ORIGINAL ARTICLE

Genome-wide copy number analysis on DNA from fetal cells isolated from the blood of pregnant women

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ABSTRACT

Objective Non-invasive prenatal testing (NIPT) based on fetal cells in maternal blood has the advantage over NIPT based on circulating cell-free fetal DNA in that there is no contamination with maternal DNA. This will most likely result in better detection of chromosomal aberrations including subchromosomal defects. The objective of this study was to test whether fetal cells enriched from maternal blood can be used for cell-based NIPT.

Methods We present a method for enriching fetal cells from maternal blood, subsequent amplification of the fetal genome and detection of chromosomal and subchromosomal variations in the genome.

Results An average of 12.8 fetal cells from 30 mL of maternal blood were recovered using our method. Subsequently, whole genome amplification on fetal cells resulted in amplified fetal DNA in amounts and quality high enough to generate array comparative genomic hybridization as well as next-generation sequencing profiles. From one to two fetal cells, we were able to demonstrate copy number differences of whole chromosomes (21, X−, and Y) as well as subchromosomal aberrations (ring X).

Conclusion Intact fetal cells can be isolated from every maternal blood sample. Amplified DNA from isolated fetal cells enabled genetic analysis by array comparative genomic hybridization and next-generation sequencing. © 2016 John Wiley & Sons, Ltd.

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Conflicts of interest: Palle Schelde is the CEO of ARCEDI Biotech, a Danish company that has developed the fetal cell enrichment and detection procedure, presented in this article. Ripudaman Singh and Lotte Hatt are both employed in this company. The Houston authors are faculty and staff at Baylor College of Medicine (BCM), which is a partial owner of Baylor Miraca Genetic Laboratories (BMGL). BMGL offers extensive genetic testing and may offer cell-based NIPT in the future. BCM holds non-patented intellectual property related to this work. ARCEDI Biotech has a patent pending for the markers used for enrichment and detection of circulating fetal cells.

INTRODUCTION

Non-invasive prenatal testing (NIPT) is currently being introduced in many countries, mainly based on the analysis of circulating cell-free fetal (cff) DNA.[1,2] However, a major problem in using cffDNA is that there is an excess of maternal DNA in plasma, making it difficult to reliably detect subchromosomal defects.[3] This problem can potentially be diminished by isolating pure fetal cells from maternal blood and performing genetic analyses on whole genome-amplified DNA from these fetal cells.[4,5] However, a major obstacle for such a strategy has been the extreme rarity of circulating fetal cells, which has resulted in many research groups over the years giving up developing NIPT based on fetal cells.[6]

For more than a decade, we have investigated the presence of fetal cells in maternal blood [7–16] with the ultimate aim of cell-based NIPT. Initially, our research strategy was to define the types of fetal cells that circulate in the maternal blood, and based on this, choose specific markers that can lead to the isolation and identification of pure fetal cells for subsequent analyses. Using gene expression arrays on pools of fetal cells collected from maternal blood, we have developed strong evidence that a major fraction of the circulating fetal cells in maternal blood is endovascular trophoblasts expressing both ectodermal and mesodermal markers.[7,11,12] By characterizing both paternal and maternal alleles in the DNA isolated from circulating trophoblastic cells, other groups have also validated the fetal origin of these cells.[17]

In this communication, we present an improved, high-throughput method by which we can extensively enrich fetal cells from maternal blood samples, spread the enriched fraction on microscope slides, and identify and pick individual fetal cells from the slides. Subsequently, we can perform whole genome amplification (WGA) on the DNA from the picked cells and finally perform array comparative genomic hybridization (array CGH) or next-generation sequencing (NGS) analyses on the amplified DNA. Using this methodology, we successfully demonstrate the gain of chromosome 21 in a female fetus with trisomy 21, as well as the expected copy number differences for the X and Y chromosomes compared with gender-mismatched reference DNA. Furthermore, we are able to detect both a loss of the X chromosome and subchromosomal telomeric deletions involving the X chromosome in separate single cells from a fetus with mosaicism for 45,X; 46,X,r(X) and rare 47,X,r(X)x2 cell lines (confirmed by karyotyping). We believe that this methodology can ultimately be developed into an NIPT assay able to reliably demonstrate even small subchromosomal aberrations.

