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Optimal Detection of Fetal Chromosomal Abnormalities by Massively Parallel DNA Sequencing of Cell-Free Fetal DNA from Maternal Blood

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BACKGROUND: Massively parallel DNA sequencing of cell-free fetal DNA from maternal blood can detect fetal chromosomal abnormalities. Although existing algorithms focus on the detection of fetal trisomy 21 (T21), these same algorithms have difficulty detecting trisomy 18 (T18).

METHODS: Blood samples were collected from 1014 patients at 13 US clinic locations before they underwent an invasive prenatal procedure. All samples were processed to plasma, and the DNA extracted from 119 samples underwent massively parallel DNA sequencing. Fifty-three sequenced samples came from women with an abnormal fetal karyotype. To minimize the intra- and interrun sequencing variation, we developed an optimized algorithm by using normalized chromosome values (NCVs) from the sequencing data on a training set of 71 samples with 26 abnormal karyotypes. The classification process was then evaluated on an independent test set of 48 samples with 27 abnormal karyotypes.

RESULTS: Mapped sites for chromosomes of interest in the sequencing data from the training set were normalized individually by calculating the ratio of the number of sites on the specified chromosome to the number of sites observed on an optimized normalizing chromosome (or chromosome set). Threshold values for trisomy or sex chromosome classification were then established for all chromosomes of interest, and a classification schema was defined. Sequencing of the independent test set led to 100% correct classification of T21 (13 of 13) and T18 (8 of 8) samples. Other chromosomal abnormalities were also identified.

CONCLUSION: Massively parallel sequencing is capable of detecting multiple fetal chromosomal abnormalities from maternal plasma when an optimized algorithm is used.

The American College of Obstetrics and Gynecology Practice Bulletin no. 77, published in 2007, supports the measurement of nuchal translucency and surrogate biochemical markers in all pregnant women in the first trimester to assess the risk of aneuploidy for Down syndrome *(1)*. These screening tests can provide only an inconclusive risk determination; they have nonoptimal detection and high false-positive rates. Today, only invasive methods, including chorionic villus sampling (CVS),[3] amniocentesis, or cordocentesis, provide definite genetic information about the fetus, but these procedures are associated with risks to both mother and fetus *(2–4)*. Therefore, a noninvasive means to obtain definite information on fetal chromosomal status is desirable.

Fan et al., in 2008, were the first to suggest counting chromosomes by mapping sequence tags as a potential quantification method for detecting fetal aneuploidy from cell-free DNA (cfDNA) obtained from maternal blood *(5, 6)*. In these studies, massively parallel DNA sequencing of cfDNA obtained from the maternal plasma yielded millions of short sequence tags that could be aligned and uniquely mapped to sites from a reference human genome. The depth of sequencing and subsequent counting statistics determine the sensitivity of detection for fetal aneuploidy *(7)*.

Although 2 recently published reports of studies with larger populations have described the successful use of sequence tag mapping and chromosome counting to detect fetal aneuploidy, these studies focused only on the classification of trisomy 21 (T21) *(8, 9)*. The algorithms used in these studies appear to be un-

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[3] Nonstandard abbreviations: CVS, chorionic villus sampling; cfDNA, cell-free DNA; T21, trisomy 21; T18, trisomy 18; NCV, normalized chromosome value; T13, trisomy 13.

able to effectively detect other aneuploidies, such as trisomy 18 (T18), that would inevitably occur in a clinical population being offered a commercially available test. In this study, we developed and tested an optimized algorithm from massively parallel sequencing data and demonstrated the potential universality of the sequence tag mapping and chromosome-quantification method for the detection of multiple chromosomal abnormalities.

Materials and Methods

BLOOD SAMPLES AND CLINICAL INFORMATION

The study was conducted by qualified clinical research personnel at 13 US clinic locations between April 2009 and July 2010 under a human participant protocol approved by institutional review boards at each institution. Informed written consent was obtained from each woman before her inclusion in the study.

