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Exploration of a Novel Noninvasive Prenatal Testing Approach for Monogenic Disorders Based on Fetal Nucleated Red Blood Cells

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BACKGROUND: Due to technical issues related to cell-specific capture methods, amplification, and sequencing, noninvasive prenatal testing (NIPT) based on fetal nucleated red blood cells (fNRBCs) has rarely been used for the detection of monogenic disorders.

METHODS: Maternal peripheral blood was collected from 11 families with hereditary hearing loss. After density gradient centrifugation and cellular immunostaining for multiple biomarkers, candidate individual fetal cells were harvested by micromanipulation and amplified by whole-genome amplification (WGA). Whole-exome sequencing/whole-genome sequencing (WGS) and Sanger sequencing were performed on the identified fNRBCs to determine the fetal genotype. The impact of single-cell and pooled WGA products on the sequencing quality and results was compared. A combined analysis strategy, encompassing whole-exome sequencing/WGS, haplotype analysis, and Sanger sequencing, was used to enhance the NIPT results.

RESULTS: fNRBCs were harvested and identified in 81.8% (9/11) of families. The results of cell-based-NIPT (cb-NIPT) were consistent with those of invasive prenatal diagnosis in 8 families; the coincidence rate was 88.9% (8/9). The combined analysis strategy improved the success of cb-NIPT. The overall performance of pooled WGA products was better than that of individual cells. Due to a lack of alternative fetal cells or sufficient sequencing data, cb-NIPT failed in 3 families.

CONCLUSIONS: We developed a novel fNRBC-based NIPT method for monogenic disorders. By combining multiple analysis strategies and multiple fetal cell WGA products, the problem of insufficient genome information in a single cell was remedied. Our method has promising prospects in the field of NIPT for the detection of monogenic disorders.

Introduction

Noninvasive prenatal testing (NIPT), which uses the fetal genetic material present in the peripheral blood of pregnant women to conduct prenatal diagnosis, is emerging as an increasingly important technique. Broadly, the methods can be divided into circulating cell-free fetal DNA (cffDNA)-based and circulating fetal cell-based. Since the discovery of cffDNA in the maternal plasma in the late 1990s (1), cffDNA has gradually become the mainstream in the field of NIPT and has been applied in the diagnosis of fetal aneuploidies (2–4), major copy number variations (5, 6), and partial monogenic disorder (7, 8). However, the highly fragmented nature and the short size of cffDNA make it technologically challenging to detect large deletions, duplications, rearrangements, and other pathogenic variants, especially for maternally inherited diseases or recessive genetic disorders (9). Such disadvantages of cffDNA have limited its widespread uptake in clinical practice.

Fetal cells from maternal blood present an alternative source of fetal DNA for NIPT (10, 11). Compared with cffDNA, intact fetal cells provide complete fetal genetic information. Theoretically, all fetal aneuploidies and monogenic disorders can be detected by analyzing these cells. Trophoblasts (12), leukocytes (13), progenitor and stem cells (14), and fetal nucleated red blood cells (fNRBCs) have been identified in the maternal circulation. Of these cell types, fNRBCs are considered to be ideal candidates for the NIPT of

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aneuploidies and monogenic disorders, as they are short-lived, are morphologically distinct from maternal blood cells, and have relatively specific fetal cell markers that were useful for identification (15). Due to the rarity of fetal cells in the maternal blood, a rapid, simple, and consistent procedure for their isolation should be developed to support their clinical application. During the past few decades, many approaches have been developed to recover fNRBCs from the maternal blood, including density gradient centrifugation (16), filtration, selective red blood cell lysis, charge flow separation, lectin-affinity separation, antibody-based separation methods such as fluorescence-activated cell sorting and magnetic-activated cell sorting (17, 18), and microfluidic chips. For example, Wang et al. were able to isolate 24 fNRBCs/mL of maternal blood in media using gelatin-coated microspheres, with anti-CD147 as a specific recognition molecule, and purify the captured fNRBCs through a spiral microfluidic chip (19). Huang et al. developed a silicon-based nanostructured microfluidic system that captures fNRBCs and extravillous cytotrophoblasts automatically (20). Gur et al. described a 2-tiered microchip system to capture and retrieve rare cells from blood samples and found that single cells can be retrieved with efficiencies and purities as high as 100% (21).

