Copy number variation sequencing-based prenatal diagnosis using cell-free fetal DNA in amniotic fluid

Qingwei Qi1*, Sijia Lu2, Xiya Zhou1, Fengxia Yao1, Na Hao1, Guangjun Yin2, Wenhui Li2, Junjie Bai3, Ning Li3 and David S. Cram4

1Department of Obstetrics and Gynecology, Peking Union Medical College Hospital (PUMCH), Beijing, China
2Yikon Genomics Co., Ltd, Beijing, China
3Becreative Lab Co., Ltd, Beijing, China
4Berry Genomics Co., Ltd, Beijing, China
*Correspondence to: Qingwei Qi. E-mail: qiqingwei@163.com

ABSTRACT

Objective The study aimed to determine whether cell-free fetal DNA (cffDNA) present in amniotic fluid supernatant can be used as a surrogate for amniocyte-based diagnosis of fetal chromosomal abnormalities.

Method Amniocentesis was performed on 28 high-risk pregnancies. Amniocytes and the cffDNA fraction were prepared from the amniotic fluid samples. Chromosomal analysis of amniocytes was performed by either karyotyping or single nucleotide polymorphism (SNP) arrays. The corresponding cffDNA samples were blindly analyzed by copy number variation (CNV) sequencing in an independent laboratory.

Results In the 28 matching amniocyte and cffDNA samples, there was a high diagnostic concordance for detection of euploidy, aneuploidy and CNVs. From ten samples referred for karyotyping, two aneuploidies (20%) were identified. From 18 samples referred for SNP array analysis, three pathogenic CNVs (16.7%) were identified. CNV sequencing of the 28 cffDNA samples also detected the two aneuploidies and the three pathogenic CNVs, giving an overall concordance rate of 100% for detection of pathogenic chromosome abnormalities. Compared with SNP array analysis, CNV sequencing returned a higher yield of benign or variants of unknown significance.

Conclusion Copy number variation sequencing of cffDNA represents an alternative approach to conventional prenatal diagnostic methods for reliable and accurate detection of clinically significant chromosomal abnormalities.

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Conflicts of interest: Mr. Sijia Lu, Mr. Guangjun Yin and Ms. Wenhui Li are employees of Yikon Genomics Co., Ltd. Mr. Junjie Bai and Ms. Ning Li are employees of Becreative Lab (Beijing) Co., Ltd. Dr David Cram is an employee of Berry Genomics. None of them has any stock or bond holdings.

INTRODUCTION

For women of advanced reproductive age or those who have returned a high-risk maternal serum screening result, cytogenetic karyotyping of amniocytes retrieved by amniocentesis at 16 weeks’ gestation is the gold standard method for diagnosis of fetal abnormalities. Karyotyping can detect a wide range of chromosomal abnormalities including trisomies, monosomies, polyploidy, balanced and unbalanced translocations, mosaicism and copy number variations (CNVs) at a resolution of >10 Mb.1 Prior to karyotyping, amniocytes are routinely cultured, and thus, cytogenetic results are normally obtained within a time frame of 10–14 days post-sampling. While cell culture of amniocytes is generally reliable, occasionally, there are issues that can compromise the diagnosis including failure of cell growth, blood and maternal cell contamination (MCC) and cell artifacts such as the appearance of low numbers of mosaic cells.2

When particular fetal genetic abnormalities are suspected, chromosomal microarray analysis (CMA) is now the preferred method for rapid and detailed high-resolution genomic analysis of uncultured amniocytes.3,4 The introduction of high-resolution oligonucleotide and single nucleotide polymorphism (SNP) arrays has revolutionized prenatal diagnosis,5 enabling more comprehensive and accurate identification of a wider spectrum of chromosome abnormalities.6 However, occasionally, variants of unknown significance (VOUS) still remain problematic to give a conclusive diagnosis.7 More recently, next-generation sequencing is emerging as an alternative to CMA for detection of pathogenic CNVs.8 Several studies have already shown that next-generation sequencing is highly reliable and accurate for detection of CNVs associated with miscarriage9 and fetal structural abnormalities.10–12
In addition to amniocytes, amniotic fluid (AF) also contains cell-free fetal DNA (cffDNA). Although cffDNA is highly fragmented (100–400 bp), it represents the complete fetal genome and is essentially free of maternal or trophoblast-derived DNA.13–15 Several promising reports have demonstrated the feasibility of using cffDNA for array comparative genomic hybridization-based prenatal diagnosis.16–19 To date, limited data exist regarding the accuracy and reproducibility of cffDNA as an alternative DNA template for molecular diagnosis. In this study, we investigate whether CNV sequencing of cffDNA can reliably and accurately detect fetal chromosomal abnormalities by benchmarking against matching amniocyte samples analyzed by gold standard karyotyping or SNP array methods.