The protocol was designed to provide blood samples and clinical data to support the development of noninvasive prenatal genetic diagnostic methods. Pregnant women age 18 years or older were eligible for inclusion. For patients undergoing clinically indicated CVS or amniocentesis, blood was collected before performance of the procedure, and fetal-karyotyping results were also collected. Peripheral blood samples (2 tubes or approximately 20 mL total) were drawn from all participants and collected into tubes containing acid citrate dextrose (Becton Dickinson). All samples were deidentified and assigned an anonymous study identification number. Blood samples were shipped overnight to Verinata Health, Inc. (San Carlos, CA) in temperature-controlled shipping containers provided for the study. The time elapsed between blood draw and sample receipt was recorded upon accessioning at the Verinata Health laboratory.

Site research coordinators used the anonymous patient identification number in entering clinical data relevant to the patient's current pregnancy and history into study case-report forms. Cytogenetic analysis of the fetal karyotype from samples obtained in invasive prenatal procedure was performed per the local laboratories, and these results were also recorded in the study case-report forms. All data obtained on the forms were entered into a clinical database at Verinata Health.

SAMPLE PROCESSING AND SEQUENCING

Cell-free plasma was obtained from individual blood tubes within 24–48 h of venipuncture via centrifugation at 1600*g* for 10 min, transfer to microcentrifuge tubes, and centrifugation at 16 000*g* for 10 min to remove residual cells. Plasma from a single blood tube was sufficient for sequencing analysis. cfDNA was extracted from cell-free plasma with the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Because cfDNA fragments are known to be approximately 170 bp in length *(10)*, a DNA-fragmentation step was not required before sequencing. For the training set samples, we sent cfDNA to Prognosys Biosciences to prepare a sequencing library (cfDNA blunt-ended and ligated to universal adapters) and for sequencing on the Illumina Genome Analyzer IIx instrumentation according to the manufacturer's standard protocols (http://www.illumina.com/). Single-end reads of 36 bp were obtained. Upon completion of the sequencing, all base-call files were transferred to Verinata Health for further analysis. For the test set samples, we prepared the sequencing libraries and carried out the sequencing on the Illumina Genome Analyzer IIx instrument at Verinata Health. For both the training and test sample sets, single-end reads of 36 bp were sequenced.

DATA ANALYSIS AND SAMPLE CLASSIFICATION

Sequence reads of 36 bases in length were aligned to the human genome assembly hg18 obtained from the University of California, Santa Cruz database (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/bigZips/). Alignments were carried out by using the Bowtie short read aligner (version 0.12.5) and allowing for up to 2 base mismatches during alignment *(11)*. Only reads that unambiguously mapped to a single genomic location were included. The genomic sites where reads mapped were counted and included in the calculation of chromosome ratios (see below). Regions on the Y chromosome where sequence tags from male and female fetuses map without any discrimination were excluded from the analysis (specifically, from base 0 to base 2×10^6, base 10×10^6 to base 13×10^6, and base 23×10^6 to the end of chromosome Y).

Intrarun and interrun sequencing variation in the chromosomal distribution of sequence reads can obscure the effects of fetal aneuploidy on the distribution of mapped sequence sites. To correct for such variation, we calculate a chromosome ratio, in which the count of mapped sites for a given chromosome of interest is normalized to counts observed on another predetermined chromosome (or set of chromosomes). To identify the optimal chromosome ratio for each chromosome of interest, we reviewed the unaffected subset of the training data (i.e., including only samples with diploid karyotypes for chromosomes 21, 18, 13, and X) and considered each autosome as a potential denominator in a ratio of counts with our chromosomes of interest. We selected denominator chromosomes that minimized the variation of the chromosome ratios within and between sequencing runs. Each chromo-

Table 1. Chromosome ratio calculation rules.

Chromosome of interest	Numerator (chromosome mapped sites)	Denominator (chromosome mapped sites)
21	21	9
18	18	8
13	13	Sum (2–6)
X	X	6
Y	Y	Sum (2–6)

some of interest was determined to have a distinct denominator (Table 1).