One of the earliest uses of enriched fNRBCs for prenatal diagnosis was reported in 1991 (22). Since then, it has been used to detect chromosomal abnormalities, fetal sex, and a few monogenic disorders such as ABO genotype (23), cystic fibrosis (24), Duchenne muscular dystrophy (25), and fetal hemoglobinopathy (26). In order to promote the clinical application of fNRBC-based NIPT for monogenic disorder detection, several challenges need to be overcome. First, a certain number of fNRBCs without maternal contamination should be obtained. Second, progresses in the single-cell whole-genome amplification (WGA) technology are desired to remove the shortcomings of low genome coverage and high allele dropout (ADO) rate. Finally, downstream sequencing and data analysis strategies need to be improved. Previous studies on fNRBCs have focused on the improvement of techniques to increase the capture rate of fNRBCs and reduce contamination of maternal cells. However, until now, fNRBCs with 100% purity cannot be achieved by enrichment technology. One alternative solution is to select individual candidate cells to avoid maternal contamination. WGA is a key step in single-cell sequencing workflows, and 4 main methods have been developed: degenerate oligonucleotide-primed polymerase chain reaction, multiple displacement amplification, multiple annealing and looping-based amplification cycles, and a combination of displacement pre-amplification and polymerase chain reaction (PCR) amplification (PicoPLEX) (27–29). The drawbacks of nonuniformity of genome coverage and ADO exist in all these WGA kits, leading to biases in the sequencing data and inaccurate downstream variant analyses. Therefore, a comprehensive sequencing and data analysis strategy needs to be explored.

In this study, we developed an NIPT approach for hereditary hearing loss based on single fNRBCs. Through single-cell PicoPLEX WGA, short tandem repeat (STR) identification, next-generation sequencing (NGS) and the integration of multiple analysis strategies, we demonstrate the feasibility of this fNRBC-based approach for the NIPT for hereditary hearing loss.

Materials and Methods

PATIENT RECRUITMENT AND SAMPLE COLLECTION

This study was approved by the Ethical Committee of the Chinese PLA General Hospital (approval reference number S2016-103-01). All enrolled participants signed an informed consent form. Pregnant women with a risk of autosomal hereditary hearing loss were recruited. Maternal peripheral blood (3 mL) was drawn in anticoagulant EDTA Vacutainer tubes (BD) for fNRBC capture and genomic DNA (gDNA) extraction during pregnancy at 8 to 25 weeks. Amniotic fluid or chorionic villous samples was collected for prenatal diagnosis. Peripheral blood (2 mL in an EDTA anticoagulant tube) from the spouse and/or proband (if applicable) was collected for gDNA extraction.

FNRBC ENRICHMENT AND ISOLATION

The capture process for fNRBCs was mainly performed as previously described by Wang et al. and Zhang et al. (19, 30) with some modification. For example, we omitted the coating process with MnO2 or gelatin as well as the cell release procedure. The concentration of the cell fixative was changed from 4% paraformaldehyde for 10 min to 0.5% paraformaldehyde for 15 min (31). The imaging of fNRBCs was done by inverted fluorescence microscopy (Nikon). Candidate single cells meeting the selection criteria (4′,6-diamidino-2-phenylindole positive, anti-ϵ-globin positive, anti-CD71 positive; Supplemental Fig. 1) were selected with custom glass micropipettes (40 μm in diameter) and transferred to individual PCR tubes containing a 1.5 μL PBS solution. Five to 10 candidate fNRBCs were selected from each family. The overall flowchart is shown in Fig. 1.

WHOLE-GENOME AMPLIFICATION AND STR ANALYSIS

The PicoPLEX Single Cell WGA Kit v3 (Takara) was used to amplify the whole genome of candidate single cells. The WGA products were purified with DNA