MATERIAL AND METHODS

Case selection

The ethical approval of this study was given by the Ethical Investigation Committee of Peking Union Medical College Hospital. All patients undertaking prenatal diagnosis provided written informed consent. From January 2013 to October 2014, a total of 28 samples of second-trimester AF were collected in a double-blind case-control study. Ten samples were referred for karyotyping based on an abnormal serum screening test or for advanced maternal age (Table 1). A further 18 samples were referred for SNP array analysis following detection of fetal ultrasonographic abnormalities (Table 2). Matching cffDNA samples were coded and analyzed blindly by CNV sequencing. Results from cffDNA samples were compared with results from amniocyte samples to evaluate the diagnostic concordance between CNV sequencing and karyotyping/SNP analysis.

Isolation of cffDNA from AF

Amniotic fluid samples (20 mL) were collected in the ultrasound unit by amniocentesis. Amniocytes for cytogenetic and molecular evaluation were isolated from the AF samples by centrifugation at 300×g for 10 min. Fresh AF supernatant samples were processed immediately upon receipt. Only samples that were free of visible maternal blood contamination were included in study. Extraction of cffDNA was performed with the Magbind free-circulating DNA kit (Cwbio, Changping, Beijing, China) according to the standard protocol provided by manufacturer. The bound cffDNA was eluted with 50 μL of elution buffer and stored at −80 °C until further processing. Before storage, the purity of the eluted DNA was assessed with the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). From 400 μL of AF supernatant, approximately 400 ng of cffDNA was routinely extracted for CNV sequencing analysis.

Karyotyping

Chromosome analysis using G-banding was performed according to standard procedures at the Laboratory of Medical Genetics, Peking Union Medical College Hospital. A total of 20 metaphase cells were analyzed at a resolution of 400 bands. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.

SNP array analysis

Single nucleotide polymorphism array analysis was performed by the Beijing Becreative Laboratory. In brief, genomic DNA was extracted from uncultured amniocytes using the Qiagen Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Genome-wide high-resolution SNP array CytoScan HD (Affymetrix, Santa Clara, CA, USA) was used containing both SNPs and oligonucleotide probes. Procedures for DNA digestion, ligation, polymerase chain reaction (PCR) amplification, fragmentation, labeling and hybridization were performed according to the manufacturer’s protocol (Affymetrix). The reporting threshold of copy number change was set at 100 kb with a marker count ≥50.

Whole genome CNV sequencing analysis of cffDNA

Copy number variation sequencing analysis of cffDNA was conducted independently on coded samples by Yikon Genomics. In brief, purified cffDNA (40 ng) was used to construct sequencing libraries with the NEB Next Ultra DNA library Prep Kit (NEB). The constructed libraries were

Table 1 Karyotyping versus CNV sequencing for detection of chromosome anomalies

<table>
<thead>
<tr>
<th>Case</th>
<th>Gestational age (weeks)</th>
<th>Indication of amniocentesis</th>
<th>Karyotype of amniocytes</th>
<th>Karyotype of cffDNA</th>
<th>Diagnostic concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>Advanced maternal age</td>
<td>47,XY,+21</td>
<td>47,XY,+21</td>
<td>Concordant</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>Abnormal maternal screening test</td>
<td>46,XX</td>
<td>46,XX</td>
<td>Concordant</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>Abnormal maternal screening test</td>
<td>46,XX</td>
<td>46,XX</td>
<td>Concordant</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>Abnormal maternal screening test</td>
<td>46,XY</td>
<td>46,XY</td>
<td>Concordant</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>Abnormal maternal screening test</td>
<td>45,X</td>
<td>45,X</td>
<td>Concordant</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>Advanced maternal age</td>
<td>46,XX</td>
<td>46,XX</td>
<td>Concordant</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
<td>Advanced maternal age</td>
<td>46,XY</td>
<td>46,XY</td>
<td>Concordant</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>Abnormal maternal screening test</td>
<td>46,XY</td>
<td>46,XY</td>
<td>Concordant</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>Abnormal maternal screening test</td>
<td>46,XX</td>
<td>46,XX</td>
<td>Concordant</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>Abnormal maternal screening test</td>
<td>46,XY</td>
<td>46,XY</td>
<td>Concordant</td>
</tr>
</tbody>
</table>

cffDNA, cell-free fetal DNA; CNV, copy number variation.
### Table 2: SNP array versus CNV sequencing for detection of chromosome anomalies