METHODS

Study design

This study focused on 111 pregnancies. These pregnancies were classified as either 'low risk' (71 samples; gestational age of 10–13 weeks) or 'high risk' (40 samples; gestational age of 10–17 weeks) of carrying fetuses with chromosomal and subchromosomal aberrations. A pregnancy was defined as low/high risk based on maternal age and/or serum screening combined with 'crown rump length' and nuchal translucency results. Based on this, a pregnancy that had a risk of carrying T21, T18, or T13 was classified as 'high risk' and was offered a chorionic villus sampling (CVS) or amniocentesis. Out of these 40 'high risk' pregnancies, only two were shown by CVS or amniocentesis to carry genomic imbalances via conventional chromosome analysis or array CGH analysis. Hence, fetal cells from these two pregnancies were used to perform the cell-based genome analyses. However, blood samples from all 111 pregnant women were used to calculate the frequency of fetal cells in maternal blood and to study the sensitivity and specificity of the fetal cell enrichment and staining markers. The samples from the two 'high risk' pregnancies were used to obtain data on genomic imbalances from fetal cells isolated from maternal blood. For both studies, the blood was processed to lyse erythrocytes, followed by magnetic enrichment and immune staining of fetal cells, smearing on slides, and localization of the fetal cells by automated scanning. In the two 'high risk' samples, after localization on the slides, the fetal cells were picked and subjected to WGA, followed by array CGH and/or NGS.

Clinical material

Following informed consent, participants were recruited from multiple referring centers, including Aarhus University Hospital, Denmark, Baylor College of Medicine, Houston, TX, USA, and other US sites. Participants included women who were pregnant at gestational age of 10–17 weeks. A total of 30 mL of blood was drawn prior to any invasive procedure in either EDTA-containing Vacutainer® tubes (Becton-Dickinson) or cell-free DNA BCT® (Streck). The project was approved by the local Danish Scientific Ethical Committee (S-20070045) and the Danish Data Protection Agency (2008-58-0035), and the Baylor College of Medicine Institutional Review Board (H-24326).

Determination of fetal gender

The gender of the fetus was determined by demonstrating Y chromosome-derived sequences in maternal plasma as previously described.[7]

Lysis of red blood cells

Red blood cells were lysed as previously described with a few modifications.[11] In short, whole blood was fixed in 'fixation buffer' [2% paraformaldehyde in phosphate-buffered saline (PBS)] with gentle mixing for 10 min followed by red blood cell lysis in 0.1% Triton X-100 in PBS for 8 min. After adding 'wash buffer' [2% bovine serum albumin (BSA) in PBS], the cells were pelleted, washed in PBS, and resuspended in 600 μL of PBS.

Magnetic enrichment and staining

Magnetic enrichment and staining of fetal cells were performed essentially as previously described.[12] The unenriched cell pellet from 30 mL blood was split in half and incubated with a cocktail of selection antibodies. ARCEDI holds a patent on the combination of antibodies used for the enrichment and subsequent staining of fetal cells (US patent number 9429520).

After 1 h of incubation, the cells were applied in parallel to two Miltenyi MS columns followed by a wash in PBS buffer. The retained cells on these columns were eluted and combined by flushing with 100 μL PBS into a single collection tube. The enriched cell pellet was washed twice in PBS. After the second wash, the enriched cell pellet was resuspended in 1 ml PBS and applied to a fresh MS column. Subsequent staining of fetal cells was performed using a cocktail of cytokeratin antibodies, on a Miltenyi MS column while in the magnet. Briefly, 500 μl 'blocking buffer' (4 x SSC, 0.5% casein, 1% BSA, 10% goat serum) was applied to the column followed by a 10 min incubation. Then the primary cytokeratin antibody cocktail was applied to the column followed by a 30 min incubation at room temperature. After washing three times with 500 μl buffer, the fluorescent secondary antibody cocktail was applied followed by a 30 min incubation at room temperature. The column was again washed three times with 500 μL PBS, and retained cells were eluted from the column into the same collection tube by flushing in 1 mL PBS. The final stained cell pellet was washed in PBS, spread on slides, and dried at room temperature overnight. The next morning, the slides were submerged in 4% formaldehyde for 10 min, washed in PBS for 5 min, and finally mounted in Vectashield with 0.6 μg/mL 4'6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Automated scanning and visual validation