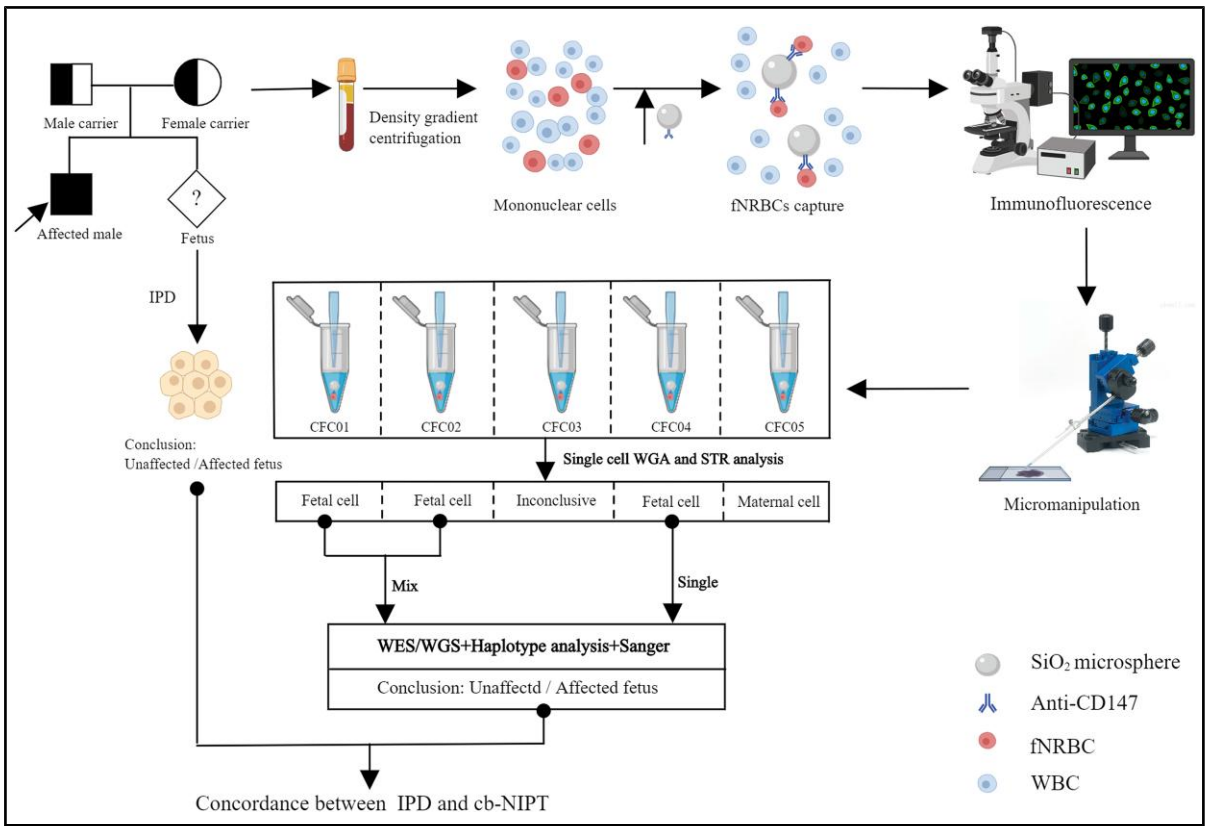

Fig. 1. Study design. The fNRBCs were obtained by density gradient centrifugation, CD147 antibody-coated SiO2 microsphere capture, tricolor immunofluorescence identification, and micromanipulation and validated by STR analysis. Single-cell or mixed WGA products were sequenced and a combined data analysis strategy (WES, WGS, haplotype analysis, Sanger sequencing) was employed. The results of NIPT were compared with those of IPD. This image was drawn using MedPeer (www.medpeer.cn).

Clean Beads and stored at −20°C for further analysis. Approximately 2.5 μg of DNA (approximately 150–1000 bp in length) was recovered after WGA. A genotyping assay was performed using 16 loci to confirm the fetal origin of the candidate single cells, and STR-PCR was performed with the WGA products and the gDNAs from the parents, as described by Chang et al. (32).

WHOLE-EXOME SEQUENCING/WHOLE-GENOME SEQUENCING

Whole-exome sequencing (WES) or whole-genome sequencing (WGS) was used to detect variants in the single-cell WGA products. The gDNA from parents, proband, amniotic fluid/chorionic villous samples, and the purified WGA products were sheared into fragments with an average size of 350 bp using the DNA Shearing System S220 (Covaris). After the processes of end-repair, 3′-adenylated, adapter-ligation, and PCR amplification for the selected fragments, DNA libraries were evaluated using an Agilent 4200 Tapestation system for insert size, quantified by Qubit, and sequenced with paired-end 150 reads on an Illumina Hiseq2500 platform according to the manufacturer's instructions. Data analysis is shown in the Supplemental material.

HAPLOTYPE ANALYSIS

The fetal haplotype was deduced using a karyomapping approach described by Handyside et al. (33). The details are described in the Supplemental material.

VARIANT DETECTION BY SANGER SEQUENCING

WGA products (2 uL) of fNRBC were amplified by PCR using the primers and the PCR conditions listed in Supplemental Tables 1 and 2. The PCR products were analyzed by Sanger sequencing (Applied Biosystems Inc.).

STATISTICAL ANALYSIS

Mean ± SD was calculated for normally distributed data, whereas the median and range were calculated for data not normally distributed.

Results

CLINICAL INFORMATION ON RECRUITED FAMILIES

In total, 11 families were enrolled. Family P02 was at risk of autosomal dominant hearing loss, and other families were at risk of autosomal recessive hearing loss. The pregnant women were between 25 and 39 (mean: 31.8, SD: ± 4.2) years old, and the gestation week at the time of blood sampling was between 8 and 25 (mean: 18.6, SD: ± 4.6). All but family P82 were naturally conceived. The couple (both *SLC26A4*-gene variant carriers) in P82 sought an in vitro fertilization solution for a healthy child. There were no probands in families P80, P82, P86, P89, P94, and P96, and there were hearing loss probands in families P88, P92, P97, P98, and P02. Details of all families are listed in Table 1.

CONFIRMATION OF FNRBCS

Sixty-nine candidate fNRBCs from 11 families (6.27 per family) were successfully obtained by micromanipulation. Overall, 28 cells (40.58%) were confirmed as fNRBCs based on the STR criteria that the number of representative paternal-specific alleles is ≥2. For example, in circulating fetal cell (CFC) CFC9705 from family P97, 56.25% (9/16) of STR loci were successfully detected and 4 paternal-specific alleles were identified by STR analysis. In CFC9706, CFC9707, CFC9709, and CFC9711, 75% (12/16), 81.25% (13/16), 56.25% (9/16), and 62.5% (10/16) of STR loci were detected, respectively, and 6, 5, 5, and 2 paternal-specific alleles were identified, respectively (Supplemental Table 3). Therefore, we can conclude that all of these 5 cells were of fetal origin. Not all STR loci can be identified from single-cell WGA products, likely because of ADO or PCR failure.