The full training set was then used to set the boundaries for sample classification. The means and SDs of chromosome ratios for the unaffected samples in the training set were determined. For each sample and chromosome of interest, a normalized chromosome value (NCV) was calculated with the equation:

$$\mathrm{NCV}_{ij} = \frac{x_{ij} - \hat{\mu}_j}{\hat{\sigma}_j},$$

where $\hat{\mu}_j$ and $\hat{\sigma}_j$ are the estimated training set mean and SD, respectively, for the j-th chromosome ratio and x_{ij} is the observed j-th chromosome ratio for sample i. When chromosome ratios are normally distributed, the NCV is equivalent to a statistical z score for the ratios. No significant departure from linearity was observed in a quantile–quantile plot of the NCVs from unaffected samples. In addition, standard tests of normality for the NCVs failed to reject the null hypothesis of normality. For both the Kolmogorov–Smirnov and Shapiro–Wilk tests, the significance value was >0.05.

For the test set, an NCV was calculated for each chromosome of interest—21, 18, 13, X, and Y—for every sample. To ensure a safe and effective classification scheme, we chose conservative boundaries for aneuploidy classification. For classification of the autosomes' aneuploidy state, we required an NCV >4.0 to classify the chromosome as affected (i.e., aneuploid for that chromosome) and an NCV <2.5 to classify a chromosome as unaffected. Samples with autosomes that had an NCV between 2.5 and 4.0 were classified as "no call."

Sex chromosome classification in the test is performed in a somewhat more complex fashion—by sequential application of NCVs for both X and Y. Specifically:

1. If the NCV for Y is greater than −2.0 SDs from the mean of male samples, then the sample is classified as male (XY).
2. If the NCV for Y is less than −2.0 SDs from the mean of male samples and the NCV for X is greater than −2.0 SDs from the mean of female samples, then the sample is classified as female (XX).
3. If the NCV for Y is less than −2.0 SDs from the mean of male samples and the NCV for X is less than −3.0 SDs from the mean of female samples, then the sample is classified as monosomy X, i.e., Turner syndrome.
4. If the NCVs do not fit into any of the above 3 criteria, then the sample is classified as a "no call" for sex.

Results

STUDY POPULATION DEMOGRAPHICS

We enrolled 1014 patients between April 2009 and July 2010. The patient demographic characteristics, the type of invasive procedure, and karyotype results are summarized in Table 2. The mean age of the study participants was 35.6 years (range, 17–47 years), and gestational age ranged from 6 weeks, 1 day to 38 weeks, 1 day (mean, 15 weeks, 4 days). The observed overall incidence of abnormal fetal chromosome karyotypes was 6.8%, with a T21 incidence of 2.5%. Of 946 participants with singleton pregnancies and a karyotype, 906 (96%) showed at least 1 clinically recognized risk factor for fetal aneuploidy before undergoing the prenatal procedure. Even after eliminating the women with advanced maternal age as their sole indication, the data demonstrate a very high false-positive rate for current screening modalities. Ultrasound findings of increased nuchal translucency, cystic hygroma, or another structural congenital abnormality were most predictive of an abnormal karyotype in this cohort.

The distribution of the diverse ethnic backgrounds represented in this study population is also shown in Table 2. Overall, the patients were 63% Caucasian, 17% Hispanic, 6% Asian, 5% multiethnic, and 4% African American. We noted that the ethnic diversity varied substantially from site to site. For example, one site enrolled 60% Hispanic and 26% Caucasian individuals, whereas 3 other clinics located in the same state enrolled no Hispanic participants. As expected, there were no discernible differences in our results with respect to different ethnicities.

TRAINING SET DATA

The training set study selected 71 samples from the initial sequential accumulation of 435 samples that were collected between April 2009 and December 2009. All participants with affected fetuses (abnormal karyotypes) in this first series of participants, as well as a random selection of nonaffected individuals with adequate sample and data, were included for sequencing. The clinical characteristics of the patients in the training set were consistent with the overall study demographics summarized in Table 2. The gestational age

Table 2. Patient demographics.