<table>
<thead>
<tr>
<th>Case</th>
<th>GA</th>
<th>Ultrasound finding</th>
<th>Molecular karyotype</th>
<th>CNV size (Mb)</th>
<th>CNV type</th>
<th>Cytogenetics/hg19 coordinates</th>
<th>Clinical significance of CNV</th>
<th>Concordance between methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>Agenesis of corpus; cerebellar hypoplasia</td>
<td>Arr(1–22)x2, (XY)</td>
<td>0.11</td>
<td>Del</td>
<td>1p33/49.570.001–49.680.000</td>
<td>Likely benign</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>Severe ventriculomegaly</td>
<td>Arr(1–22)x2, (XY)</td>
<td>0.85</td>
<td>Dup</td>
<td>15q11.2/22.410.001–23.260.000</td>
<td>VOUS</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>Severe ventriculomegaly</td>
<td>Arr(1–22)x2, (XY)</td>
<td>0.18</td>
<td>Del</td>
<td>15q14/34.690.001–34.870.000</td>
<td>Likely benign</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>Atrioventricular septum defect; cleft lip</td>
<td>Arr(1–22)x2, (XY)</td>
<td>0.16</td>
<td>Del</td>
<td>4q13.2/70.100.001–70.260.000</td>
<td>Likely benign</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>Meningocele</td>
<td>Arr(1–22)x2, (XY)</td>
<td>3.43</td>
<td>Del</td>
<td>1p11.22q11.23/25.403.868–28.438.826</td>
<td>VOUS</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>Cleft lip, cleft palate</td>
<td>Arr(1–22)x2, (XY)</td>
<td>0.13</td>
<td>Del</td>
<td>15q14/34.690.001–34.820.000</td>
<td>Likely benign</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>7</td>
<td>23</td>
<td>Cerebellar hypoplasia; fetal growth restriction</td>
<td>Arr(1–22)x2, (XY)</td>
<td>1.68</td>
<td>Dup</td>
<td>Xp22.31/6.455.151–8.135.568</td>
<td>VOUS</td>
<td>Fully concordant</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>Absent nasal bone; echogenic bowel; fetal growth restriction</td>
<td>Arr(1–22)x2, (XY)</td>
<td>1.85</td>
<td>Dup</td>
<td>Xp22.31/6.320.001–8.170.000</td>
<td>VOUS</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>9</td>
<td>24</td>
<td>Hydrocephaly; severe fetal growth restriction</td>
<td>Arr(1–22)x2, (XY)</td>
<td>0.58</td>
<td>Dup</td>
<td>21p11.2/9.830.001–10.410.000</td>
<td>VOUS</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>Ventricular septum defect; nasal bone dysplasia</td>
<td>Arr(1–22)x2, (XY)</td>
<td>0.58</td>
<td>Dup</td>
<td>15q15.1/28.400.001–28.980.000</td>
<td>VOUS</td>
<td>Partially concordant</td>
</tr>
<tr>
<td>11</td>
<td>18</td>
<td>Skeletal dysplasia</td>
<td>Arr(1–22)x2, (XY)</td>
<td>17.4</td>
<td>Dup x2</td>
<td>12p13.33p11.1/173.786–34.835.641</td>
<td>Pallister–Killian syndrome</td>
<td>Fully concordant</td>
</tr>
<tr>
<td>12</td>
<td>24</td>
<td>Severe ventriculomegaly; several fetal growth restriction</td>
<td>Arr(1–22)x2, (XY)</td>
<td>0.121</td>
<td>Dup</td>
<td>9q31.3/11.3.431.222–11.3.552.613</td>
<td>VOUS</td>
<td>Fully concordant</td>
</tr>
<tr>
<td>Case</td>
<td>Chromosome</td>
<td>Cytogenetic Abnormality</td>
<td>Description</td>
<td>CNV Coordinates</td>
<td>Significance</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>22</td>
<td>Complex cardiac anomaly</td>
<td>Arr(1-22)x2, (XY)</td>
<td>4.09 Dup 11p11.2-q11/55.290.145</td>
<td>VOUS Fully concordant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>18</td>
<td>Absent nasal bone; echogenic bowel; single umbilical artery; fetal growth restriction</td>
<td>Seq(1-22)x2, (XY)</td>
<td>4.53 Del 2p11.2/90.030.001-90.410.000</td>
<td>Likely benign</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>15</td>
<td>20</td>
<td>Tetralogy of Fallot</td>
<td>Arr(1-22)x2, (XY)</td>
<td>1.55 Del Xq28/153.681.800-155.233.098</td>
<td>Xq28 microduplication syndrome Fully concordant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>Omphalocele; clubfoot; clenched hand</td>
<td>Seq(1-22)x2, (XY)</td>
<td>1.59 Del Xq28/153.290.001-154.880.000</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>17</td>
<td>24</td>
<td>Severe fetal growth restriction</td>
<td>Arr(1-22)x2, (XY)</td>
<td>0.51 Del 2p11.2/89.900.001-90.410.000</td>
<td>Likely benign</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>Skeletal dysplasia</td>
<td>Arr(1-22)x2, (XY)</td>
<td>0.106 Del 2q13/110.873.834-110.980.295</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Seq(1-22)x2, (XY)</td>
<td>0.13 Del 2q13/110.850.000-110.980.000</td>
<td>Carrier of NPHP1 gene deletion Fully concordant</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CNV, copy number variation; GA, gestational age (weeks); SNP, single nucleotide polymorphism; VOUS, variants of unknown significance.