We successfully obtained fNRBCs from 81.82% (9/11) of families. No fNRBCs were found for families P80 and P86. Considering that only 5 candidate cells were selected from these 2 families, increasing the number of candidate cells may lead to positive findings.

THE NGS PERFORMANCE OF WGA PRODUCTS

Two or 3 cells with the most paternal-specific alleles and total STR loci amplified were selected for subsequent sequencing. For example, we selected CFC8201, CFC8202, and CFC8204 from P82 and CFC8808 and CFC8810 from P88 for WES or WGS. The ID of the selected cells for other families can be seen in Table 1.

Given that a low number of initiation DNA templates may affect the downstream analysis, resulting in undetectable or erroneous results, we specifically designed the "MIX" group, which was composed of multiple WGA products with equal DNA input. For example, the CFC92MIX was a mixture of CFC9202, CFC9204, CFC9205, and CFC9210, and the CFC96MIX was a mixture of CFC9601, CFC9602, CFC9603, and CFC9618. These mixed WGA products were also subjected to WES or WGS testing. To prevent maternal contamination, the criterion of paternal-specific STR loci ≥2 should be strictly followed when selecting the members of the MIX group.

The performance of WGA samples and gDNA samples in WES is shown in Table 2 and Supplemental Table 4. Coverage ≥ 1× (%) refers to the proportion of target regions covered by sequencing; the average was 48.4% in the single-cell group and 76.7% in the MIX group. Coverage ≥20× (%) refers to the percentage of target areas with read depth more than 20× in the total areas; the average was 37.5% in the single-cell group and 60.2% in the MIX group. To detect single-nucleotide polymorphism (SNP) coverage in the WGA samples, we selected approximately 9000 SNPs as the target region. Compared with coverage of more than 99% in gDNA samples, the target SNP coverage of single-cell samples with read depth > 0 averaged 53.15%, whereas that of MIX samples was 92.1%. The number of SNPs with a read depth > 0 was 4083.9 ± 1956.1 for single fNRBCs and 6488.2 ± 1695.9 for mixed samples. In terms of the ADO rate, the average ADO rate of single-cell WGA samples was 42.1% and that of mixed WGA samples was 17%. In terms of WGS, sequencing quality revealed a similar pattern: the sequencing quality of mixed WGA products was better than that of single-cell WGA products (Table 2).

To compare the different characteristics of single maternal cells and single fetal cells in the peripheral blood, we selected some maternal cells (CMC8001 from P80, CMC8205 from P82, CMC8608 from P86, and CMC8907 from P89) for WES. The 1× coverage and SNP numbers of WGA products of single maternal cells in WES were larger than that for single fNRBCs, which may be because fetal cells are more susceptible to degradation in the peripheral blood environment (Table 2).

NONINVASIVE PRENATAL TESTING FOR MONOGENIC DISORDERS

Families with successful NIPT. The results of cell-based-NIPT (cb-NIPT) in 8 families were consistent with that of invasive prenatal diagnosis (IPD) through the use of the combined analysis strategy.

Table 1. Summary of case characteristics and cb-NIPT results.

		Variants[b]				cb-NIPT results							
Family	Gene/ transcript number[a]	Mother	Father	Proband	Sample ID	Variants tested by WES	WES haplotype[c]	Variants tested by WGS	WGS haplotype	Variants tested by Sanger	Comprehensive results	IPD results[d]	Concordance
P82	*SLC26A4*/ NM_000441.2	c.919-2A>G het	c.919-2A>G het	NA	CFC8201	c.919-2 not detected	M: M2; P: uncertain	ND	ND	Fail	c.919-2 no variation	c.919-2 no variation	Yes
					CFC8202	c.919-2 not detected	M: uncertain; P: uncertain	ND	ND	c.919-2A no variation			
					CFC8204	c.919-2A no variation	M: M2; P: P2	ND	ND	c.919-2A no variation			
					CMC8205*	c.919-2A>G het	NA	ND	ND	ND	c.919-2A>G het	c.919-2A>G het	Yes
P88	*TMC1*/ NM_138691.2	c.1334G>A het	c.627C>T het	Compound heterozygous	CFC8808	c.1334 not detected; c.627 not detected	M: uncertain; P: uncertain	ND	ND	Fail	c.1334G no variation; c.627 uncertain	c.1334G no variation; c.627C>Thet	No
					CFC8810	c.1334 not detected; c.627 not detected	M: M2; P: uncertain	ND	ND	Fail			
					CFC88MIX	ND	ND	c.1334 not detected; c.627 not detected	M: M2; P: uncertain	ND			
P89	*GJB2*/ NM_004004.6	c.235delC het	c.235delC het	NA	CFC8902	c.235C no variation	NA	ND	ND	c.235C no variation	c.235C no variation	c.235C no variation	Yes
					CMC8907*	c.235delC het	NA	ND	ND	c.235delC hom	c.235delC het	c.235delC het	Yes
P92	*SLC26A4*/ NM_000441.2	c.917insG het	c.2168A>G het	Compound heterozygous	CFC9202	c.917 not detected; c.2168 not detected	M: M2; P: uncertain	c.917 not detected; c.2168 not detected	M: M2; P: P2	c.917T and. c.2168A no variation	c.917T and c.2168 no variation	c.917T and c.2168 no variation	Yes
					CFC9210	c.917T no variation; c.2168 not detected	M: uncertain; P: uncertain	c.917 not detected; c.2168 not detected	M: M2; P: P2	Fail			

