Genome-wide cfDNA screening: clinical laboratory experience with the first 10,000 cases

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Purpose: Invasive diagnostic prenatal testing can provide the most comprehensive information about the genetic status of a fetus. Non-invasive prenatal screening methods, especially when using cell-free DNA (cfDNA), are often limited to reporting only on trisomies 21, 18, and 13 and sex chromosome aneuploidies. This can leave a significant number of chromosomal and subchromosomal copy-number variations undetected. In 2015, we launched a new genome-wide cfDNA screening test that has the potential to narrow this detection gap.

Methods: Here, we review the results from the first 10,000 cases submitted to the Sequenom clinical laboratory for genome-wide cfDNA screening.

Results: The high-risk indication for this cohort differed compared with standard cfDNA screening. More samples were submitted with

ultrasound indications (25% compared with 13% for standard cfDNA screening) and fewer for advanced maternal age (51% for genome-wide screening versus 68% for standard cfDNA screening). A total of 554 positive calls were made, of which 164 were detectable only via genome-wide analysis.

Conclusion: This reports indicates a difference in utilization compared with standard cfDNA screening, where positivity rates are higher and a large subset of positive calls could not have been made using standard cfDNA screening.

Genet Med advance online publication 15 June 2017

Key Words: cfDNA; genome-wide; karyotype; NIPS; prenatal

INTRODUCTION

Today, the most comprehensive prenatal information about the genetic health of a fetus can be obtained by invasive testing, such as chorionic villus sampling or amniocentesis, combined with karyotype and/or microarray analysis.¹ The importance of making this comprehensive information available to all women, without restrictions for age or other specific risk factors, has been recognized in professional society guidelines since 2007.² More recently, this has been reaffirmed by the expansion of this recommendation to include microarray analysis in cases where ultrasound abnormalities are present.³

Although the procedure-related risks are small,⁴ a considerable number of patients prefer to forgo invasive testing.⁵ Furthermore, in some cases, invasive testing might not be available due to technical or clinical considerations. In part motivated by these challenges, noninvasive methods for prenatal testing have been in development since the early 1980s. Since its introduction in 2011, noninvasive cell-free DNA (cfDNA) testing has become widely accepted as a screening tool, where screen-positive tests are followed by diagnostic confirmation through invasive sampling and subsequent analysis by karyotype and/or microarray analysis. These “traditional” cfDNA screening tests are typically limited to a selected subset of chromosomal abnormalities, including trisomies 21, 18 and 13, as well as sex chromosome aneuploidies; some also screen for a selected set of

microdeletions.^{6–10} However, cfDNA screening need not be limited to only a subset of chromosomal abnormalities.¹¹ Recent data suggest that approximately 80% of pregnancies with abnormal chromosomal representation from a general obstetric population can be identified using traditional cfDNA tests, leaving a significant 20% detection gap between traditional cfDNA screening and serum screening with invasive confirmation.¹²

In 2015, we introduced a new cfDNA screening test that aims to narrow this detection gap of noninvasive testing by enabling genome-wide analysis of copy-number variations equal to or larger than 7 Mb, as well as a select group of microdeletions smaller than 7 Mb in size.¹³ This screening test is offered as an alternative to standard cfDNA screening for cases when more information is desired. After nearly one year of experience in the clinical laboratory, we report here on the first 10,000 cases examined, providing an observational summary of the clinical utilization of the test and a general discussion of the results reported thus far. A more detailed analysis of confirmed positives and discordant results will be published at a later date, when sufficient outcome data are available.

MATERIALS AND METHODS

Sample cohort

Data reported here were generated from clinical use of the MaterniT GENOME laboratory-developed test in our Clinical

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Submitted 17 October 2016; accepted 20 March 2017; advance online publication 15 June 2017. doi:10.1038/gim.2017.56

Laboratory Improvement Amendments–certified and College of American Pathologists–accredited laboratory from September 2015 to May 2016. Indications for testing were designated by ordering clinicians on the test requisition form as one or more of the following: advanced maternal age (AMA), family or personal history, ultrasound abnormalities, abnormal serum screening, and “other.” Gestational age was determined by last menstrual period or ultrasound, as reported by the ordering clinician. Samples were accessioned into the laboratory and results reported to the ordering clinician. Samples were tested for genome-wide copy-number variations ≥ 7 Mb in size, and for a selected group of microdeletions < 7 Mb in size associated with 1p36 deletion, Wolf–Hirschhorn, Cri-du-chat, Langer–Giedion, Jacobsen, Prader–Willi, Angelman, and DiGeorge syndromes. The 7 Mb cutoff is a feature of the MaterniT GENOME test and was not customized for this analysis.

