Review

Non-Invasive Prenatal Diagnosis: A Comparison of Cell Free Fetal DNA (cffDNA) Based Screening and Fetal Nucleated Red Blood Cell (fnRBC) Initiated Testing

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Current prenatal diagnosis uses non-invasive procedures of maternal serum screening and ultrasound exam to evaluate the risk of chromosomal abnormalities and invasive procedures of chorionic villus sampling and amniocentesis for the diagnosis of cytogenomic abnormalities and gene mutations. The discovery of cell free fetal DNA (cffDNA) in maternal blood prompted the application of massive parallel sequencing to screen fetal aneuploidies. The multi-center large-scale validation of cffDNA based prenatal screening has resulted in rapid integration of this close-to-diagnostic non-invasive procedure into clinical application. Further improvement of this approach could lead to the screening of pathogenic copy number variants and known disease-causing gene mutations. The success from cffDNA fuels efforts in isolating circulating fetal nucleated red blood cells (fnRBCs) for direct non-invasive prenatal testing of fetal genetic disorders. Various isolation and enrichment methods based on the physical and biologic features of the fnRBCs have been developed but the analytic and clinical validities have not yet been established. The cffDNA based prenatal screening has significantly reduced unnecessary invasive procedures. Future breakthrough on fnRBC initiated prenatal testing will further shift the paradigm toward non-invasive prenatal diagnosis.

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INTRODUCTION

Current prenatal diagnosis uses both invasive and non-invasive procedures to detect fetal anomalies and genetic abnormalities. Invasive procedures include chorionic villus sampling (CVS) and amniocentesis, in which probes or needles are inserted into the uterus to obtain the diagnostic samples. Non-invasive procedures like maternal serum screening for risk of chromosomal abnormalities and ultrasound examination of fetal anomalies have been the standard of practice. Because of the added anxiety to pregnant women and slightly increased risk of miscarriage and fetal injury associated with the invasive procedures, a non-invasive procedure is a preferred option whenever possible. Therefore, there have been continuous efforts in developing reliable and accurate fetal DNA-based or cell-initiated non-invasive prenatal diagnosis (NIPD) to detect chromosomal abnormalities and gene mutations.

In 1969, fetal nucleated hematopoietic cells were found circulating within the maternal blood and male fetal cells can be detected by karyotype analysis.[1] Flow sorted fetal nucleated red blood cells (fnRBCs) were used to determine Y chromosome sequences by polymerase chain reaction (PCR) and to detect trisomy 21 by fluorescence in situ hybridization (FISH).[2,3] These studies provided the proof-of-concept that fnRBCs can be isolated in a research setting, but effective enrichment and detection techniques of fnRBC for clinical use still remain a technical challenge.[4] In 1997, the discovery of cell-free fetal DNA (cffDNA) in maternal plasma and serum provided a promising alternative option.[5] PCR amplification of cffDNA sequences has been used to detect mutations and single nucleotide polymorphism (SNP) haplotypes for cystic fibrosis, congenital adrenal hyperplasia and β-thalassemia in the fetus.[6-8] In 2006, trisomy 18 was screened in maternal plasma by epigenetic allelic ratio analysis[9] In 2008, shotgun sequencing and massive parallel sequencing (MPS) of cffDNA from maternal plasma were used to detect fetal aneuploidies.[10,11] The genome-wide genetic and mutational profile of the fetus was revealed using maternal plasma DNA sequencing.[12] Recently, several large-scale validation studies have demonstrated that cffDNA based prenatal screening can offer close-to-diagnostic screening for common aneuploidies.[13-15] This review aims to

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highlight the technical development and clinical applications of cffDNA based screening and fnRBC initiated testing and provide insight for the paradigm shift of prenatal diagnosis in the near future.