Enriched and cytokeratin-stained cells were located on the slides by automatic scanning using a MetaSystems scanner in MetaCyte mode and with an in-house-developed 'classifier'. The classifier consisted of a computer algorithm that identified positive fetal cells based on custom parameters such as cell size, morphology, and staining intensity. For the automated scanning, a 10x objective was used. Green fluorescent cells picked out by the microscope scanner were finally validated manually using a 40x objective based on a set of in-house-developed criteria. Both the classifier criteria and the criteria for final visual validation were established on samples from women carrying a male fetus by performing X and Y chromosome fluorescence *in situ* hybridization (FISH) on cells identified as fetal.

X and Y chromosome fluorescence *in situ* hybridization

X and Y chromosome FISH was performed as previously described with slight modifications.[9] Chromosome-specific repeat probes, DXZ1 (CEP X alpha satellite) labeled with spectrum green and two DYZ1 (CEP Y satellite III) probes labeled with spectrum orange and spectrum aqua (Abbott Molecular), respectively, were used. After FISH, slides were mounted in Vectashield with 0.6 μg/mL DAPI.

Picking of fetal cells

Fetal cells on the scanned slides were picked individually or in groups using either the CellCelector from ALS Automated Lab Solutions GmbH (Jena, Germany) or the CytePicker from RareCyte (Seattle, USA) following the manufacturer's protocols. DNA from the collected fetal cells was then amplified, and subsequent array CGH analyses were performed using the method described by Bi *et al.*,[18] with slight modifications.

Whole genome amplification

Whole genome amplification on the picked cell and quality control of the resulting amplification products was performed using the Ampli1™ WGA kit and Ampli1™ QC kit from Silicon Biosystems, using the manufacturer's protocols.

Array comparative genomic hybridization

Array CGH was performed on WGA DNA using SurePrint G3 human CGH 4 × 180K arrays from Agilent Technologies as described previously.[18] Briefly, 1 μg of WGA DNA was labeled per hybridization, using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) for quantification. Fetal DNA was labeled with dCTP-Cy5, and reference DNA was labeled with dCTP-Cy3, for 3 h at 37 °C using the SureTag DNA labeling kit (Agilent). A multiscreen-PCRμ96 filter plate (Millipore, Billerica, MA, USA) was used to remove unincorporated nucleotides. Hybridizations were carried out at 65 °C for 40–72 h, after which they were washed and scanned using an Agilent microarray scanner (PN G2565BA). Data were extracted using Agilent feature extraction software (version 9.5.3.1) and analyzed using Agilent cytogenomics edition 2.5.8.11. The DNA used as a reference for each fetal cell WGA product was a pool of WGA DNA from multiple (5–10 single cells) WGA reactions from either male or female lymphoblast reference cell lines. Gender-mismatched references were used unless otherwise indicated.

Bioinformatics analysis of single-cell next-generation sequencing data

Paired-end sequencing reads were obtained for each sample. Reads were mapped to human reference genome hg19 using Burrows–Wheeler aligner.[19] Base quality recalibration and local realignment were performed using the genome analysis tool kit.[20] Samtools[21] was used to sort and index the resultant binary alignment/map (BAM) files.