Sample laboratory processing

Testing was performed using whole-blood samples collected in cfDNA blood collection tubes (Streck, Omaha, NE) or on processed plasma that was shipped and received frozen. cfDNA was extracted from plasma using an automated extraction method with MyOne Dynabeads (ThermoFisher Scientific, Waltham, MA). Plasma DNA was used to create indexed sequencing libraries as described by Tynan *et al.*¹⁴ Sequencing libraries were multiplexed, clustered, and sequenced on HiSeq 2000 or HiSeq 2500 instruments (Illumina, San Diego, CA), as described by Lefkowitz *et al.*¹³ Sequencing results were normalized and analyzed for fetal fraction; chromosome 21, 18, and 13 trisomy; sex chromosome aneuploidies; and other genome-wide whole-chromosome and subchromosome copy-number variants, using bioinformatics algorithms as previously described.^{11,13,15}

Data review

Clinical laboratory directors reviewed sequencing data from each sample before the final reporting of results to the ordering clinician. When necessary, clinical laboratory directors had access to indication and clinical information provided on the test requisition form. Samples with insufficient fractional fetal DNA concentration were classified as “quantity not sufficient” using a previously described method, and no report was issued. Samples failing other laboratory quality control metrics, including library concentration and sequencing-specific metrics, were classified as “other not reportable.”

The data analyzed for this retrospective study were obtained from de-identified and not individually identifiable patient data collected on the test requisition form. Furthermore, all patient-specific data that were generated as a result of the MaterniT GENOME laboratory-developed test were de-identified in accordance with the Health Insurance Portability and Accountability Act and the April 2005 Food and Drug Administration guidance document “Informed Consent for In vitro Diagnostic Device Studies Using Leftover

Human Specimens That Are Not Individually Identifiable” and combined for analysis. This report describes the overall clinical usage and findings with the test; it does not provide detailed descriptions of individually identifiable patient cases.

RESULTS

Risk indications for noninvasive prenatal screening

From September 2015 to May 2016, 10,272 samples were submitted to the clinical laboratory for genome-wide assessment of copy-number variations with the MaterniT GENOME laboratory-developed test. The distribution of gestational age at the time of submission was comparable to that for traditional cfDNA screening by the MaterniT 21 PLUS laboratory-developed test, with a slight but not statistically significant increase in the relative proportion of samples collected at 20 to 21 weeks gestation (data not shown). This might indicate increased usage later in pregnancy due to positive ultrasound findings, a hypothesis that is further supported by the distribution of high-risk indications found for the submitted samples. **Figure 1** describes the distribution of risk factors provided upon sample submission for genome-wide as well as traditional cfDNA testing (risk indications are further detailed in **Supplementary Figures 1–3**). The risk factors are divided into the following categories on the test requisition form: AMA, abnormal ultrasound finding, abnormal serum screening, a personal or family history of chromosomal abnormalities, or “other.” The most recognizable differences for traditional cfDNA testing are in the group of samples submitted for AMA and for abnormal ultrasound findings. The proportion of samples submitted for “AMA only”

Figure 1 Distribution of risk indications in the tested population. Values are based on the information provided in the sample requisition form by the ordering physician per test. The inner circle shows the risk indications of patients using MaterniT 21 PLUS ($n > 500,000$) and the outer circle shows the risk indications from MaterniT GENOME ($n > 10,000$). AMA, advanced maternal age; AS, abnormal serum screening result; HIST, personal and/or family history; US, abnormal ultrasound finding.

dropped from approximately 68% in traditional cfDNA testing to approximately 51% in genome-wide cfDNA testing. This reduction was almost entirely compensated by samples that had abnormal ultrasound findings either as the sole high-risk indication, or as part of multiple high-risk indications (13% in traditional cfDNA testing and 25% in genome-wide cfDNA testing).