Demographic characteristics	Total enrolled (n = 1014)	Training set (n = 71)	Test set (n = 48)
Dates of enrollment	Apr 2009 to Jul 2010	Apr 2009 to Dec 2009	Jan 2010 to Jun 2010
Patients enrolled, n	1014	435	575
Maternal age			
Mean (SD), years	35.6 (5.66)	36.4 (6.05)	34.2 (8.22)
Minimum/maximum, years	17/47	20/46	18/46
Not specified, n	11	3	0
Ethnicity, n (%)			
Caucasian	636 (62.7)	50 (70.4)	24 (50.0)
Hispanic	167 (16.5)	6 (8.5)	13 (27.0)
Asian	63 (6.2)	6 (8.5)	5 (10.4)
Multiethnic (>1)	53 (5.2)	6 (8.5)	1 (2.1)
African American	41 (4.0)	1 (1.3)	3 (6.3)
Other	36 (3.6)	2 (2.8)	1 (2.1)
Native American	9 (0.9)	0 (0.0)	1 (2.1)
Not specified	9 (0.9)	0 (0.0)	0 (0.0)
Gestational age, weeks, days			
Mean	15, 4	14, 5	15, 3
Minimum/maximum	6, 1/38, 1	10, 0/23, 1	10, 4/28, 3
No. of fetuses, n			
1	982	67	47
2	30	4	1
3	2	0	0
Prenatal procedure, n (%)			
CVS	430 (42.4)	38 (53.5)	28 (58.3)
Amniocentesis	571 (56.3)	32 (45.1)	20 (41.7)
Not specified	3 (0.3)	1 (1.4)	0 (0.0)
Not performed	10 (1.0)	0 (0.0)	0 (0.0)
Fetal karyotype, n (%)			
46,XX	453[a] (43.9)	22[a] (29.7)	7[a] (14.6)
46,XY	474[a] (45.9)	26[a] (35.1)	14 (29.2)
47,+21 (both sexes)	25[a] (2.4)	10[a] (13.5)	13 (27.1)
47,+18 (both sexes)	14 (1.4)	5 (6.8)	8 (16.7)
47,+13 (both sexes)	4 (0.4)	2 (2.7)	1 (2.1)
45,X	8 (0.8)	3 (4.1)	3 (6.3)
Complex, other	18[a] (1.7)	6 (8.1)	2 (4.2)
Karyotype not available	36 (3.5)	0 (0.0)	0 (0.0)
Prenatal screening risks for karyotyped singletons	**Nonsequenced (n = 834), n (%)**	**Analyzed training set (n = 65), n (%)**	**Analyzed test set (n = 47), n (%)**
AMA[b] only (≥35 years)	445 (53.4)	27 (41.5)	21 (44.7)
Screen positive (trisomy)[c]	149 (17.9)	18 (27.7)	9 (19.1)
Increased NT	35 (4.2)	3 (4.6)	5 (10.6)
Cystic hygroma	12 (1.4)	5 (7.7)	4 (8.5)
Cardiac defect	14 (1.7)	0 (0.0)	4 (8.5)
Other congenital abnormality	78 (9.4)	4 (6.2)	3 (6.4)
Other maternal risk	64 (7.7)	5 (7.7)	1 (2.1)
None specified	37 (4.4)	3 (4.6)	0 (0.0)

[a] Includes results of fetuses from multiple gestations.
[b] AMA, advanced maternal age; NT, nuchal translucency.
[c] Assessed and reported by clinicians.

for the samples in the training set ranged from 10 weeks, 0 days to 23 weeks, 1 day. Thirty-eight patients underwent CVS, 32 underwent amniocentesis, and 1 patient did not have the type of invasive procedure specified (an unaffected karyotype, 46,XY). The patients were 70% Caucasian, 8.5% Hispanic, 8.5% Asian, and 8.5% multiethnic. Six sequenced samples were removed from this set for the purposes of training—4 samples from individuals with twin gestations (further discussed below), 1 sample with T18 that was contaminated during preparation, and 1 sample with fetal karyotype 69,XXX—leaving 65 samples for the training set.