cffDNA BASED PRENATAL SCREENING

Several studies have demonstrated that about 11% of total cell free DNA (cfDNA) in maternal plasma is from fetus.[10,12] Theoretically, a pregnant woman carrying a fetus with trisomy 21 will have a slightly higher amount of chromosome 21 cffDNA in the plasma. The use of next-generation sequencing technologies on cfDNA has enabled accurate mapping of sequencing data onto the human genome and reliable calculation of relative amount of cffDNA for the detection of common aneuploidies such as trisomies 21, 18 and 13. This non-invasive approach has been validated and rapidly integrated into the current prenatal diagnosis.[10-15]

Analytical and Clinical Validities

The technical procedures for cffDNA based prenatal screening include extraction of cfDNA from maternal plasma, construction of cfDNA library for sequencing and statistical analysis of sequencing data. Several technical challenges need to be resolved. Firstly, the total amount of cfDNA in maternal plasma is low and ranges from 1.6 to 28.1 ng/ml plasma by different isolation methods;[16] secondly, the fraction of cffDNA among the total cfDNA in the first trimester varies from 4% to 25% and the proportion of pregnancies with fetal fraction below 4% increases with maternal weight;[17] thirdly, the statistical model to calculate the risk of aneuploidy needs to be built on a set of sequencing data from normal and abnormal cases.[10-15] Quantitative PCR-based methods were introduced to evaluate the fraction of cffDNA among extracted cfDNA from maternal plasma. For instance, half of fetuses are males and their Y chromosome sequences like the SRY and DYS14 genes were amplified by PCR to examine the fraction of cffDNA among the total cfDNA.[5,10] The promoter region of the RASSF1A gene is hypermethylated in cffDNA but not in maternal cfDNA. So after digestion of cfDNA by enzymes that recognize unmethylated DNA, the remained fraction of cffDNA was detectable by PCR.[18] Polymorphic markers like short tandem repeat sequences can be used to determine fetal paternal and maternal haplotypes.[7] SNP alleles were analyzed so that the maternal plasma cfDNA will have three alleles instead of two if cffDNA exists.[8,12] The implementation of effective quality control measures to evaluate the fetal gender, cffDNA fraction, genomic DNA contamination and cfDNA library is crucial to the success of this screening. Any sample that has no or little cffDNA should be excluded from further analysis.

A common statistical method, "z-score", was introduced to analyze the MPS data.[13-15] Briefly, a training set of known euploid samples was counted for the ratio of fragments from targeted chromosome 21 (or 18 and 13) to the total fragments (or selected reference chromosomes). The obtained ratios were treated as a normal distribution, and the mean with standard deviation (SD) for the targeted chromosome was calculated from the training set. For the test samples, the ratio from targeted chromosome 21 can be used to calculate the "z-score" which is defined by the difference between the means from the test samples and the normal training set divided by the SDs of reference controls. A z-score bigger than 3 suggested a 99% chance statistically significance for trisomy 21. Therefore, the higher a z-score is, the more likely a trisomy happens. In order to improve the sensitivity and specificity, the cut-off value of z-score should be carefully calculated from sufficient number of euploid and aneuploid samples. A laboratory without a big training set to begin with can set a stringent cut-off value of z-scores. For all samples with a grey-zone z-score or unreportable result, a diagnostic invasive cytogenetic test should be recommended.