To identify copy number variations, first, the human genome was divided into slide windows according to the control sample. The aligned reads of the control sample were binned into variable-length windows, and the number of mapped reads in every window was the same. Then the aligned reads of the case sample were counted in each window, and the log2 ratio between the case and control was calculated. For trisomy detection, there were 4000 mapped reads within each window, and the total number of windows across the genome was around 2100. The segmentation and copy number estimation were performed using CGHweb R package.[22]

RESULTS

Fetal cell enrichment and detection

An overview of the fetal cell isolation procedure and the time required for each step is shown in Table 1, together with the enrichment factor (expressed as the approximate number of remaining maternal cells). The procedure, including the WGA step that gives DNA for array CGH, takes around 20 h of

Table 1 Approximate number of maternal blood cells after the different steps in the procedure and time consumption

Procedure step	Approximate number of maternal cells after each step	Time required per sample
A. Blood sampling – 30 mL whole blood from a pregnant woman	1.3×10^{11} RBC, $1.0–3.0 \times 10^{8}$ WBC	10 min
B. Blood processing – fixation and RBC lysis	$1.0–3.0 \times 10^{8}$ WBC	3 h
C. Fetal cell separation, staining, and spreading on the slides	120 000 WBC	4 h
D. Drying of slides	No change	6 h
E. Automated scanning	100–200 WBC	2 h
F. Visual selection of true fetal cells	0	15 min

RBC, red blood cells; WBC, white blood cells.

technical time. The initial enrichment step involved a selective lysis of red blood cells resulting in a pellet of maternal white blood cells and fetal cells (Table 1),[7] followed by magnetic-activated cell sorting enrichment of fetal cells, and their subsequent staining on the magnetic column using a cocktail of cytokeratin staining markers (Table 1). This resulted in a 10 000-fold enrichment of fetal cells. Following enrichment, we were left with approximately 100–200 'positive events' from each sample, the majority of which were artifacts (Table 1). These artifacts were easily discounted by manual inspection of each event based on a set of criteria for fetal cell morphology.[7,11,12] Apart from the few fetal cells with very fragmented nuclei, two types of cytokeratin-stained fetal cells could be recognized, namely, fetal cells with an even cytoplasmic stain, and those with the cytokeratin stain localized in small intense 'bubbles', mainly around the nucleus.

Marker sensitivity

After establishing the criteria to define fetal cells, we used the fetal cell enrichment and identification procedure to estimate the frequency of fetal cells in maternal blood by analyzing samples from 111 pregnancies (gestational age 10–13 weeks). We found 1422 fetal cells in total with a mean of 12.8 cells per sample, and a median of 11.0. Furthermore, we found a high level of variation in the number of fetal cells among pregnant women, with a range of 1–45 fetal cells per 30 mL of blood (Figure 1).

Marker specificity

Using the FISH approach described previously, we were also able to estimate the specificity of our marker combination for identification of fetal cells. Using only pregnancies known to be carrying a male fetus, 148 stained cells identified as likely fetal from 11 pregnancies were subsequently subjected to X and Y chromosome FISH. Out of these, 116 cells had signals for the Y probes and one for the X probe, while none had two signals for the X probe. The remaining cells were either lost (eight cells) or gave no FISH signals (23 cells) because of very condensed nuclei. In the 11 samples, the fetal cells confirmed by X and Y chromosome FISH were found in the range of 5–20. Figure 2 shows a series of pictures of the fetal cells, both after cytokeratin staining (A) and after subsequent X and Y chromosome FISH (B).

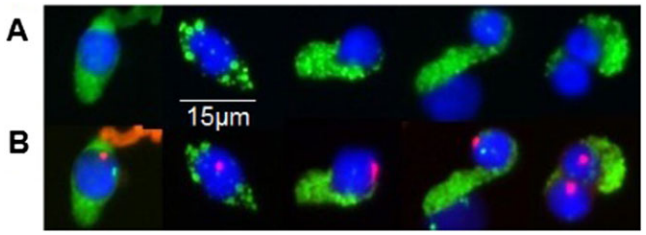

Figure 2 A gallery of fetal cells enriched and stained by the present method. Row (A) shows fetal cells from different pregnancies enriched and stained using the present method. In row (B), the same cells are shown after X and Y chromosome fluorescence in situ hybridization. The Y chromosome is depicted by a red spot in the nucleus

Detection of chromosome aberrations and fetal cells

Using the pregnancy risk assessment described previously, and by subsequent CVS/amniocentesis, two pregnancies were shown to carry abnormal fetuses. Figure 3 shows the result of an array CGH and NGS analysis of two pooled fetal cells (B) from a pregnancy (HR33) carrying a female fetus with trisomy 21 compared with opposite gender reference DNA. The gender difference and the gain of chromosome 21 (enlarged in C) are clearly seen and are detected by the standard

