RESEARCH LETTER

A robust second-generation genome-wide test for fetal aneuploidy based on shotgun sequencing cell-free DNA in maternal blood

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The proof-of-principle of diagnosing fetal aneuploidy by shotgun sequencing cell-free DNA from maternal blood was demonstrated independently by two groups in 2008.[1,2]

The strength of the initial approach was the fact that it was genome-wide by design, yet it was exclusively or mainly limited in scope to the detection of trisomy 21 (T21). Follow-up studies then substantiated the lower sensitivity of the approach for autosomal trisomies other than T21.[3] Fan and Quake[4] showed that the inherent guanine-cytosine (GC) bias was a key factor limiting the sensitivity of the assay. Once the GC bias is removed, the sensitivity is determined only by counting statistics and by the fetal DNA fraction.

Despite unresolved technical restrictions, a number of studies pursued the way toward clinical implementation of the technology. The study by Bianchi *et al.*[5] conceptually demonstrated the efficacy of the approach for the common aneuploidies. However, the weaknesses of the study were first, some aneuploidies, including T13 and Turner syndrome, were less accurately called; second, in 3% of the cases, no fetal DNA was detected; and third, 3–7% of complex anomalies, partially overlapping with the samples lacking fetal DNA, were excluded from analysis. The objective of this study was to develop a genome-wide test capable of replacing invasive testing for the major aneuploidies. The requirements of such a test include the robust detection of the fetal DNA fraction, GC-bias removal and appropriately powered counting metrics, and rigorous quality control testing at all levels of the procedure.

The first part of the study consisted in a prospective plasma sample collection arm whose central aim was to constitute a reference set of 81 samples from singleton pregnancies with normal male or female karyotypes (Effinger Fetal Medicine Center, Bern, Switzerland). A set of 19 pathological samples was included in order to train discrimination between normal samples and aneuploidy. The second phase was double-blind and retrospective in design, with 88 euploid and 88 aneuploid pregnancies, and served the purpose to test the robustness of the assay for the detection of the most prevalent fetal aneuploidies (Fetal Medicine Centre, King's College Hospital, London, UK). The combined set of 276 samples included 107 aneuploidies and 169 euploid controls, which conferred adequate statistical power for sensitivity and specificity analysis.[5]

An average of 8 mL ethylenediaminetetraacetic acid blood was processed by the double-spin procedure. Cell-free DNA was extracted from 2 mL of plasma, with an average yield of 30 ng DNA/mL of plasma (PicoGreen). Real-time polymerase chain reaction (PCR) assays to quantitate the total and fetal DNA fraction based on the presence of *SRY* were performed. The fetal fraction was not measured for female fetuses, and an exclusion threshold based on the fetal fraction was not applied, because sequencing was much more accurate than qPCR, particularly for fetal fractions <5% (Supplementary Figure 1).

Input DNA (≤10 ng) was used for library construction using a ChIP-Seq protocol and the Illumina reagents. Libraries were massively parallel sequenced on a HiSeq 2000 (Illumina) with the TruSeq SBS Kit v3 (Illumina). An average of 27×10^6 (27.3 ± 8.3) total reads and an average of 20×10^6 (20.5 ± 6.3) unique exact reads (UER), that is, single reads genome-wide with no mismatches to the hg19 reference, per sample were produced.

Two main algorithms were used, one based on the *z*-score statistics,[2,3] and the other based on Fan *et al.*[1,4] For both, individual samples were compared with the complete reference set of normal karyotypes, with a mean ± 6.8 SD used for discrimination of normal and aneuploid samples.

The training set provided the thresholds to discriminate normal from aneuploid samples. In the validation set, we initially analyzed the group of autosomal trisomies. We re-sequenced four libraries from the training set, one each of T18 and T13, and two normal male samples, which were correctly called, confirming that the thresholds were appropriately set (not shown). On the basis of these conditions, we correctly identified all autosomal trisomies except one case of T18 that was classified as normal (Table 1). To set the lower bound threshold for X-chromosomal anomalies, we had only one single 45,X reference sample that had been analyzed outside the current study and was now used in combination with the set of normal female karyotype samples to set the thresholds for X-chromosomal dosage anomalies. On the basis of these settings, the test correctly identified all cases of Turner syndrome and triple X syndrome. The sensitivity for the 81 autosomal trisomies was 98.76% (95% CI 92.3–99.9%) and specificity was 100% (95% CI 97.59–100%). The combined sensitivity and specificity rates for the X-chromosomal anomalies Turner and Triple X syndromes were 100% (95% CI for sensitivity 79.9–100%, 95% CI for specificity 98.1–100%).

To improve the initial resolution (Supplementary Figure 2) of the assay, we performed a correction for the GC bias.[4] This resulted in first, improved discrimination between T21 and euploid cases; second, correct identification of all cases of T18 and T13; third, detection of rare autosomal trisomies, such as the single T22 (Figure 1a–d); and fourth, as shown in Supplementary Figure 3, detection of structural anomalies smaller than full chromosome size, such as terminal deletion of the small arm of chromosome 4 and a complex form of the Cri-du-chat syndrome. The results of the array comparative genomic hybridization (CGH) performed with fetal DNA are shown; the size of the terminal Wolf–Hirschhorn syndrome deletion was 23 Mb, the Cri-du-chat terminal deletion was 19 Mb and the interstitial duplication was 30 Mb. This size bias explains why the assay reported the duplication.