Positivity rates

Screen-positive test results were reported in 554 cases, leading to a screen-positive rate of approximately 5.4% (compared with 2.3% in traditional cfDNA screening).¹⁶ Samples submitted with abnormal ultrasound findings, as the sole indication or in combination with other high-risk factors, had a high screen-positive rate of ~11%, while samples submitted for personal or family history had a lower screen-positive rate at 4% (Figure 2). Some subgroups of samples with specific combinations of high-risk indications showed very high screen-positive rates. For example, in samples submitted for AMA and abnormal ultrasound findings together, the positivity rate was 23%. These screen-positive rates are higher than what is expected in a general high-risk population. This can probably be attributed to a subjective selection process that patients have undergone before submission of their sample by the clinician. Taken together, these data indicate that during this initial phase of clinical adoption, providers appear to preferentially choose this test for cases that are at very high risk for chromosomal abnormalities.

To investigate the benefits of genome-wide screening, we break down the positive results into findings that could have been obtained by traditional cfDNA screening ($n=390$) (includes trisomies of chromosomes 13, 18, and 21 and sex chromosome aneuploidies) and findings that are discoverable only by genome-wide cfDNA screening ($n=164$). Findings exclusive to genome-wide cfDNA testing constitute approximately 30% of all screen-positive results and include large (≥ 7 Mb) subchromosomal and/or whole-chromosome aneuploidies across the entire genome. While some indications for

testing have minimal influence on the frequency of these uniquely discoverable findings, other indications show a significant influence. However, this analysis is complicated by the fact that many patients may have more than one indication for testing. Because patients can be referred with multiple risk indications, a stratification based on the many possible combinations of individual risk indications is suboptimal. For the purposes of this analysis, we assigned patients to four meaningful categories. Three of these groups consisted of patients with one or multiple risk indications but at least one of the following: (i) abnormal ultrasound findings, (ii) an abnormal serum screen, or (iii) a personal or family history. The last group, of a type that has proven useful in other studies, consisted of patients whose only high-risk indication was (iv) AMA. The frequency of positive results that can be obtained exclusively through genome-wide testing varies between these groups. In samples with personal or family history, approximately 50% of the findings are discoverable only with genome-wide screening. In samples with AMA this proportion is 38%. It is well known that ultrasound and serum screening are purposefully designed to identify pregnancies specifically at high risk of trisomies 18 and 21 (and, to a lesser extent, trisomy 13). In fact, positive samples in this study with abnormal ultrasound findings and/or abnormal serum screen indications are enriched for the three common autosomal aneuploidies (58% for abnormal ultrasound findings and 51% for abnormal serum screen compared with 47% for AMA, and 29% for family and/or personal history). Thus, the relative contributions of uniquely discoverable genome-wide findings in these two groups decrease slightly from the overall frequency of 30% in the entire cohort to 25% for samples with abnormal serum screen results and 24% for samples with abnormal ultrasound findings.

Genome-wide finding location and size distribution

A total of 80 samples were reported to screen positive for aneuploidies of autosomes other than chromosomes 21, 18, and 13. Most often affected were chromosome 16 (15 cases),

Figure 2 Positivity rates by risk indicator and finding type. (a) Positivity rate stratified by risk indicator and grouped by type of positive finding. (b) Contribution of each positive finding type to the positive cohort per risk group. The study cohort average genome-wide contribution of 30% is indicated by a gray line at 0.7. AMA, advanced maternal age; AS, abnormal serum screening result; HIST, personal and/or family history; US, abnormal ultrasound finding. Subgroups of each barchart column comprise T21/T18/T13 (bottom), sex chromosome aneuploidies (middle), and genome-wide (top) positive findings.

chromosome 7 (11 cases), and chromosome 3 (10 cases). Other than 45X, no monosomies were reported, and no trisomies were reported for chromosomes 5, 6, 17 and 19.