The number of unique sequence sites (i.e., tags identified with unique sites in the genome) increased from 2.2×10^6 in the early phases of the training set study to 13.7×10^6 in the latter phases because of improvements in the sequencing technology over this period. To monitor for any potential shifts in the chromosome ratios over this 6-fold range in unique sites, we ran different unaffected samples at the beginning and the end of the study. For the first run of 15 unaffected samples, the mean number of unique sites was 3.8×10^6, and the mean chromosome ratios for chromosomes 21 and 18 were 0.314 and 0.528, respectively. For the last run of 15 unaffected samples, the mean number of unique sites was 10.7×10^6, and the mean chromosome ratios for chromosomes 21 and 18 were 0.316 and 0.529, respectively. There was no statistically significant difference in the chromosome ratios over the time of the training set study for chromosome 21 or for chromosome 18.

The training set NCVs for chromosomes 21, 18, and 13 are shown in Fig. 1. These results are consistent with an assumption of normality, in that approximately 99% of the diploid NCVs fall within ±2.5 SDs of the mean. Of this set of 65 samples, 8 samples with clinical karyotypes indicating T21 had NCVs between 6 and 20. Four samples with clinical karyotypes indicative of fetal T18 had NCVs between 3.3 and 12, and the 2 samples with karyotypes indicative of fetal trisomy 13 (T13) had NCVs of 2.6 and 4. The spread in the NCVs in affected samples is due to their dependence on the percentage of fetal cfDNA in the individual samples.

Similarly to the autosomes, the means and SDs for the sex chromosomes were established in the training set. The sex chromosome thresholds allowed 100% of the male and female fetuses in the training set to be identified.

TEST SET DATA

Having established chromosome ratio means and SDs from the training set, we selected a test set of 48 samples from 575 samples collected between January 2010 and June 2010. One of the samples from a twin gestation was removed from the final analysis, leaving 47 samples in the test set. The personnel preparing samples for sequencing and operating the equipment were blinded to the clinical karyotype information. The range of gestational ages was similar to that of the training set (Table 2). Fifty-eight percent of the invasive procedures were CVS, higher than the percentage of the overall procedural demographics, but similar to that of the training set. The participants were 50% Caucasian, 27% Hispanic, 10.4% Asian, and 6.3% African American.

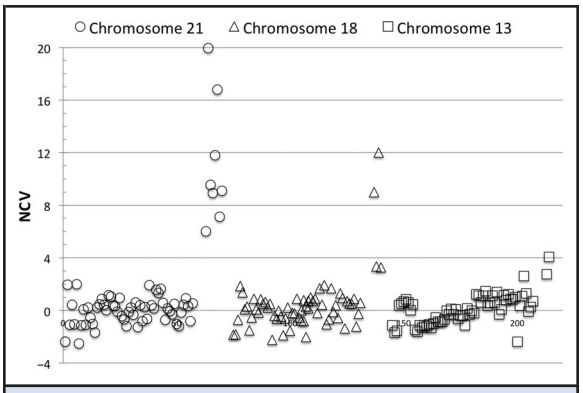

Fig. 1. NCVs for the 65 samples in the training set. The last 8 samples in the chromosome 21 data set (NCVs 6–20) have T21 karyotypes. The last 4 samples in the chromosome 18 data set (NCVs 3.3–12) have T18 karyotypes. The last 2 samples in the chromosome 13 data set (NCVs 2.6 and 4) have T13 karyotypes.

In the test set, the number of unique sequence tags varied from approximately 13×10^6 to 26×10^6. For unaffected samples, the chromosome ratios for chromosomes 21 and 18 were 0.313 and 0.527, respectively. The test set NCVs for chromosomes 21, 18, and 13 are shown in Fig. 2, and the classifications are given in Table 3. In the test set, 13 of 13 individuals with clinical karyotypes indicating fetal T21 were correctly identified, with NCVs between 5 and 14. All 8 individuals with karyotypes indicating fetal T18 were correctly identified, with NCVs between 8.5 and 22. The single sample with a karyotype classified as T13 in this test set was classified as a "no call," with an NCV of approximately 3.

For the test data set, all male samples were correctly identified [including a sample with complex karyotype, 46,XY plus a marker chromosome (unidentifiable by cytogenetics); Table 3]. Nineteen of 20 female samples were correctly identified; 1 female sample was categorized as a "no call." Two of 3 samples in the test set with a karyotype of 45,X were correctly identified as monosomy X. The third sample was classified as a "no call" (Table 3).

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