpha fetoprotein levels (Hay *et al.*, 1979; Dallaire *et al.*, 1980; MacLennan *et al.*, 1994). Samura and colleagues (2003) measured the amount of cff DNA in maternal circulation after amniocentesis and found that it increased significantly in 79% of post-procedure maternal samples.

For CVS, Pelikan and colleagues performed a study investigating whether this procedure causes proportional increases in alpha-fetoprotein (AFP) and fetal RBCs in maternal blood (Pelikan *et al.*, 2006). While AFP increased post-procedure, fetal RBCs did not. Therefore they suggested that the risk of maternal immunization following CVS is small but not negligible.

The results of these studies suggested to us that further assessment of CVS using a sensitive technique to measure the transfer of fetal cellular material into the maternal circulation was warranted. The purpose of this study was to quantify the amount of cff DNA in maternal blood before and immediately after CVS, and to determine specific factors that might affect levels and infer placental damage as a result of the procedure.

Methods

This study was approved by the Institutional Review Board at Tufts Medical Center. Fifty-two pregnant women were recruited prior to clinically indicated CVS at 10 5/7 to 13 2/7 weeks. Written consent was obtained prior to drawing blood. Using ultrasound guidance, transabdominal CVS was performed once (i.e. a single insertion) with a 20 gauge needle by different perinatologists. Multiple needle passes were performed to obtain chorionic villi using a single insertion site. Maternal peripheral venous blood was collected in an EDTA tube before and within 15 minutes after CVS. Plasma was obtained by centrifugation of whole blood at 1,600 rpm for 10 minutes at 4°C. Supernatant was removed and then centrifuged again at 14,000 rpm for 10 minutes. The supernatant was once again collected, stored in fresh tubes, and stored at -80°C.

DNA was extracted from 400 μ L of plasma using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the blood and body fluid protocol. DNA was eluted in 50 μ L of the elution buffer. Real time quantitative PCR amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a marker of total DNA was performed as previously described (Johnson *et al.*, 2004). PCR amplification of *DYS1*, a Y chromosome sequence, was used as a marker for male fetuses and was performed as previously described (Watanagana *et al.*, 2004a). All samples were analyzed in triplicate. Analysis was

blinded, and a female investigator processed and handled all samples so that there was no risk of contaminating samples with male DNA. Fetal gender was confirmed by obtaining the karyotype of the chorionic villi.

Statistical assessment was performed using analysis of covariance (ANCOVA) of log transformed absolute DNA differences between paired pre- and post-procedure samples, as well as the proportional change between pre-procedure (baseline) and post-procedure levels with adjustment for baseline fetal and total DNA levels, gestational age (GA), sample weight, placental location (i.e. anterior vs. posterior), and individual operator experience. All statistical analyses were performed using SAS/STAT software (SAS Institute, Inc., Cary, NC). Statistical significance was assigned when $p < 0.05$.

Results

Twenty-two fetuses were female; 30 were male. Four male fetuses were aneuploid, including one case each of trisomies 13, 18, and 21 and one case of mosaicism (92, XYY[7]/46, XY [13]). The other 26 male fetuses were euploid. Due to the small number of aneuploid cases, these fetuses were not analyzed separately from the euploid cases. *DYS1* amplification was undetectable in all female fetuses (specificity = 100%). The sensitivity of *DYS1* detection when the fetus was male was 100%. In male fetuses, the mean concentration of the *DYS1* sequence pre CVS was 13.1 genome equivalents (GE)/mL (range: 1.2 to 122 GE/mL) and 12.9 GE/mL (range: 1.1 to 67.3 GE/mL) post CVS (Table 1).

Overall, there were no statistically significant differences in absolute quantities of total (*GAPDH*) DNA pre-and post-procedure, nor were there statistically significant effects of any of the variables that were studied (Table 1). Similarly, there were no overall statistically significant differences in the level of fetal (*DYS1*) DNA levels between paired samples (Table 1). However, there were differences in the number of patients with relative increases or decreases in cell-free DNA relative to baseline (i.e. pre-procedure) levels following CVS, as well as differences in the magnitude of these changes. For fetal DNA, there were 20 (67%) women who had an increase in *DYS1* following the procedure, compared to 10 (33%) who had a decrease. The greatest percentage increase in fetal DNA was 907.1%, while the greatest decrease was 77.4%. The overall median percent change of *DYS1* was 26.6%. There were no obvious trends among the aneuploidy cases that were analyzed. In addition, while there were only four women who showed a decrease of greater than 50% in fetal DNA, 11 women had increases of greater than 50%.

