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SHORT REPORT

The fetal fraction of cell-free DNA in maternal plasma is not affected by *a priori* risk of fetal trisomy

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Objective: To determine the relationship between *a priori* risk for fetal trisomy and the fraction of fetal cell-free DNA (cfDNA) in maternal blood. *Methods*: A comparative analysis on fetal cfDNA amounts was performed in subjects stratified into *a priori* risk groups based on maternal age, prenatal screening results, or nuchal translucency measurement. *Results*: Across the highest and lowest deciles within each group, there were no significant differences in the fetal cfDNA fraction. *Conclusions*: These data support the concept that non-invasive prenatal test performance as determined by fetal cfDNA fraction is not predicted to be different based on patient risk classification.

Keywords: Non-invasive, prenatal diagnosis, cell-free DNA, trisomy risk

Introduction

The recent introduction into clinical practice of non-invasive prenatal tests (NIPT) for detection of trisomy 21, 18 and 13 using cell-free DNA (cfDNA) is supported by several clinical validation studies utilizing pregnant populations enriched for fetal trisomy [1–4]. While not all studies specified identical inclusion criteria, all subjects underwent invasive diagnostic testing (chorionic villus sampling (CVS) or amniocentesis) for definitive characterization of fetal karyotype. While the published literature to date has shown robust performance in high-risk populations enriched for trisomy 21, the performance and thus the clinical appropriateness for NIPT in the general population has been questioned as this group has not been specifically studied.

Both maternal and fetal cfDNA are found in the plasma of pregnant women in small fragments (200–300 base pairs [5]) and it is likely that fetal DNA is released from fetal and/or placental cells undergoing apoptosis [6]. Most assay approaches to date utilize the latest high fidelity sequencing technology for chromosome identification through counting of cfDNA fragments. Whether this is done by directing sequencing runs to chromosome selective fragments [7,8] or by the random sequencing of cfDNA by shotgun methods [2–4], a critical determinant of the ability to detect trisomy and therefore generate a clinically useful result is the fraction of fetal cfDNA. On average, the amount of fetal cfDNA in plasma from a pregnant woman is approximately 10% [1] but there is a large variance in the fraction of fetal cfDNA between patients. The higher the fraction of fetal cfDNA, the more effective NIPT is at distinguishing fetal trisomy from a euploid fetus. Currently published thresholds for accurate detection of trisomy are a fetal cfDNA fraction of 4% or greater [1,2].

Little is known about the clinical factors that affect fetal cfDNA percentage in maternal blood. A recent publication limited to high risk pregnancies in the first trimester using multivariate analysis showed no significant independent contribution to fetal fraction from factors such as fetal karyotype, crown-rump length, nuchal translucency or a number of maternal characteristics [9]. However, fetal fraction increased with serum PAPP-A and free β-hCG and decreased with maternal weight. In addition, it is reported that approximately 2–4% of patients will not receive any result from cfDNA testing, in most cases due to a low fraction of fetal cfDNA [1,2,4]. A deeper understanding of the specific clinical factors that influence this variance, including those commonly associated with *a priori* trisomy risk would be helpful in understanding the clinical application and utility of NIPT in a broad patient population.

We performed a *post hoc* analysis on a cohort from a previously reported multicenter study (NICE Study) of cfDNA testing for fetal aneuploidy [1]. We had previously reported that between 10 and 22 weeks gestational age, there was no statistical difference in fraction of fetal cfDNA. Our goal was to assess *a priori* clinical risk factors and their potential influence on the fraction of fetal cfDNA as determined in this study.

Methods

Study population

Pregnant women 18 years and older with a singleton pregnancy of at least 10 weeks gestational age who were planning to undergo invasive prenatal diagnosis for any indication were prospectively enrolled as part of the NICE Study. Institutional Review Board approval was obtained at all participating centers and informed consent was obtained from all subjects.

Sample collection and preparation

Blood samples were collected prospectively from subjects prior to invasive testing. Samples were collected into Cell-free DNA™ BCT (Streck, Omaha, NE) and received by the laboratory of Ariosa Diagnostics, Inc. (San Jose, CA) within 7 days of collection. Plasma was isolated from blood via a double centrifugation protocol and then cfDNA was isolated from plasma using a modified Dynabeads® Viral NA DNA purification beads (Dynal, Grand Island, NY) protocol as previously described [7,8].