Trisomies involving chromosomes 21, 18, and 13 are most frequently nonmosaic, whereas most other autosomal aneuploidies are more likely to associate with mosaicism and/or be confined to the placenta.^{17–19} A known limitation of both chorionic villus sampling and cfDNA testing is that they assume the genetic makeup of the placenta is identical to that of the fetus, but in rare cases there is discordance, which may be due to confined placental mosaicism. For cfDNA measurements, no methods exist today to predict accurately a placental mosaicism. However, comparison of two independent fetal fraction measurements at the time of testing can suggest that the placenta may be mosaic (**Figure 3**). The first measurement, sequencing-based fetal fraction (SeqFF), is a fetal fraction estimation based on sequencing data from different genomic regions and is independent of the aneuploidy status of the fetus.¹⁵ The second measurement, affected fraction, is applied in cases where an aneuploidy has been detected; this method calculates the fraction of affected DNA necessary to cause the observed gain (or loss) in sequence counts of that particular affected region. In the case of a nonmosaic trisomy, these values are highly concordant. In the case of a mosaic placenta, however, the affected fraction value will be noticeably smaller than the SeqFF estimate, indicating that not all of the placentally derived cfDNA is affected by the aneuploidy. In this data set, the mean ratio of affected fraction to SeqFF for standard trisomies 21, 13, and 18 was 1.06 (s.d. = 0.27) with only 5% of samples having ratios lower than 0.54; these observations support the notion that most of these trisomies involve the placenta in its entirety. In contrast, the ratios observed for other autosomal trisomies showed bimodal distribution, and more than 50% of samples showed ratios lower than 0.54, indicating that in these cases only a fraction of the placental DNA was affected by the

trisomy. Thus, the relative ratio of fetal fraction estimated in the chromosome of interest (affected fraction) divided by the fetal fraction estimated for the entire genome (SeqFF) appears to be a useful metric that can assist with the prediction of the mosaic versus nonmosaic status of the placenta. Especially at later gestational ages, awareness of the likelihood of placental mosaicism may be increasingly important to the clinician since it is more likely that the mosaicism would be confined to the placenta. Consequently, the well-known clinically significant adverse effects of confined placental mosaicism may be monitored.²⁰

Subchromosomal events were reported on all autosomes with the exception of chromosomes 19 and 17. To allow interpretation of the predicted copy-number variation sizes, some of the assay constraints have to be considered. This assay was designed to predict genome-wide copy-number gains and losses only if they are larger than 7 Mb (the typical level of resolution for G-banded chromosome analysis), to assure high analytical sensitivity and minimize interpretation challenges. Smaller events are reported only when associated with a select set of clinically relevant microdeletions, or when discovered as an incidental finding associated with a prediction for a larger deletion or duplication (as may be seen with an unbalanced translocation) and after in-depth review by the laboratory director. The resulting distribution of estimated sizes shows that smaller copy-number variations are more common than larger ones (**Figure 4**). This is in line with previous findings from invasive testing studies.^{1,21} The set of very large copy-number variations often involves the terminal end of a chromosome. In this data set, predicted deletions tend to be smaller than predicted duplications (median size for deletions: 13 Mb; median size for duplications: 31 Mb).

Other findings

In five cases, a deletion in the 22q11 region was predicted to be maternal in origin. Two of these cases had abnormal

Figure 3 Independent fetal fraction estimates. Concordance between SeqFF and the fetal fraction estimation based on the deviation of the affected chromosome from the population median. Parallel lines highlight the 95% confidence interval of the regression line describing the relationship between the two fetal fraction estimates.

Figure 4 Size distribution of subchromosomal copy-number variations. The histogram shows the prevalence of copy-number variations in each group size among the positive samples.

ultrasound findings while three had only AMA as the sole risk indication.

For another subset of patients, two or more subchromosomal copy-number variations were predicted. We have seen several cases in the laboratory where the co-occurrence of two events, especially when located at the terminal ends of the affected chromosomes, is indicative of an unbalanced translocation event. It is important to note that, to date, no rigorous validation data exist for cfDNA analysis and this association is currently based on a limited subset of karyotype-confirmed samples. In this context, it is interesting to note that samples with a personal or family history as the risk indication were three times more likely to present with such complex test findings.