For total DNA, the results with respect to percentage change were similar to those of fetal DNA. There were 18 (60%) women who exhibited an increase in *GAPDH* following the procedure, compared to 12 (40%) who showed a decrease. The greatest percentage increase in total DNA was 368.3%, while the greatest decrease was 72.1%. The overall median percent change for *GAPDH* was 10.3%. There were three women who showed a decrease of greater than 50% in total DNA, while seven women had an increase of greater than 50%. Interestingly, the three women who had the greatest percentage increase in fetal DNA also had the greatest percentage increase in total DNA.

There was a trend toward an inverse association between post-procedure fetal DNA levels and gestational age ($p=0.053$) resulting, on average, in 23% lower post-procedure levels for each additional week of gestation after the adjustment for pre-procedure levels. No association of sample weight, placental location (i.e. anterior vs. posterior), or individual operator experience with changes in DNA levels with regard to the procedure were detected.

Discussion

Circulating cell-free DNA levels variably increase following CVS. While our results do not demonstrate a consistent, statistically significant overall increase in all women, more women have increased than decreased fetal and total DNA levels following the procedure. The variables analyzed in this study (gestational age, placental location, and individual operator technique and experience) did not predict changes in DNA levels. These results suggest either that as-yet undiscovered variables may influence the extent of post-procedure DNA release, or that the study did not have sufficient power (e.g. study sample size) to achieve statistical significance. Because of these results, cell-free DNA measurement is not likely to be a useful alternative to the currently used Kleihauer Betke (KB) test to quantify fetomaternal hemorrhage.

This is the first study to examine cell-free DNA levels before and immediately after CVS. The results, however, are similar to prior studies of cell-free DNA levels before and immediately after amniocentesis (Samura *et al.*, 2003) and elective termination of pregnancy (Wataganara *et al.*, 2004c; 2005). Interestingly, in both the current and past studies, some women showed a decrease in cell-free DNA levels after the procedure. Samura and colleagues (2003) found that 21% of the study population had a decrease in the amount of fetal DNA following amniocentesis; we found that 33% of women showed a similar decrease following CVS. This decrease is most likely due to chance variation in the levels of cell-free DNA before and after the procedure. Samura *et al.* hypothesized that this decrease was related to uterine contraction after the procedure, which delayed the release of fetal DNA. However, other studies have shown that levels of cff DNA have been noted to rise in preterm labor (Shimada *et al.*, 2004; Lo *et al.*, 1998; Leung *et al.*, 1998), suggesting that contractions increase the levels of cell-free DNA. Thus, no firm conclusions regarding uterine contractions and levels of cell-free DNA can be made. Furthermore, fetal DNA that is transferred into the maternal circulation following an iatrogenic breach of the fetomaternal placental barrier may not be protected in membrane-bound vesicles (apoptotic bodies), as it is normally under physiologic conditions (Bischoff *et al.*, 2005). Thus, the DNA released as a result of the procedure may be more vulnerable to destruction (Wataganara *et al.*, 2004c).

Since a subset of women showed a dramatic percentage increase in fetal and to a slightly lesser extent total DNA, further assessment of the effect of CVS on levels of cff DNA in the maternal circulation is warranted. This could be achieved by enrolling a larger number of women and by comparing amounts of cff DNA with timing of post-procedure blood draw to further characterize the dynamics of the release and clearance of cff DNA into and from the maternal circulation, as we have done with elective termination of pregnancy (Wataganara *et al.*, 2004b,c; 2005). Body mass index could also be taken into account, as there is an inverse correlation between maternal obesity and cff DNA levels (Wataganara *et al.*, 2004d). In the present study, smoking status was not recorded; this affects total DNA levels (Urato *et al.*, 2008). Also, it is important to investigate whether the magnitude and direction of change in fetal and total DNA levels pre- and post-CVS may predict a pregnancy outcome.