Test methods

cfDNA from each subject sample was isolated and quantified using the DANSR™ assay, which has been described in detail

elsewhere [8]. Briefly, this method uses ligation of locus-specific oligonucleotides to produce a sequencing template only from selected genomic loci. To assess fetal fraction, designed assays against a set of 192 SNP-containing loci on chr1-12, were used to query each SNP. SNPs selected for use in the DANSR assay were optimized for minor allele frequency in the HapMap 3 dataset (http://hapmap.ncbi.nlm.nih.gov/). A maximum likelihood estimate using the binomial distribution was employed to determine the most likely fetal fraction based upon measurements in which fetal alleles differed from maternal alleles.

Data analysis

Subjects were stratified in *post hoc* analysis into risk groups for having a fetus with trisomy. As the definition of "high risk" (HR) and "low risk" (LR) for fetal aneuploidy can vary, we performed multiple comparative analyses based on maternal age (-MA), aneuploidy screening results (-SC), and NT measurements (-NT). For comparative analysis, the highest and lowest decile values for the LR and HR groups were used and included all subjects at the decile cut-off values. In the SC category, the lowest or highest risk value for either trisomy 21 or trisomy 18 was used for stratification and was not averaged for the individual subject. A given patient may be represented more than one group if, for example, a single patient was low risk based on maternal age (LR-MA) but high risk based on NT and/or serum screening (HR-NT and HR-SC). Statistical analyses were performed using R version 2.15.1. Fetal percent comparisons were analyzed using analysis of variance (ANOVA) controlling for gestational age. As shown previously, fetal fraction does not vary significantly by gestational age from 10 to 22 weeks but increases thereafter [1]. To control for gestational age, fetal percent was first fitted to a linear model involving only gestational age, and the resulting fitted value was subtracted from its original value.

Results

Within the NICE Study cohort, there were 3007 subjects in which fetal fraction of cfDNA was measured. NT values and/or prenatal screening risks were available for 965 and 1351 patients, respectively. For the maternal age (MA) comparison, the average HR-MA and LR-MA in the highest and lowest risk deciles were 42.9 years (range: 42–50; $n = 214$) and 20.4 years (range: 18–23; $n = 274$), respectively. For the aneuploidy screening result (SC) comparison, the average HR-SC and LR-SC results were 1 in 6 (range: 1 in 3 to 1 in 14; $n = 106$) and 1 in 33,000 (range: 1 in 6500 to <1 in 100,000; $n = 135$), respectively. For the NT measurement comparison, the average HR-NT and LR-NT were 5.2 mm (range: 3.4–15.9 mm; $n = 91$) and 1.0 mm (range: 0.1–1.2 mm; $n = 87$), respectively. Table I shows the distribution and lack of statistical difference of fetal fraction of cfDNA between the HR and LR groups for each risk variable analyzed.

All trisomy 21 cases were correctly identified in LR and HR groups regardless of risk variable. The one false negative case of trisomy 18 was found in the HR-NT, HR-SC, and LR-MA group. There were no false positive test results in any of the subgroups analyzed.

Discussion

This study demonstrates that there is no difference in the fraction of fetal cfDNA between patient groups stratified by clinical risk factors for fetal trisomy. The primary inclusion criteria for the enrolled cohort from the NICE study were patients with

Table I. Fraction of fetal cfDNA values across risk categories.

Variable	n	Variable average value and range	Average fetal fraction (%) (SD)	p Value
Maternal age (MA)	High risk (HR) 214	42.9 years (42–50 years)	11.0 (4.6)	0.60
	Low risk (LR) 274	20.4 years (18–23 years)	10.7 (6.1)	
Prenatal screening result (SC)	High risk (HR) 106	1 in 6 (1 in 3 to 1 in 10)	11.4 (6.0)	0.34
	Low risk (LR) 135	1 in 33,000 (1 in 6500 to 1 in 100,000)	10.8 (5.7)	
Nuchal translucency measurement (NT)	High risk (HR) 91	5.2 mm (3.4–15.9 mm)	10.8 (4.0)	0.36
	Low risk (LR) 87	1.0 mm (0.1–1.2 mm)	11.4 (5.1)	