Among the class of structural anomalies in the combined data sets, we correctly assigned the size class of 20–30 Mb to the short arms of chromosomes 4 and 5 and to the distal part of the long arm of chromosome 6. This allowed determining the break points with a precision equivalent to a medium-to-high resolution microarray analysis (Agilent 4x180K; Supplementary Table 1). However, we missed two structural anomalies that were not characterized with microarrays and hence of not precisely determined size: a terminal, *bona fide* 6 Mb deletion of the short arm of chromosome 6 and a *bona fide* 20 Mb duplication of the terminal long arm of chromosome 10.

The findings of this study demonstrate the feasibility of developing a genome-wide assay for fetal aneuploidy based on shotgun sequencing cell-free DNA in maternal plasma capable of replacing invasive testing for major aneuploidies. This method has a spatial resolution and molecular precision for structural anomalies that is better than that of karyotyping. Only one type of anomaly, polyploidy, was systematically excluded from the study because of lack of appropriate *in silico* analytical method to detect it. The test was robust in detecting the fetal DNA fraction and produced no technical failure with all samples enrolled in the study being classified as euploid or aneuploid.

Three findings in the examination of the training set are of note. First, the T22 detected was in fact a confined placental mosaicism type I, confirming that cell-free DNA is likely derived from cytotrophoblastic cells.[6,7] Second, irrespective of the statistics used, our assay does not have sufficient statistical power to detect polyploidies (bona fide 23 times less power to detect triploidy in comparison with a single trisomy). Third, we had no single technical failure among the consecutively analyzed samples.

In the combined data sets, we correctly identified all cases of autosomal trisomy except one case of trisomy 18. There was no obvious explanation for the failure to detect this case of T18, because first, the two key factors determining fetal DNA fraction, gestational age and maternal weight[8] were not different from the complete set of trisomy samples and second, we had successfully replicated the correct identification of each one of the T18 and T13 samples under limiting conditions ($\leq 3 \times 10^6$ UER), which was one full order of magnitude (10×) lower than the UER used for this individual sample (30×10^6 UER).

The test correctly detected all cases of Turner and Triple X syndromes with 100% specificity and also one of the three partial chromosome anomalies. Concerning structural anomalies, clearly more work is required to precisely define what regions combined with what size classes will be detected or not. For instance, one major limiting factor is the quality of the current genome annotation (hg19), on which the counting statistics is based – a

Table 1 Performance of screening for the autosomal trisomies and sex chromosome aneuploidies in the combined training and validation sets

Aneuploidy	Sensitivity	Specificity
Trisomy 21 (*n* = 39)	39 (100%, 95% CI 88.8–100)	237/237 (100%, 95%CI 98.0–100)
Trisomy 18 (*n* = 24)	23 (95.8%, 95%CI 76.8–99.7)	252/252 (100%, 95%CI 97.0–100)
Trisomy 13 (*n* = 15)	15 (100%, 95%CI 74.6–100)	261/261 (100%, 95%CI 98.1–100)
Trisomy 16 (*n* = 1)	1 (100%, 95%CI 5.4–100)	275/275 (100%, 95%CI 98.2–100)
Trisomy 22 (*n* = 2)	2 (100%, 95%CI 19.7–100)	274/274 (100%, 95%CI 98.2–100)
45,X (*n* = 15)	15 (100%, 95%CI 74.6–100)	261/261 (100%, 95%CI 98.1–100)
47,XXX (*n* = 5)	5 (100%, 95%CI 46.2–100)	271/271 (100%, 95%CI 98.2–100)

CI, confidence interval.