Conclusion

In summary, CVS does not lead to a statistically significant overall increase in fetal or total cell-free DNA levels post-procedure. The procedure does, however, appear to affect the release of cell-free DNA in a subset of pregnant women. Our results provide further support that measurements of cell-free DNA in maternal plasma may serve as an effective indicator of both fetal and maternal cellular damage following invasive prenatal diagnostic procedures.

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Table 1
Effects of variables on cell-free DNA quantities in pre- and post-CVS maternal plasma samples

Comparison	Marker	N	Median (interquartile range)			p values ¹
			Pre, GE/mL	Post, GE/mL	(Post-Pre)/Pre, %	
Overall	<i>DYSI</i>	30	7.2 (3.2,13.6)	9.4 (4.3,16.1)	27 (-17,91)	(0.24 - 0.10) ²
Overall	<i>GAPDH</i>	30	150 (80,224)	123 (89,220)	10 (-21,38)	(0.74) ²
Placental location: Anterior	<i>DYSI</i>	20	5.6 (2.4,13.1)	8.1 (3.5,19.9)	39 (-12,96)	0.81
Placental location: Posterior	<i>DYSI</i>	10	7.9 (6.1,21.4)	9.9 (6.6,14.5)	12 (-44,66)	
Placental location: Anterior	<i>GAPDH</i>	20	150 (87,221)	123 (99,222)	16 (-21,58)	0.88
Placental location: Posterior	<i>GAPDH</i>	10	126 (51,242)	141 (86,214)	4 (-15,22)	
Gestational age > 12 weeks	<i>DYSI</i>	13	4.9 (1.4,17.5)	4.8 (3.5,10.4)	35 (-35,77)	0.14 (0.053) ³
Gestational age ≤ 12 weeks	<i>DYSI</i>	17	8.4 (4.2,12.7)	10.1 (7.1,16.5)	18 (-15,91)	
Gestational age > 12 weeks	<i>GAPDH</i>	13	155 (87,224)	122 (103,203)	1 (-23,18)	0.30
Gestational age ≤ 12 weeks	<i>GAPDH</i>	17	118 (80,196)	135 (89,221)	26 (-12,64)	
Sample weight > 40 g	<i>DYSI</i>	12	8.3 (5.0,13.5)	9.4 (4.0,14.3)	-1 (-40,27)	0.28
Sample weight ≤ 40 g	<i>DYSI</i>	18	5.5 (1.9,13.6)	9.1 (4.8,16.1)	52 (9,147)	
Sample weight > 40 g	<i>GAPDH</i>	12	175 (112,221)	129 (87,211)	-5 (-35,14)	0.13
Sample weight ≤ 40 g	<i>GAPDH</i>	18	103 (53,242)	123 (103,224)	24 (-12,64)	
Operator experience < 1yr	<i>DYSI</i>	6	5.2 (1.4,14.4)	9.3 (3.5,23.4)	43 (-9,151)	0.61
Operator experience > 1yr	<i>DYSI</i>	24	7.7 (3.3,13.1)	9.4 (4.6,15.3)	27 (-40, 79)	
Operator experience < 1yr	<i>GAPDH</i>	6	81 (53,155)	100 (80,224)	21 (13,51)	0.86
Operator experience > 1yr	<i>GAPDH</i>	24	158 (87,230)	129 (106,219)	3 (-21,36)	
Operator ID: SC ⁴	<i>DYSI</i>	15	7.1 (3.2,21.4)	8.2 (3.7,14.5)	35 (-56,67)	0.30
Operator ID: all others	<i>DYSI</i>	15	8.1 (1.9,13.6)	9.6 (4.3,25.9)	18 (-15,147)	
Operator ID: SC	<i>GAPDH</i>	15	154 (86,237)	122 (103,219)	-12 (-48,64)	0.27

Comparison	Marker	N	Median (interquartile range)			p values ¹
			Pre, GE/mL	Post, GE/mL	(Post-Pre)/Pre, %	
Operator ID: all others	<i>GAPDH</i>	15	119 (74,201)	135 (86,224)	20 (1,38)	

Median pre- and post-procedure cell-free fetal and total DNA quantities were calculated separately for each category within each variable GE: genome equivalents

¹ p values from linear regression model of log-transformed post-procedure levels adjusted for pre-procedure levels unless specified otherwise

² p values from Wilcoxon signed rank test

³ Statistical significance when included in the model as continuous variable

⁴ SC was the operator with the most experience in performing CVS