aNumbers in brackets represent hg19 reference genome coordinates.

The karyotype of this case is 47,XY,+i(12)(p10).
sequenced on an Illumina HiSeq 2500 platform to generate a total of ~10 million reads. Chromosomal CNV analysis was performed as previously described. Read numbers were counted and displayed with a bin size of 10 kb along the whole genome. Copy number gain from two to three copies results in a 50% increase in read counts in a genome segment, while copy number loss from two to one results in a 50% decrease in read counts in a genome segment. After GC correction, mapped reads were normalized to relative read numbers at 30–35 reads per 10-kb bin for CNV analysis. Relative read numbers in test and control bins were compared to calculate CNV. CNV Z-score analysis using the Z-test was performed as described previously. CNV analysis was conducted using a set of proprietary Perl scripts, and CNV visualization was processed with a proprietary R script. In this study, data from 10-kb sequencing bins were used for generating chromosome CNV plots.

All pathogenic CNVs were verified by quantitative fluorescence polymerase chain reaction (QF-PCR). QF-PCR target regions were chosen based on the CNV Z-score analysis results, and one bin for each chromosome arm was chosen as the amplification target region using two sets of QF-PCR primers designed to different regions within the selected bin. Three housekeeping genes (beta-actin, beta-tubulin and GAPDH) were used as internal controls for QF-PCR. The cycle threshold (Ct) values of housekeeping genes were used to normalize input DNA between samples. Ct values of target regions in each sample were compared with Ct values of housekeeping genes of the same sample for estimation of CNVs. QF-PCR was performed in a 20-μL volume containing 10-μL 2 × SYBR® Premix Ex Taq™ (TaKaRa, Dalian, Liaoning, China), 2 μL of genomic DNA (100 ng), 0.15 μL of each gene-specific primer (10 μM) and 8 μL ddH2O. The PCR conditions were as follows: 95 °C for 3 min, 40 cycles of 95 °C for 15 s, 59 °C for 15 s and 72 °C for 20 s. Three replicates were used for each sample. Reactions were conducted on a CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). All data were analyzed using the CFX Manager Software (Bio-Rad).

Interpretation of the results of SNP array and CNV sequencing analysis

For the interpretation of CNVs detected by either the SNP array or the CNV sequencing, the following databases were interrogated: database of genomic variants (http://projects.tcag.ca/variation/), DECIPHER database (http://decipher.sanger.ac.uk), Online Mendelian Inheritance in Man (http://www.omim.org), UCSC (http://genome.ucsc.edu/hg19), the International Standards for Cytogenomics Arrays (http://www.iscaconsortium.org) and CAG database (http://www.cagdb.org). All pathogenic CNVs revealed by CNV sequencing were further confirmed by QF-PCR. The significance of the detected CNVs was determined according to the guidelines of American College of Medical Genetics and Genomics.