Continued

Table 1. (continued)

Family	Gene/ transcript number[a]	Variants[b]			Sample ID	cb-NIPT results						IPD results[d]	Concordance
		Mother	Father	Proband		Variants tested by WES	WES haplotype[c]	Variants tested by WGS	WGS haplotype	Variants tested by Sanger	Comprehensive results		
					CFC92MIX	c.917 not detected; c.2168A no variation	M: M2; P: uncertain	c.917T and c.2168A no variation	M: M2; P: P2	c.917T and c.2168A no variation			
P94	*GJB2*/ NM_004004.6	c.109G>A het	c.235delC het	NA	CFC9407	c.109 no variation; c.235 no variation	NA	ND	ND	c.109 no variation; c.235 no variation	c.109 no variation; c.235 no variation	c.109 no variation; c.235 no variation	Yes
					CFC94MIX	c.109 no variation; c.235 no variation	NA	ND	ND	c.109 no variation; c.235 no variation			
P96	*GJB2*/ NM_004004.6	c.235delC het	c.235delC het	NA	CFC9601	c.235delC het	NA	ND	ND	c.235delC hom	c.235delC het	c.235delC het	Yes
					CFC9618	c.235delC het	NA	ND	ND	c.235delC hom			
					CFC96MIX	c.235delC het	NA	ND	ND	c.235delC hom			
P97	*GJB2*/ NM_004004.6	c.235delC het	c.235delC het	c.235delC hom	CFC9707	c.235delC hom	M: M2; P: uncertain	c.235delC hom	M: M2; P: P1	c.235delC hom	c.235delC het	c.235delC het	Yes
					CFC9709	c.235delC hom	M: M1; P: uncertain	c.235delC hom	M: M2; P: P1	c.235delC hom			
					CFC97MIX	c.235delC het	M: M2; P: uncertain	c.235delC het	M: M2; P: P1	c.235delC hom			
P98	*MYO7A*/ NM_000260.4	c.6545G>C het	c.721C>T het	Compound heterozygous	CFC9818	c.721C no variation; c.6545 not detected	M: uncertain; P: uncertain	c.721C no variation; c.6545 not detected	M:uncertain; P: uncertain	c.721C no variation; c.6545 fail	c.721 no variation; c.6545 no variation	c.721 no variation; c.6545 no variation	Yes
					CFC98MIX	c.721 no variation; c.6545 no variation	M: uncertain; P: uncertain	c.721 no variation; c.6545 no variation	M: uncertain; P: uncertain	c.721C and c.6545G no variation			
P02	*GJB2*/ NM_004004.6	Noncarrier	Noncarrier	c.223C>T het (AD[h])	CFC0208	c.223 no variation	ND	ND	ND	c.223 no variation	c.223 no variation	c.223 no variation	Yes
					CFC02MIX	c.223 no variation	ND	ND	ND	c.223 no variation			

Continued

Table 1. (continued)

Family	Gene/ transcript number[a]	Variants[b]			cb-NIPT results						IPD results[d]	Concordance
		Mother	Father	Proband	Sample ID	Variants tested by WES	WES haplotype[c]	Variants tested by WGS	WGS haplotype	Variants tested by Sanger	Comprehensive results		
P80	*GJB2*/ NM_004004.6	c.235delC het	c.235delC het	NA	CMC8001*	c.235delC het	NA	ND[g]	ND	c.235delC het	c.235delC het	c.235delC het	Yes
P86	*GJB2*/ NM_004004.6	c.257C>G het	c.35insG het	NA	CMC8608*	c.257C>G het	NA	ND	ND	c.257C>G het	c.257C>G het	c.257C>G het	Yes

[a]Genome build is GRCH38.

[b]Variants: het means heterozygous; hom means homozygous; NA means not applicable.

[c]Haplotype analysis: M refers to maternal; P refers to paternal; M2 and P2 means the wild-type; M1 and P1 means the variant-type; Amniotic fluid was used in P82 to conduct haplotype analysis.