The validity of cffDNA based prenatal screening has been evaluated by its sensitivity and specificity in a clinical setting. Clinical sensitivity refers to the probability that a test will be positive in patients with the condition; clinical specificity refers to the probability that a test will be negative in subjects without the condition.[19] Several clinical validation studies using MPS in pregnancies with high-risk indicators or mix risk factors and routinely screened first-trimester populations have been reported.[13-15,20-25] The results showed a sensitivity of 99%–100% and a specificity of 97.9%-99.97% for detecting trisomies 21 and 18,[13-15,20-23] and a sensitivity of 91.7% and a specificity of 99.1% for trisomy 13.[15] Sample pooling is a common practice to reduce the cost of MPS. A comparison between a 2-plex (two-sample pool for one sequencing run) and an 8-plex (eight-sample pool for one sequencing run) protocols for detecting trisomy 21 noted significantly reduced sensitivity from 100% to 79.1%.[13] A modified sequencing method termed digital analysis of selected regions (DANSR) has been reported with a capacity of pooling and sequencing 96 indexed samples.[21] In the DANSR method, region-specific cfDNA will be selected by hybridizing to locus-specific oligonucleotides and amplified by universal PCR. PCR products are then sequenced to calculate the risk of aneuploidies. The depth of sequencing in DANSR is less than 5% of that required by whole genome sequencing. The DANSR is aimed to improve the mapping efficiency and sample throughput as well as decrease the sequencing expense. Recently, the extension of MPS screening for sex chromosome aneuploidies (45,X, 47,XXY, 47,XXX and 47,XYY) showed a sensitivity of 85.5%-96.2% and a specificity of 99.7%~99.9% with noted non-reportable rate of 5%.[25-26] A prospective multi-center study further confirmed the efficacy of cffDNA based prenatal screening and recommended the incorporation of this approach to reduce the unnecessary invasive procedures.[27]

Practical Guidelines and Considerations

The rapid integration of cffDNA based prenatal screening demands policy and guideline from peer experts and professional organizations. Rapid response statements have been reported from the International Society for Prenatal Diagnosis and the American College of Medical Genetics and Genomics (ACMG).[28,29] In these statements, the purpose of cffDNA test is specified for prenatal screening of autosomal aneuploidies and possibly sex chromosome changes. The limitations from this procedure are clearly defined. Key elements in pretest and posttest genetic counseling have been addressed.

The cffDNA based prenatal screening has its strengths and limitations. The obvious strength is its high sensitivity and specificity that made it a close-to-diagnosis screening. The other strengths include the release of anxiety of pregnant women and the elimination of miscarriage risk associated with an invasive procedure. With the future improvement in data analysis, it may be able to screen for other genetic disorders like pathogenic copy number variants and known disease-causing gene mutations. Currently the cffDNA-based prenatal screening is limited to the detection of autosomal trisomies and sex chromosome abnormalities.[27] The fraction of cffDNA among the total cfDNA is crucial for the screening performance. If the fetal fraction is lower than 4%, it is more likely to return an inconclusive result.[16] Additionally, this screening is not suitable for twin or multiple pregnancies and singletons with triplody, tetraploidy and balanced translocations. **Table 1** summarizes the technical capacity and some practical considerations of cffDNA based prenatal screening.

Table 1. A Comparison of cffDNA-based screening and fnRBC-initiated testing.*

	cffDNA	fnRBC
Sample Preparation	0.1~1.4 ng DNA/ml maternal blood[16] (equivalent to 15~210 cells)	0.05~168 cells/ml maternal blood[32-38] (equivalent to 0.0003~1 ng DNA)
Analytical Procedures	**Purify cfDNA from maternal plasma**[16] **Sequencing Analysis** MPS genome-wide sequencing[13-15] Selected region sequencing (DANSR)[21] **Data Analysis** Z-score and risk calculation[13-15] T-score and L-score[23]	**Isolation and Enrichment of fnRBCs** MACS[32] Density gradient[33] Density gradient/Lectin[35] Osmolality/Double density gradient[37] Microfludic chip/magnetic enrichment[38] **Whole Genome Amplification (WGA)**[43,44] PCR-based, MDA, OmniPlex
Clinical Use	**Prenatal Screening for**[27] Trisomy 21 (Se: 100%, Sp: 100%) Trisomy 18 (Se: 97.2%, Sp: 100%) Trisomy 13 (Se: 78.6%, Sp: 100%) Turner 45,X (Se: 93.8%, Sp: 99.8%) Female XX (Se: 100%, Sp: 99.5%) Male XY (Se: 100%, Sp: 100%)	**Reported Applications** Male detection by karyotyping[1] Trisomy 21 detected by FISH[3] Male detection by Y-specific PCR[33] Male detection by FISH[36] Male XY FISH (Se: 74.4%, Sp: 95~99%)[34]
Advantages	Close-to-diagnosis screening, release of maternal anxiety and reduction of invasive procedure, potential for detecting copy number variants and gene mutations.	Direct analysis of fetal genome, Possible non-invasive diagnosis for aneuploidies, copy number variants and mutations.
Disadvantages	Limited to chromosome aneuploidies, cautious for mosaicism, not applicable to balanced rearrangements, tri-/tetra-ploid, and twin/multiple pregnancies.	Technically challenging, pending clinical feasible fnRBC isolation and enrichment procedures.