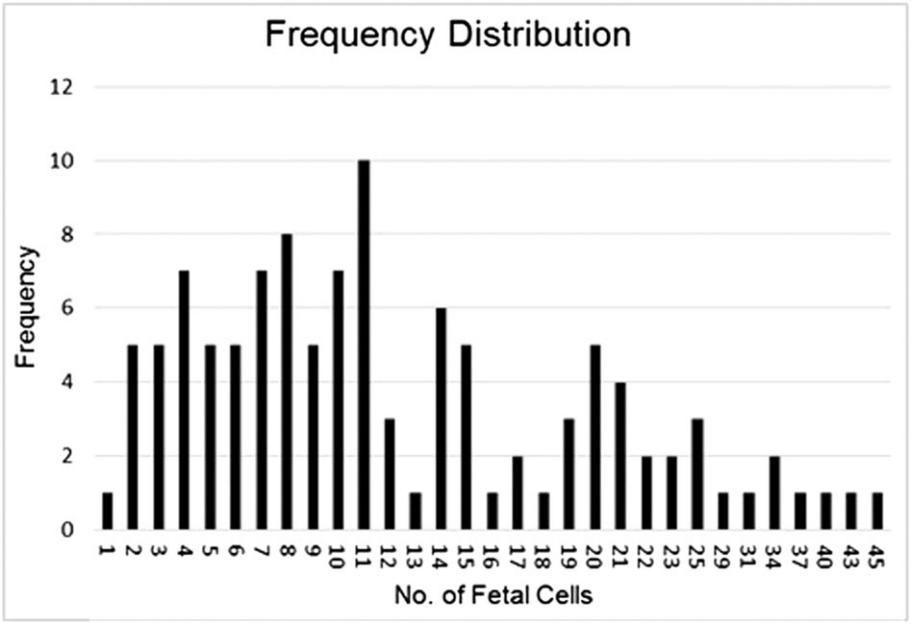

Figure 1 Frequency distribution of fetal cells in 111 samples

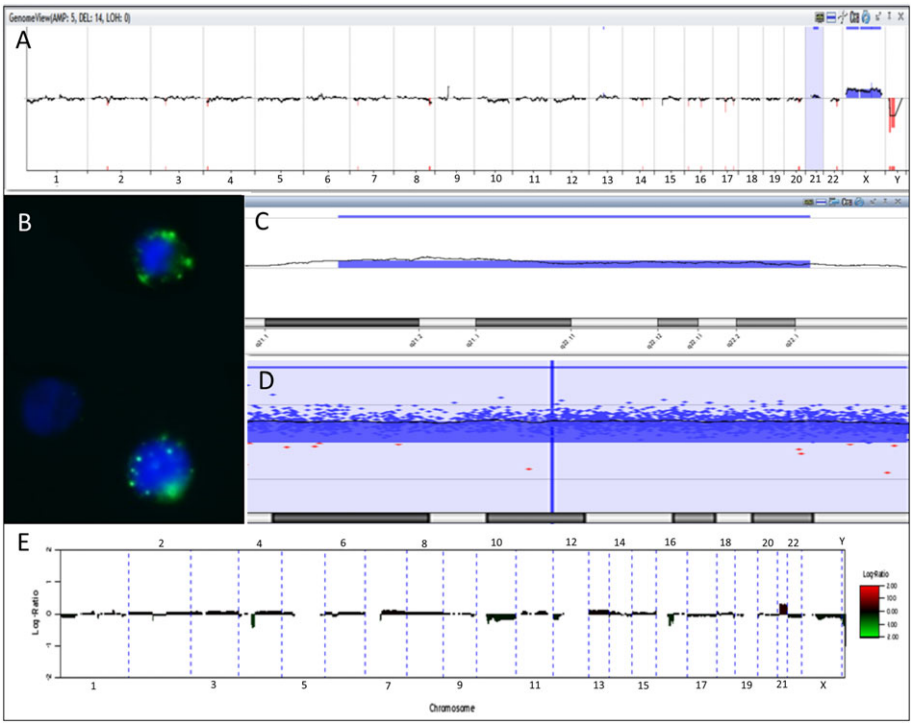

Figure 3 Array comparative genomic hybridization and next-generation sequencing (NGS) on two fetal cells from a trisomy 21 fetus. (A) Whole genome plot from two pooled fetal cells (B) derived from a female fetus with trisomy 21, hybridized with a normal male reference. The array shows the expected gain of chromosome 21 (enlarged in C). This is corroborated by the array results from the chorionic villus sampling (D) and NGS results from the two pooled cells (E)