DISCUSSION

An evolving debate in cfDNA screening revolves around how best to expand test content beyond trisomies 21, 13, and 18 and sex chromosome aneuploidies. One viewpoint suggests screening for a small but well-defined set of chromosomal regions, which are associated with known clinical conditions, such as the deletion of 22q11 (DiGeorge syndrome). Since each of the conditions taken by itself is very rare, it is important to compile a sufficiently large set of conditions, such that the cumulative prevalence is high enough to justify adoption. One downside of multiple-condition panels, especially when using targeted cfDNA tests, is a high overall false-positive rate due to unavoidable problems brought on by multiple-hypothesis testing. Our genome-wide cfDNA screen avoids multiple-hypothesis testing by using mathematical algorithms that result in low false-positive rates.^{13,15} Ultimately, a clinical study is needed to determine the true false-positive rate for genome-wide cfDNA analysis. For the purpose of this discussion, we assume that previously established sensitivity and specificity values of > 97% and > 99.9%, respectively, remain unchanged in this cohort. In addition, genome-wide cfDNA screening does not limit the regions that are being analyzed, thus enabling detection of esoteric deletions, duplications, and aneuploidies that would not have been suspected *a priori*. One challenge for genome-wide cfDNA screening is comparable to a challenge for microarray analysis: for some copy-number variations, a clinical correlation is difficult to determine. However, the laboratory-developed test described herein largely avoids this issue by reporting only on copy-number variations larger than 7 Mb. Historically, this has been a lower limit of resolution for the visible deletions or duplications reported by G-banded chromosome analysis karyotyping. Interestingly, positive findings were observed on every chromosome throughout the genome, underlining the benefit of a true genome-wide screen.

At 5.4%, the overall screen-positive rate was approximately twice as high as that reported for traditional cfDNA screening in a high-risk population (2–3%). This indicates that, in this initial phase of adoption, the genome-wide cfDNA test has been preferentially used for pregnancies in which the prevalence of chromosomal abnormalities is much higher compared with the conventional high-risk population. This

interpretation is aided by the distribution of test indications. The test has been ordered less frequently for women for whom AMA is the only risk indication and more frequently for women who have an abnormal ultrasound finding, compared with the distribution of test indications in traditional cfDNA testing. Interestingly, around 30% of all positive findings would not have been detectable with traditional cfDNA screening. Among those are many samples with more than one deletion and/or duplication, such as might be expected with unbalanced translocations. Although an unbalanced translocation cannot be detected directly (cfDNA screening only detects over- or underrepresentation of genomic material, not structural chromosomal abnormalities), they do appear to follow a specific pattern. Samples that simultaneously harbor a terminal deletion as well as a terminal duplication are typically associated with an unbalanced translocation. Further studies are necessary to evaluate to what degree this pattern is truly predictive.

This report provides the first description of clinical experience with genome-wide cfDNA analysis for prenatal screening, within the first year of the test's introduction. Because outcome information is not yet available for most pregnancies tested, these observational findings do not allow assessment of performance characteristics, including sensitivity or positive predictive values for the detection of genome-wide copy-number variations. Nonetheless, this report provides, for the first time, essential information about the use of genome-wide cfDNA testing in a large clinical cohort. Most strikingly, genome-wide cfDNA screening uniquely contributed 30% of the clinically relevant abnormalities found in this cohort. It is important to point out that current guidelines do not recommend using genome-wide cfDNA screening as a routine testing option. A recently updated position statement from the American College of Medical Genetics and Genomics recommended the use of an invasive procedure with chromosomal microarray testing to detect genome-wide copy-number variations.²² To frame this essential discussion, it is important to point out that we agree that no cfDNA screening can serve as a substitute for diagnostic testing and that the most comprehensive information about the genetic health of a fetus is obtained by microarray analysis of fetal DNA. Whether the information obtained from genome-wide cfDNA testing will benefit an individual woman depends on a variety of factors, such as medical history, prior risk, desire to obtain genome-wide information, and willingness to undergo diagnostic testing. These factors should be evaluated and a decision should be rendered on a case-by-case basis by the pregnant woman, in close communication with her healthcare provider, to truly enable personalized care and to respect her autonomy.²³

The role of genome-wide cfDNA screening in the average-risk population is an interesting topic, but this cannot be adequately evaluated based on the current data as the vast majority of submitted samples were classified as high risk. It is hoped that future work will provide further insights on this front.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

DISCLOSURE

All authors were employed at Sequenom, a subsidiary of Laboratory Corporation of America Holdings, at the time of submission.

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