*Se, sensitivity, Sp, specificity; numbering given as listed in the reference.

fnRBC INITIATED DIAGNOSIS

Isolation and Enrichment of fnRBC

The presence of fetal cells in maternal body and its underlying pathophysiology of pregnancy has been demonstrated with the first observation described more than a century ago.[30] This has fueled a quest of developing a method for noninvasive prenatal diagnosis using circulating fetal cells. Three types of fetal cells in the maternal blood, namely lymphoblasts, nucleated erythroblasts and trophoblasts, have been employed as the targets for prenatal diagnosis. After circulating for a few years in the maternal circulatory system, some lymphoblasts may have originated from present or past pregnancies. The rapid clearance of fetal trophoblasts by maternal pulmonary circulation limits its application of prenatal diagnosis.[31] The fetal erythroblasts, fetal nucleated red blood cells (fnRBCs), have long been considered a perfect candidate because they have a distinct morphology from other nucleated cells, a particular type of hemoglobin and limited life span.[32,33] However, the extreme scarcity of fnRBC in maternal blood has limited its clinical application.[34]

In 1995, using negative magnetic activated cell sorting (MACS), fnRBCs were enriched and found at a range of 0.05-11.5 cells per ml of maternal blood in 37 of the 40 pregnant women.[32] A discontinuous density gradient method using Percoll isolated 0.5-11 fnRBCs/ml of maternal blood and fetal sex was predicted accurately by Y-specific PCR in 10 out of 11 samples.[33] Using density gradients and subsequently galactose-specific lectin, the yield of fnRBCs was 0.16-13.66 fnRBCs/ml maternal blood and the number of fnRBCs was correlated with gestational age.[35] Further

evaluation using FISH on lectin-enriched cells confirmed that fnRBCs were present in maternal blood and that 30.4% of nucleated RBCs in maternal blood were fetal in origin.[36] Using a combined method of optimal osmolality and a double-density gradient system, the yield of fnRBCs was 0.2-4.8 cells/ml maternal blood.[37] In 2008, a device using a microfluidic chip for size-based cell separation and a magnetic enrichment of hemoglobin cells was developed. This device removes non-targeted red blood cells and white blood cells at a very high efficiency (99.99%) and successfully collected 0.37-168 fnRBCs/ml maternal blood.[38] However, the lack of further analytic and clinical validity for all the above methods indicated that effective isolation and enrichment of fnRBCs for clinical use remains a technical challenge.

Several studies were conducted to characterize more physical and biological features for fnRBCs to facilitate effective isolation and enrichment. For example, fnRBC-specific lightscattering spectroscopic signatures were noted and could be used to separate fnRBCs from other cells.[39] A proteomic and bioinformatic approach was used to identify 12 plasma membrane proteins and eight proteins with transmembrane domains unique to fnRBC.[40] Specific monoclonal antibodies to antigens (CD36, CD71, GPA, antigen-i, and galactose) expressed exclusively on fnRBCs have been selected.[41] Other on-going technology development includes the individual cell sorting with DEPArray by Silicon Biosystem (www.silicon biosystem.com) and CellScape fetal cell technology by CellScape (www.cellscapecorp.com).