software. Figure 4 shows array CGH and NGS results from a pregnancy (NIPT413) carrying a fetus with mosaicism for a monosomy X cell line and a cell line containing 1–2 copies of a ring X chromosome: 45,X[10]/46,X,r(X)(p21.3q26.3)[9]/47, X,r(X)(p21.3q26.3)x2[1]. Two single fetal cells were isolated from the maternal blood for this case. The first cell (A) harbors the 45,X chromosome complement as shown by the loss of the entire X chromosome (B, E, and J), whereas the second cell (C) harbors the 46,X,r(X) chromosome complement as shown by the loss of the distal short arm (p) and distal long arm (q) on the X chromosome (D, F, and K). This is corroborated by the array CGH and karyotype results on the cells from the amniotic fluid (Figure 4G–I).

DISCUSSION

The presented method is able to enrich a highly purified sample of fetal cells, and from these cells produce whole genome-amplified fetal DNA of sufficient quantity and quality to do array CGH and NGS analyses. The data also show that an accurate copy number assessment can be made from only one to two cells, although our data suggest that larger numbers of cells might be available for analysis in most cases (Figure 2). It is noteworthy that most of the fetal cells that are enriched by our method are the so-called 'bubble' cells, which are described by the 'inclusion type' of cytokeratin-staining pattern. This 'bubble' type of cytokeratin staining has also been described in circulating tumor cells from breast cancer by Mèhes *et al.*,[23] who also performed terminal deoxynucleotidyl transferase dUTP nick end labeling assays and found positive staining in cells with large 'bubbles' and very condensed nuclei. Hence, they concluded that the circulating tumor cells with large 'bubbles' were apoptotic. We assume that our fetal cells that show inclusion type of cytokeratin staining do so because they are undergoing epithelial to mesenchymal transitions.[12] Some fetal cells might also be in the early stages of apoptosis. However, this does not appear to interfere with the downstream analysis (WGA and subsequent array CGH and NGS).

As previously mentioned, we found on average 12.8 fetal cells in 30 mL of maternal blood. Few data exist about the exact number of fetal cells circulating in maternal blood, because only a few groups have directly counted fetal cells after XY FISH in unenriched or only modestly enriched populations of nucleated cells from the blood of pregnant women carrying a male fetus.[24–29] Of these, Hamada *et al.* have performed the analysis down to gestational week 12 and found one to two fetal cells per milliliter of maternal blood,[25] while Emad *et al.*[24] estimated slightly higher numbers. In comparison, we found a mean of 0.43 fetal cells per milliliter of blood with our markers and were able to reach the value of over one cell per milliliter of blood in seven out of 111 samples, suggesting that some fetal cells are not being efficiently captured by this method. This could be due to technical limitations of the enrichment method, or due to the fact that we enrich only a specific cell type, namely, endovascular trophoblasts,[12] and there may be other fetal cell types such as fetal-nucleated red blood cells in the maternal blood stream.[30] Also, the fact that we found no cells with two X signals among the 148 putative