singleton pregnancies undergoing an invasive diagnostic procedure, and the study did not include only those at high risk for trisomy. The NICE cohort included individuals who were undergoing invasive procedures for a variety of indications, not all of which were related to trisomy risk (actual or perceived), and thus provided a significant number of subjects who would otherwise be considered at the low end of the risk spectrum. While it may be difficult to arrive at consensus definitions for "high" or "low" risk among prenatal diagnosticians, we stratified the cohort based on the extremes of risk for trisomy. We have previously reported from the entire cohort that fetal fraction did not vary with race or ethnicity, maternal age, or trisomy type. In addition, there was no statistical difference in fraction of fetal cfDNA in pregnancies of gestational age between 10 and 22 weeks [1]. This latter point is in general agreement with other reports [2,9].

When considering the performance characteristics of any aneuploidy screening test, prevalence of trisomy in the population under examination is a critical determinant of the true positive and true negative rate, although it does not impact the sensitivity or specificity of the cfDNA assay. The amount of fetal cfDNA in maternal plasma is a key determinant of assay performance, as distinguished from final test performance. If there is sufficient fetal cfDNA in the sample and quality control metrics are met, then the assay can provide accurate counting of the available chromosome fragments. The larger the fraction of fetal cfDNA that is present, the better the ability to distinguish euploid from aneuploid fetuses, thereby the better the test performance. A unique feature of the cfDNA assay under study in this report (the Harmony™ Prenatal Test) is the FORTE™ algorithm, which accounts for prevalence of trisomy in the final risk calculation using the specific individual *a priori* risk (maternal age and gestational age [8]). It is therefore important to assure that the fraction of cfDNA does not vary with *a priori* aneuploidy risk.

As opposed to other studies of cfDNA in the detection of fetal aneuploidy, the NICE study included any patient undergoing invasive prenatal testing, rather than only those at high risk of fetal aneuploidy. Therefore, the study included a subset of patients at low risk for aneuploidy but electing CVS or amniocentesis due to indications such as risk for single gene disorders or maternal choice. Thus our ability to assess test performance in this low risk sub-population offers a unique opportunity to address some of the important concerns regarding the use of cfDNA in this group.

This study is not without limitations. This represents a secondary analysis of a larger cohort study that was not designed to specifically address the issues discussed in this current report. The number of patients at the most extremes of risk (lowest and highest) that

we report on is relatively small. In this study, the only low risk subgroup that included trisomy cases was that based on maternal age (LR-MA),and included ten cases of fetal trisomy 21 and 2 cases of fetal trisomy 18. While these patients were young, most were high risk based on screening results. All fetal trisomy 21 cases were correctly identified in both high and low risk groups regardless of clinical risk factors. The one fetal trisomy 18 case not detected was found in the high risk NT, high risk aneuploidy screening and low risk maternal age groups. No false positive cell free DNA-based test results were found in any of the low risk or high risk subgroups in this analysis. This study adds important information to the literature regarding the relationship between clinical risk factors and fetal cfDNA percentage; factors that could impact test and assay performance if applied to a broader, average risk population.

NIPT currently offers improved detection of fetal trisomy as compared to conventional screening, and can be performed as early as 10 weeks of gestation. Numerous peer reviewed studies support the high performance characteristics of cfDNA assays for NIPT [1–4]. Despite excellent detection and low false positive rates, NIPT using cfDNA should still be regarded as a high performance screening test, as published data demonstrate less than 100% sensitivity and specificity. However, the improved performance over conventional serum screening for aneuploidy makes NIPT an attractive alternative for the general prenatal population [10]. With detection rates greater than 99% for trisomy 21 and false positive rates as low as 0.1%, the potential to reduce the number of unnecessary invasive diagnostic procedures with the use of NIPT using cfDNA is profound. Our findings indicate that important characteristics of cfDNA testing for aneuploidy risk are constant across a wide range of risk categories in the prenatal patient population; thus supporting the performance of this test as a population based screen.

Acknowledgements

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