[d]IPD results: refer to results from chorionic villi sample or amniotic fluid. For maternal cells CMC8001*, CMC8205*, CMC8608* and CMC8907* , this column refers to the mother's genotype.

ND, not done; AD, autosomal dominant.

For families without probands, we used the strategy of combining WES of single-cell WGA products, WES of pooled WGA products, and Sanger sequencing to detect gene variants. For families with probands, we added NGS-based haplotype analysis and WGS.

The results of cb-NIPT in family P92 are shown in Fig. 2. CFC9202, CFC9204, CFC9205, and CFC9210 were identified as fetal cells by STR analysis (Fig. 2A). CFC9202, CFC9210, and CFC92MIX were subjected to NGS. The WES target gene loci of CFC9202 were *SLC26A4*: c.917 not detected and c.2168 not detected; the results of CFC9210 were *SLC26A4*: c.917T no variation and c.2168 not detected; and the results of CFC92MIX were *SLC26A4*: c.917 not detected and c.2168A no variation (Fig. 2D and E). The WES haplotype analysis of CFC9202 and CFC92MIX showed that the maternal haplotype was the wild-type, but the paternal haplotype was uncertain (Fig. 2B). The WES haplotype analysis of CFC9210 failed due to the lack of key SNP information. The Sanger sequencing results of CFC9202 and CFC92MIX were both *SLC26A4*: c.917T no variation and c.2168A no variation (Fig. 2F). Based on the combined analysis of the WES, it can be inferred that the fetus was unaffected, with *SLC26A4*: c.917T no variation and c.2168A no variation. For WGS data, the target gene loci of CFC92MIX were detected as *SLC26A4*: c.917T no variation and c.2168A no variation. The haplotype analysis results of CFC9202, CFC9210, and CFC92MIX showed that both maternal and paternal haplotypes were the wild-type (Fig. 2C). WGS data can provide more key SNP sites upstream and downstream of variant sites, making it more advantageous for haplotype analysis. The cb-NIPT results of this family were confirmed by amniocentesis.

Details of the results for the other 7 families (P82, P89, P94, P96, P97, P98, P02) are in the Supplemental material (Supplemental Figs. 2, 4–9).

Families with NIPT failure. In this study, cb-NIPT failed for 3 families. In 2 families, there were no optional fetal cells for testing, and in the other family, a conclusion could not be drawn due to insufficient data.

In families P80 and P86, no cells met the screening criteria for fetal origin by STR analysis. We selected the maternal cells CMC8001 and CMC8608 for WES testing to assess the accuracy of NGS for maternal single-cell WGA products. The WES target gene loci of CMC8001 and CMC8608 were the same genotype as the mother (Supplemental Fig. 3).

In the P88 family, CFC8807, CFC8808, and CFC8810 were identified as fetal cells by STR analysis (Fig. 3A). The WES results of analysis of single-cell WGA products from CFC8808 and CFC8810 both showed that *TMC1*: c.1334 and c.627 were not

Table 2. The NGS performance of gDNA and WGA products (mean ± SD).

Type		Data, (Gb)	Q30 (%)	Coverage ≥ 1X (%)	Coverage ≥ 20 × (%)	Average read depth	Read depth > 0 SNPs (n)[a]	ADO rate (%)	ADI[b] rate (%)
WES	gDNA	12.4 ± 2.0	92.3 ± 0.9	99.9 ± 0.1	99.7 ± 0.3	142.2 ± 27.0	8749.2 ± 828.4	—	—
	WGA (Single-cell)	12.1 ± 5.4	90.9 ± 2.4	48.4 ± 24.3	37.5 ± 18.6	114.9 ± 78.9	4083.9 ± 1956.1	42.1 ± 1.6	1.0 ± 0.5
	WGA (Mixture)	14.3 ± 5.7	92.5 ± 3.3	76.7 ± 16.5	60.2 ± 17.4	151.1 ± 103.2	6488.2 ± 1695.9	17.0 ± 0.0	1.0 ± 0.0
	mWGA (Single-cell)[c]	7.3 ± 3.7	92.7 ± 0.7	70.3 ± 15.5	48.5 ± 15.9	76.8 ± 46.9	6479.5 ± 1387.3	—	—
WGS	gDNA	109.1 ± 16.2	92.5 ± 1.0	97.03 ± 0.33	NA[d]	30.0 ± 8.4	45 130.1 ± 20 821.0	—	—
	WGA (Single-cell)	103.1 ± 7.7	89.5 ± 5.0	44.91 ± 19.91	NA	26.3 ± 7.0	25 226.2 ± 14 407.4	ND[e]	ND
	WGA (Mixture)	122.4 ± 14.7	93.8 ± 1.3	72.59 ± 10.38	NA	32.4 ± 6.7	38 010.8 ± 20 371.5	ND	ND