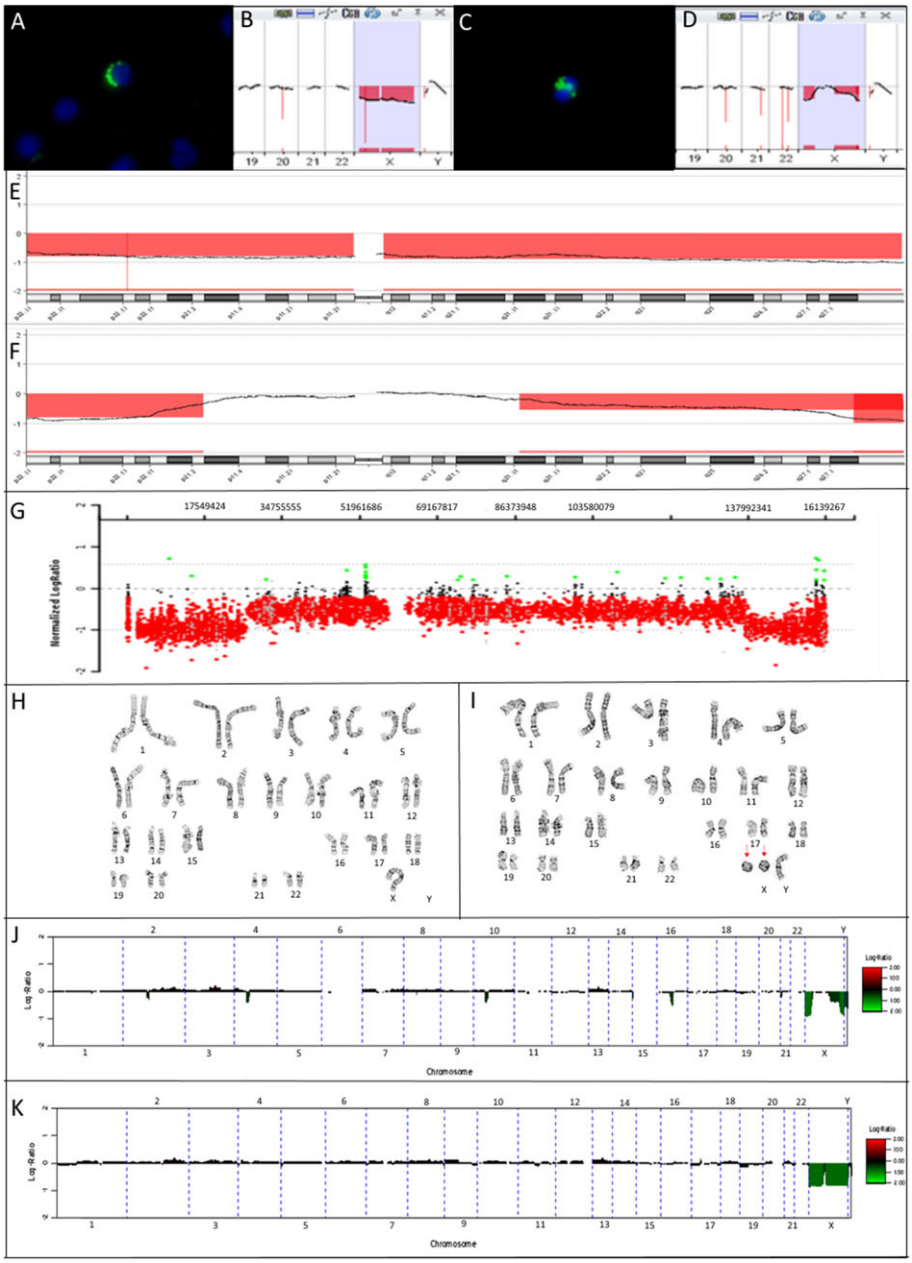

Figure 4 Chromosomal analyses on two single fetal cells and amniotic fluid from a fetus with mosaicism for 45,X; 46,X,r(X) and rare 47,X,r(X) x2 cell lines. Karyotype, array comparative genomic hybridization (CGH), and next-generation sequencing (NGS) data from a pregnancy (non-invasive prenatal testing 413) carrying a fetus with mosaicism for 45,X; 46,X,r(X) and rare 47,X,r(X)x2 cell lines. Two single fetal cells were isolated and analyzed by array CGH and NGS. The first cell (A) harbors the 45,X chromosome compliment as shown by the loss of the entire X chromosome (B, enlarged in E), whereas the second cell (C) seems to harbor the 46,X,r(X) chromosome complement as shown by the loss of the distal short arm (p) and long arm (q) on the X chromosome (D, enlarged in E). (G) shows array results on direct amniotic fluid showing combined copy number for the mixed population of 45,X; 46,X,r(X) and rare 47,X,r(X)x2 cells. (H) and (I) show the karyotypes for 45,X and 47,X,r(X)x2, respectively. This is also supported by the NGS results from the two cells (J and K, respectively)

fetal cells from male pregnancies indicates a very high fetal cell specificity of our marker combination.