RESULTS

A total of 28 prenatal samples were included in the study with 10 referred for karyotyping and 18 for SNP array analysis. In parallel, the cfDNA from the matching AF samples was coded and then analyzed by CNV sequencing. Following diagnosis, the CNV sequencing results were unblinded and compared with their respective karyotyping and SNP array results. The results of karyotyping and CNV sequencing for the ten matched samples are summarized in Table 1. Diagnoses by karyotyping and CNV sequencing were fully concordant, comprising two aneuploid samples with trisomy 21 and monosomy X (Figure 1) and eight euploid samples.

Comparative results for SNP array and CNV sequencing analysis of the remaining 18 samples from pregnancies with ultrasonographic fetal abnormalities are summarized in

A. Down Syndrome

![Down Syndrome](image)

B. Turner Syndrome

![Turner Syndrome](image)

Figure 1 Detection of aneuploidy using copy number variation (CNV) sequencing. Sequencing results are plotted as copy number (y-axis) versus chromosome (x-axis). (A) 47,XY,+21 and (B) 45,X. A red box indicates chromosome copy number changes. The diagnosis by CNV sequencing was fully concordant with conventional karyotyping.
Table 2. Of the 18 matching samples, six were completely concordant, and 12 were partially concordant for different types of CNVs. Importantly, two samples with pathogenic CNVs involving tetraploidy of 12p (Pallister–Killian syndrome) and a 1.55-Mb Xq 28 duplication (Xq28 microduplication syndrome) were identified by both methods (Figure 2). In addition, a 106-kb deletion in one copy of the NPHP1 gene associated with carrier status for nephronophthisis type 1 disease was also identified by both methods (Figure 2). For all three CNVs, the defined interval of the duplication or deletion was very similar between SNP array and CNV sequencing.

The three other concordant samples involved a 4-Mb 11p polymorphism, a 1.68-Mb Xp22.31 duplication and a 121-kb 9q31.3 duplication (Table 2) and were classed as VOUS. In 11 of the 12 partially concordant samples, the discordance was due to the additional detection by CNV sequencing of one or more CNVs that were either benign or VOUS. In the remaining partially concordant sample, only SNP array detected a 3.43-Mb Yq11.22 duplication in the azoospermia factor region, which is essential for male infertility.

DISCUSSION

In this study, we investigated the performance of CNV sequencing of cffDNA for detection of chromosomal aneuploidies that were previously identified in matching amniocyte samples by standard prenatal diagnostic methods. Overall, we demonstrated a high degree of concordance for detection of chromosome abnormalities between CNV sequencing and karyotyping/SNP arrays. From the analysis of 28 matching cffDNA and amniocyte samples collected from high-risk pregnancies, four pathogenic fetal chromosomal abnormalities were co-identified by both methods, including trisomy 21 (Down syndrome), monosomy X (Turner syndrome), a 12p tetrasomy (Pallister–Killian syndrome) and a 1.55-Mb Xq28 duplication (Xq28 microduplication syndrome). While further validation of a larger number of cffDNA samples by CNV sequencing is warranted, our findings suggest that cffDNA is a useful alternative template to amniocytes for reliable and accurate molecular identification of fetal chromosome abnormalities.

There were 12 instances of partial concordance between the two diagnostic methods that involved benign CNVs or VOUS located in intragenic regions of the genome. In 11 of these cases, CNV sequencing identified CNVs, which were not detected by SNP array. One possible explanation is that CNV sequencing may be more sensitive than array comparative genomic hybridization, but less specific when applied to a poorer quality DNA template like cffDNA. However, we believe that this is unlikely because, firstly, all the CNVs were sample specific, appearing only once, and, secondly, the altered copy number of each CNV was consistent in the majority of sequencing bins across the entire CNV interval. We speculate that the low-level CNV discordance is most likely to be attributed to fundamental differences in the design of two diagnostic platforms used for CNV detection. For SNP arrays,
although the position of SNPs is fixed throughout the genome, a sufficient density of SNP probes can be selected to provide high genome coverage.6 However, there are some regions of the genome with only a paucity of SNPs, and thus, some small CNVs <1 Mb in size may not be confidently detected.7 On the other hand, CNV sequencing generates random genome-wide sequencing reads with a relatively uniform genome coverage.10 Thus, when sequencing reads are allocated to small bin sizes, there are sufficient data points to reliably identify small CNVs at virtually any genome position.