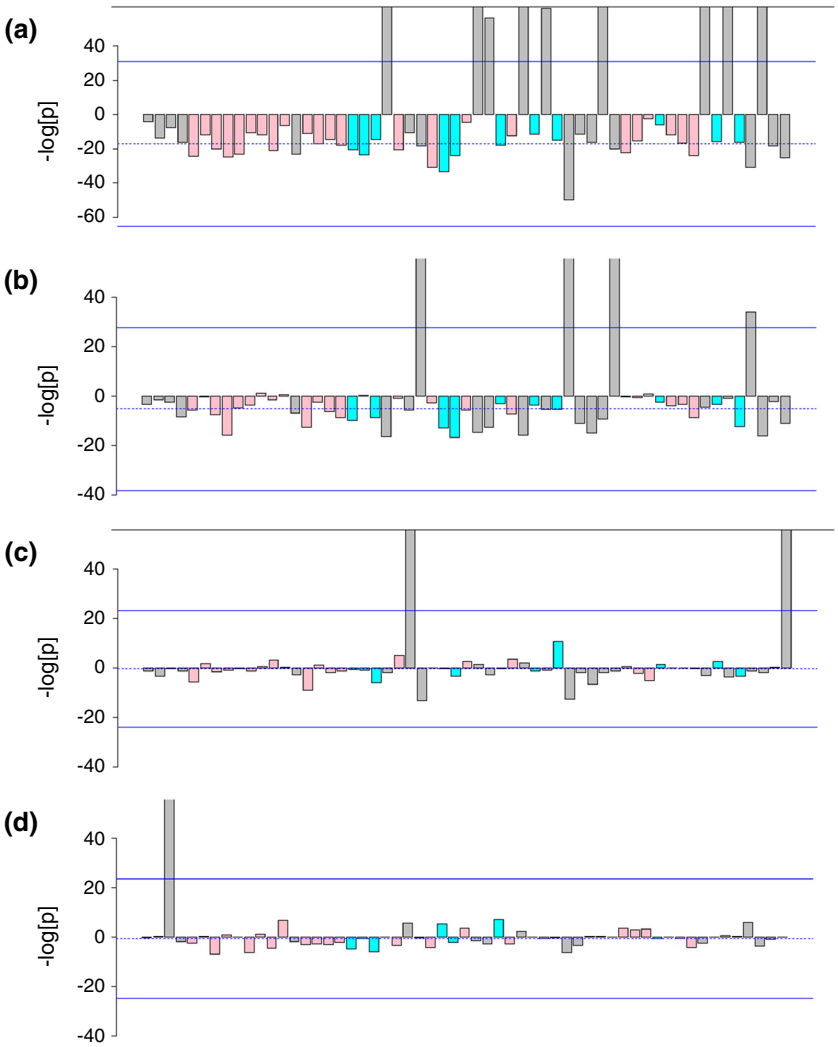

Figure 1 Chromosome-wise statistical difference after GC correction between the means of 100 plasma samples in comparison with the reference set for chromosomes (a) 21, (b) 18, (c) 13 and (d) 22. The threshold to detect anomalies (blue lines) was empirically set as the mean ± 6.8 SD, as it led to 100% specificity and 100% sensitivity for the training set

quality that differs among chromosomes, and varies for a given chromosome within different subregions.[9] This issue can be solved with large prospective studies collecting hundreds of plasma samples from fetuses affected by such structural anomalies that need to be characterized by high-resolution array CGH.

The test described is suitable for clinical application under conditions of continuing monitoring and rigorous ongoing quality control. This assay has the adequate robustness and the appropriate sensitivity/specificity performance to replace invasive karyotyping in principle; larger studies are required to confirm this. Although in its current version, it does not detect polyploidy, it has a better spatial resolution and molecular precision than karyotyping for partial structural anomalies that currently require a two-tiered diagnostic approach involving karyotyping followed by locus-specific fluorescent *in-situ* hybridization analysis and particularly array CGH.[10]

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Non-invasive genome-wide screening of fetal aneuploidy by shotgun sequencing cell-free DNA in maternal blood has been shown to effectively identify fetal trisomy 21, but the performance of screening for other aneuploidies is variable.

WHAT DOES THIS STUDY ADD?

- Optimizing all individual steps in the procedure and performing rigorous quality control provides a test capable of replacing invasive testing for the major aneuploidies.

REFERENCES

1. Fan HC, Blumenfeld YJ, Chitkara U, *et al*. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci USA 2008;105:266–71.
2. Chiu RW, Chan KC, Gao Y, *et al*. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci USA 2008;105:20458–63.
3. Chen EZ, Chiu RW, Sun H, *et al*. Noninvasive prenatal diagnosis of fetal trisomy 18 and trisomy 13 by maternal plasma DNA sequencing. PLoS One 2011;6:e21791.
4. Fan HC, Quake SR. Sensitivity of noninvasive prenatal detection of fetal aneuploidy from maternal plasma using shotgun sequencing is limited only by counting statistics. PLoS One 2010;5:e10439.
5. Bianchi DW, Platt LD, Goldberg JD. Genome-wide fetal aneuploidy detection by maternal plasma DNA sequencing. Obstet Gynecol 2012;119:890–901.
6. Faas BH, de Ligt J, Janssen I, *et al*. Non-invasive prenatal diagnosis of fetal aneuploidies using massively parallel sequencing-by-ligation and evidence that cell-free fetal DNA in the maternal plasma originates from cytotrophoblastic cells. Expert Opin Biol Ther 2012; (Suppl 1):S19–26.
7. Choi H, Lau TK, Jiang FM, *et al*. Fetal aneuploidy screening by maternal plasma DNA sequencing: 'false positive' due to confined placental mosaicism. Prenat Diagn. 2013;33:198–200.
8. Ashoor G, Poon L, Syngelaki A, Mosimann B, Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: effect of maternal and fetal factors. Fetal Diagn Ther 2012;31:237–43.
9. Yandell M, Ence D. A beginner's guide to eukaryotic genome annotation. Nat Rev Genet 2012;18(13):329–42.
10. Shaffer LG, Rosenfeld JA, Dabell MP, *et al*. Detection rates of clinically significant genomic alterations by microarray analysis for specific anomalies detected by ultrasound. Prenat Diagn 2012;32:986–95.