It was encouraging to see that fetal genome analyses could be performed on as few as one fetal cell. We chose to pool the two fetal cells from HR33 for the analyses. However, because NIPT413 carried a fetus with mosaicism for 45,X; 46, X,r(X) and 47,X,r(X)x2, we decided to do genome analyses on single fetal cells. WGA is an essential step to provide the sufficient amount of DNA for the downstream analysis. A previous study has shown that WGA performed on few cultured fetal cells from amniotic fluid did have a sufficient genome coverage to provide accurate detection of major fetal chromosome abnormalities.[31] Some of the authors of this article have previously shown that WGA on single cells could be used to detect copy number changes larger than 1 MB using array CGH.[18,32]

The modest time required for the individual steps of the enrichment procedure (Table 1) is encouraging, and we believe

that the fetal cell isolation procedure, with proper automation, can be reduced to less than 48 h. The duration of the downstream portions of the procedure, including WGA, array CGH, and NGS, can most likely be reduced to 3 days or less, altogether making it realistic on a routine basis to perform the total procedure in less than a week.

We believe that our current method will be well suited for noninvasive detection of aneuploidy and even large subchromosomal deletions and duplications in a prenatal screening program, and the method could replace cffDNA-based NIPT in time. Furthermore, an important advantage of the cell-based approach will be the absence of contaminating maternal DNA that will enable detection of smaller microdeletions that are currently beyond the limits of detection of cffDNA-based NIPT.

However, in order to launch this method as a routine prenatal test, a number of additional conditions must be fulfilled. First, a method of this complexity has to be centralized, making sample transportation critical.

In the current study, two out of 40 'high-risk' pregnancies were found to carry abnormal fetuses by invasive procedures (CVS/amniocentesis). The fetal cells from these two 'high-risk' pregnancies were used to check whether the genomic information about the fetus from the cells was corroborated with the data from CVS/amniocentesis. In addition to the stability of the samples, we also feel that it is important to extensively validate the performance of cell-based NIPT. Therefore, we have planned a clinical validation study on high-risk pregnancies, where prenatal genetic analyses will be performed on CVS samples and compared with cell-free NIPT and cell-based NIPT.

MATERIALS AVAILABILITY

An aliquot of selection and staining antibody cocktails can be obtained for research purposes upon request.

ACKNOWLEDGEMENTS

This article is dedicated to Professor Steen Kølvraa (first author) who spent many years developing and leading the project in Denmark. He also developed the microscopic classifiers and did the largest portion of the writing. Professor Kølvraa passed away on 31 March 2016.

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Authorship

R. S. designed and performed experiments, analyzed the data, and contributed to writing. E. A. N. and S. Q. designed and performed experiments and analyzed data; I. B. V. was responsible for human subjects approval and provided expertise and advice in Houston; L. J. provided expertise and advice to the Houston project over many years; L. H. designed and performed experiments and analyzed data; P. S. designed and performed experiments and analyzed data, managed the project in Denmark, and spent considerable time in Houston to make the collaboration successful; N. U. collected the clinical material in Denmark; E. M. V. performed array CGH analysis on CVS samples. L. Z., R. C., and C. A. S. performed the NGS analysis on fetal cells. A. M. B. co-led the project in Houston, designed and performed experiments, analyzed the data, and contributed to writing; A. L. B. was the project leader in Houston and contributed to writing.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Fetal cells circulating in maternal blood can be enriched with high specificity using markers specific for extravillous trophoblasts.

WHAT DOES THIS STUDY ADD?

- Enriched fetal cells from maternal blood can be used for performing whole genome amplification, followed by array CGH and next-generation sequencing paving the way for cell-based NIPT.

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