However, the higher yield of small benign or VOUS CNVs returned by CNV sequencing may unnecessarily complicate prenatal diagnosis. Firstly, the CNV data requires careful assessment against known databases to determine high or low risk for pathogenicity, and for VOUS, this remains challenging.7,24 Secondly, at the clinical level, reporting of VOUS creates a difficult conundrum because it raises unnecessary concerns for clinicians, genetic counselors and patients.25 Therefore, once more data are accumulated from CNV sequencing in a prenatal setting, it will be essential to set firm guidelines for interpretation and reporting of benign and VOUS CNV results.

In the remaining case of partial concordance, SNP array identified a 3.43-Mb Yq11.22 duplication that was missed by CNV sequencing. This CNV region was designated VOUS because only deletions of Yq11.22 are known to cause male infertility.23 The reason why CNV sequencing failed to detect this CNV is largely attributable to the low number of sequencing reads that can be accurately mapped to Y chromosome because of extensive regions with highly repetitive sequences and genes with multiple copy numbers.26 In fact at best, CNV sequencing can only accurately determine the copy number of Y, enabling discrimination of 46,XY from 46,XXY and detection of sex chromosomal aneuploidies such as 45,X, 47,XXX and 47,XXX, including mosaic variants.27 In contrast, SNP arrays can selectively incorporate a sufficient density of single-copy SNPs located in specific regions of Yp and Yq, enabling not only aneuploidy detection but also detection of Y-specific CNVs.

While the use of cultured or uncultured amniocytes for karyotyping or SNP array analysis is highly reliable to provide a fetal diagnosis, there are occasions where prenatal test failure occurs and results are not reportable. These situations usually arise because of either cell culture failure or retrieval of a low number of amniocytes,2 particular in late gestation amniocentesis procedures where only small volumes of AF can be safely collected.28 In addition, technical failures of CMA can occur because of insufficient genomic DNA, poor quality DNA or background noise after hybridization and washing. In one recent study, where CMA results were not obtainable because of poor-quality genomic DNA samples, the repeat application of CNV sequencing on the same samples enabled a conclusive diagnosis in all cases.29 Nonetheless, based on the promising results from this study, cffDNA should be able to provide a source of fetal DNA as a backup when results from karyotyping or SNP arrays are compromised by cell culture failure, low cell numbers or technical issues.

In addition to cell culture failure or low cell yield, amniocentesis samples can sometimes be compromised by blood and MCC. For genetic diagnosis by CMA, MCC levels exceeding 5% can lead to uninterpretable results.2 While not demonstrated here, the use of cffDNA may offer a solution to overcome MCC and obtain a conclusive result. This is because centrifugation of AF samples with MCC should pellet both the amniocytes and the contaminating maternal cells, leaving a supernatant essentially free of any extraneous maternal DNA. In support of this notion, one recent study of 49 bloody low-volume AF samples collected late in gestation showed that the purified cffDNA fraction was indeed free of any detectable MCC, enabling the accurate detection of aneuploidies by quantitative fluorescent PCR.30 Therefore, given these clinical scenarios where a 'no result' is returned because of compromised samples or a technical issue, this strongly advocates for banking of the cffDNA as a backup to provide a diagnosis for the patient and avoid a repeat amniocentesis procedure, which exposes the pregnancy to further risk.28 In our study, we found that after purification of the cffDNA, all 28 AF samples provided a minimum of 1-ng DNA per microliter, indicating that cffDNA prepared from a 20-mL AF sample is a plentiful source of high-quality cffDNA for multiple genetic analyses. Therefore, for test failures, the cffDNA would be a sufficient template for molecular analysis of fetal chromosome abnormalities because CNV sequencing and SNP array diagnostic platforms require a minimum of 50 and 500 ng of DNA, respectively, for accurate 24 chromosome profiling at a resolution of 0.1 Mb.10

CONCLUSION
In this pilot study, CNV sequencing of cffDNA detected all clinically significant abnormalities detected by conventional amniocyte-based prenatal diagnostic methods. At a minimum, cffDNA sequencing has clinical utility as a backup in occasional cases where traditional amniocyte-based diagnosis by either karyotyping or arrays fails because of compromised amniocentesis samples or technical issues.

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?
- Amniotic fluid is a source of amniocytes for traditional prenatal genetic diagnosis.
- Discarded amniotic fluid contains cell-free fetal DNA.

WHAT DOES THIS STUDY ADD?
- Direct sequencing of cell-free fetal DNA in amniotic fluid supernatant represents a promising alternative approach for prenatal diagnosis of fetal chromosome abnormalities where traditional amniocyte-based diagnosis fails.
REFERENCES