[a]Read depth > 0 SNPs (n) means the number of SNPs with read depth greater than 0.
[b]allele dropin.
[c]mWGA(Single-cell) refers to WGA product of maternal single cell.
[d]not applicable.
[e]not done.

detected, possibly because the WES did not cover the target gene loci region. The WES-based haplotype analysis of CFC8808 showed that both the maternal and paternal haplotype were uncertain (Fig. 3B). The WES haplotype analysis of CFC8810 showed that the maternal haplotype was wild-type and the paternal haplotype was uncertain (Fig. 3B). The Sanger sequencing of the target gene loci failed. When we added CFC88MIX samples to WGS, the target gene loci were also undetected, likely due to the poor quality of the WGA product. The WGS-based haplotype analysis of CFC88MIX showed that the maternal haplotype was wild-type and the paternal haplotype could not be determined because there were no available paternal key SNP loci upstream of the mutant gene (Fig. 3C). Although the direct variant detection failed, it can be inferred from the haplotype analysis that *TMC1*: c.1334G was wild-type, excluding the condition of an affected fetus. IPD of amniotic fluid showed that *TMC1*: c.1334 had no variation and c.627C > T was heterozygous. The cb-NIPT of this family only obtained a defined diagnosis of one gene locus, possibly due to poor quality data from NGS. The 1× coverage rate of CFC8808 and CFC8810 in WES data was 31.71% and 51.20%, respectively. The 1× coverage rate of CFC88MIX in WGS data was 57.99% (Table 2). We can conclude that insufficient data coverage and the limited detection of SNP loci can affect the judgment of monogenic disease in cb-NIPT.

EVALUATION OF CONSISTENCY BETWEEN NIPT AND IPD

The results of WES and Sanger sequencing showed good consistency, except for heterozygosity in *GJB2:* c.235delC. WES and WGS showed good consistency in the detection of all target gene loci. The mixture of WGA products from multiple fetal cells had a better success ratio in detecting target gene loci than single-cell products. In addition, WGS was significantly better than WES in haplotype analysis because it had more key informational SNPs in the upstream and downstream regions of the target gene loci. When various results were not consistent, data quality should first be checked, and results with good data quality were more reliable. On the basis of qualified quality control, the principle that the minority is subordinate to the majority should be followed. The comprehensive conclusion for cb-NIPT was drawn by 2 researchers followed blinded methods.

In summary, the results of cb-NIPT were consistent with IPD in 8 of 9 families with fNRBCs successfully isolated; the coincidence rate was 88.9%. The cb-NIPT of family P88 was inconclusive due to the lack of sufficient sequencing data. The sequencing results of single maternal cells in P80, P82, P86, and P89 were consistent with the respective maternal genotype. If calculated according to the alleles, the loci coincidence rate of cb-NIPT was 94.1% (16/17), the sensitivity was 85.7% (6/7), the missed diagnosis rate was 14.3%, the specificity was 100%, the misdiagnosis rate was 0%, and the Youden index was 85.7% (Table 3).

Discussion

We explored the application of fNRBCs in the field of NIPT on monogenic diseases, represented by hereditary hearing loss, and achieved satisfactory results. The capture process of fNRBCs in this work was based on Zhang et al.'s and Wang et al.'s work (19, 30); we simplified the process and verified its stability and strong operability. Wang et al. (19) used gelatin-coated SiO2 microspheres, and Zhang et al. (30) used MnO2-coated SiO2 microspheres, with subsequent coating removal and steps for cell release. The study by Zhang et al. (30) showed that the cell capture efficiency of MnO2 silica spheres was approximately 75% and that of bare silica spheres was approximately 50%. We used bare SiO2 microspheres, so there was no need to include steps for coating removal, and we also eliminated steps for cell release. Although the cell capture rate of the bare silicon spheres was reduced, in consideration of the more simplified experimental process, this method is easier to perform and can likely be completed in most laboratories. It is worth mentioning that the bare silica sphere-based method can still capture a sufficient number of candidate fNRBSs. It is estimated that 100 candidate nucleated red blood cells can be captured from 2 milliliters of maternal peripheral blood, of which approximately 40 are genuine fNRBCs. But we did not select all candidate fetal cells, mainly considering the time and economic cost. According to our experience, 10 to 20 single candidate fNRBCs per family are enough for subsequent analysis.

More importantly, we optimized and innovatively explored several steps after cell capture, including attempts to create a mild cell fixation method, increasing the purity of fNRBCs, screening out a suitable WGA amplification kit, and exploring an effective analysis strategy to promote the application of fNRBCs in NIPT.