Single Cell Analysis through Whole Genome Amplification

To overcome the technical hurdle of extreme scarcity of fnRBCs from maternal blood, whole genome amplification (WGA) of the fetal DNA for further diagnostic applications could be practical based on the experience from preimplantation genetic diagnosis (PGD).[42-44] Current WGA methods are classified into three categories: 1) PCR-based thermal-cycle amplification,[45] 2) Multiple displacement isothermal amplification (MDA),[46,47] and 3) OmniPlex library methods. Each method usually has its own characteristics in template dependence and amplification biases. High amplification efficiency and high fidelity with minimal locus bias (sequence coverage) and allelic bias (nucleotide fidelity) are the two important factors in the selection of a WGA method for clinical use.

Various PCR-based amplification methods such as primer extension preamplification (PEP), degenerate oligonucleotide primed PCR (DOP-PCR), and ligation mediated PCR (LM-PCR) have been used in prenatal diagnosis and also PGD.[43,44] These methods gave efficient amplification of targeted genomic sequences from DNA samples extracted from CVS, amniocytes and fnRBCs and provided sufficient amplicons for subsequent sequencing analysis of gene mutations. However, these methods showed serious locus bias (low coverage) and allelic bias (such as allele drops and add-in).[46] PCR-based methods are widely used for locus- or gene-specific analysis. Using DOP-PCR amplified DNA of blastocysts at the single cell level to detect copy number variations by low coverage MPS has been reported.[48]

MDA-based methods, including the REPLI-g single cell kit (Qiagen Inc.) and GenomiPhi (GE Healthcare), greatly overcome the problem of the PCR-based methods.[46] Further improved version of REPLI-g kit showed a 98% sequence coverage and ~98% nucleotide fidelity by exome sequencing.[47] MDA methods have been successfully applied to sequencing mutations in prenatal diagnosis and PGD.[42] For example, DNA from individual blastocysts was amplified by GenomiPhi and a neurofibromatosis type I (NF-1) gene mutation was detected.[49]

The OmniPlex library method starts with a reformation of the entire genome into a library of randomly overlapped and fully covered small molecules flanked by a universal sequence at its two ends, which are then uniformly amplified by PCR. The GenomePlex single cell whole genome amplification kit (Sigma Aldrich) and recent MALBAC method constitute a two-step process including random priming and universal amplification, showed a high coverage of the genome for single cells. In one report, cffDNA was size selected and amplified with GenomePlex kit.[50] Recently, genome-wide amplification of a single human cell by the MALBAC method demonstrated a 93% genome coverage for the detection of single nucleotide and copy number variations.[51]

As a summary, a technical breakthrough on effective isolation and enrichment of fnRBCs is needed before considering them as a reliable source for prenatal testing. Newly developed WGA technologies can be used to amplify fetal genome from fnRBCs at the single cell level. Table 1 outlines estimated yield of fnRBCs from maternal blood and examples of fnRBC initiated prenatal testing by various methods.

CONCLUSION

The cffDNA based prenatal screening has been validated and rapidly integrated into current prenatal diagnosis. There are on-going continuous efforts in developing effective fnRBC initiated prenatal testing. As noted in many prenatal cytogenetic laboratories, the improved high resolution ultrasound examination and the integrated close-to-diagnosis cffDNA based prenatal screening for common aneuploids have resulted in significant reduction of invasive CVS and amniocentesis procedures.[23,52] At this stage, chromosome analysis on cultured villi cells or amniocytes will still be the gold standard for detecting numerical and structural abnormalities, especially for Robertsonian translocations, balanced rearrangements, tri-/tetraploidies, and mosaicism. As continuous efforts building into the development of novel effective isolation of fnRBCs and WGA of fetal genomic DNA, prenatal diagnosis will be revolutionized by achieving fully non-invasive detection of chromosomal abnormality, pathogenic copy number variants and gene mutations.[53,54] This foreseeable paradigm shift from invasive prenatal diagnosis to NIPD will have great impact on clinical management and genetic counseling of genetic disorders.

CONFLICT OF INTEREST
None.

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