Fetal cells obtained by noninvasive methods are often plagued by the problem of insufficient purity of cells. Until now, candidate fetal cells collected by the multiple cell collection strategy cannot achieve 100% cell purity, which may cause maternal cell contamination and interfere with subsequent data analysis. According to previous reports, the purity of the captured fetal cells was between approximately 50% and 87% (34, 35). Additionally, the methods of cell origin identification

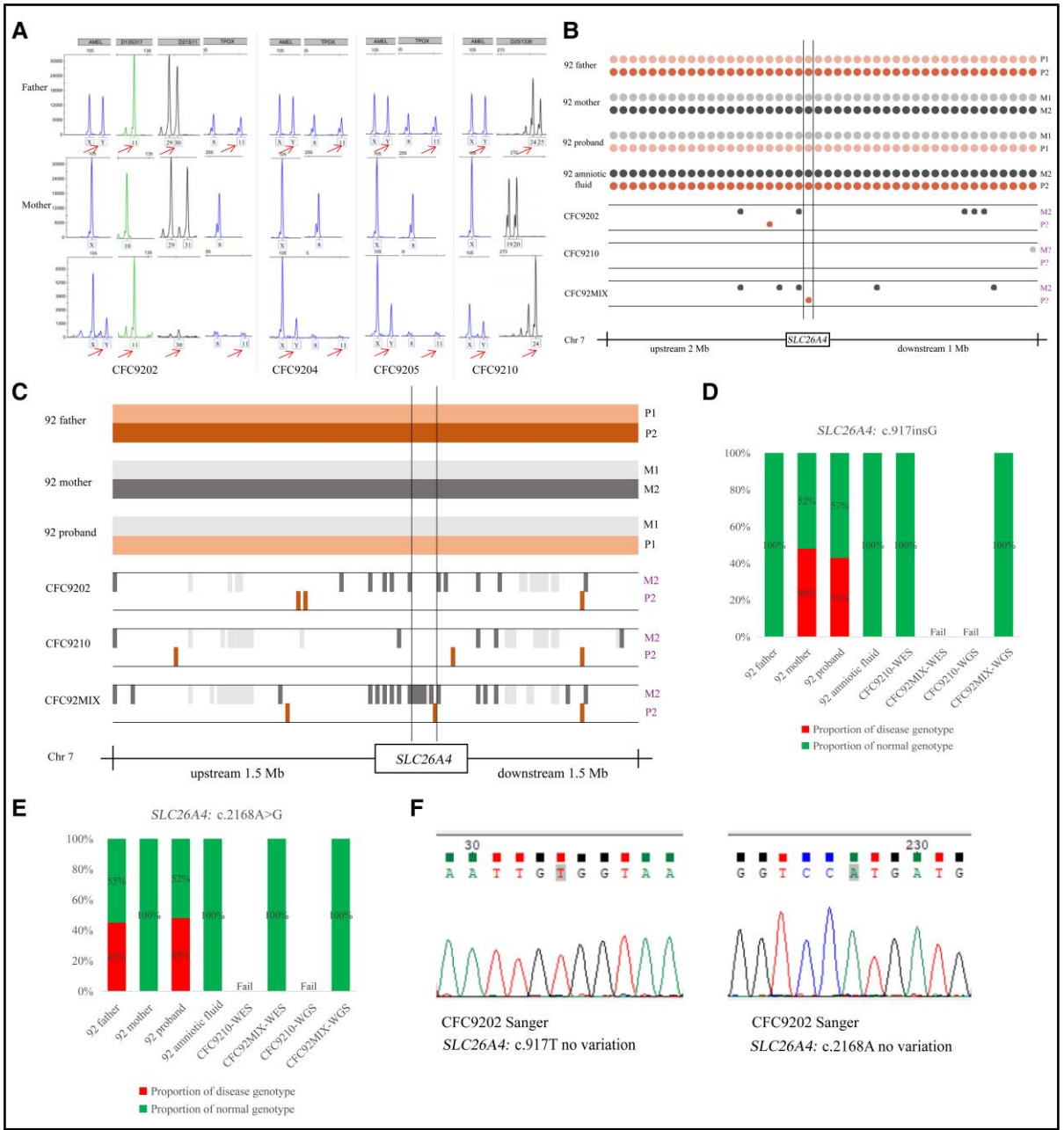

Fig. 2. cb-NIPT results from P92. (A), STR analysis. Red arrows indicate the paternal specific STR loci; (B), WES haplotype analysis. Each dot represents an informative SNP. Red indicates P2, pink indicates P1, black indicates M2, grey indicates M1, purple represents the inferred results for the key SNPs, and vertical lines indicate the location of the target gene loci; (C), WGS haplotype analysis. Each small rectangular block represents an informative SNP. Light orange indicates P1, brown indicates P2, grey indicates M1, black indicates M2, and purple represents the inferred results from key SNPs; (D) and (E), Variant allele fraction (%); (F), Sanger sequencing of the WGA products.

mainly rely on immunofluorescence, fluorescence in situ hybridization, Y-chromosome specific sequence testing, or STR, but these identification methods are not 100% accurate. Therefore, some of the cells that were judged as "positive" by the previously mentioned methods were still nonfetal. In view of this, we abandoned the

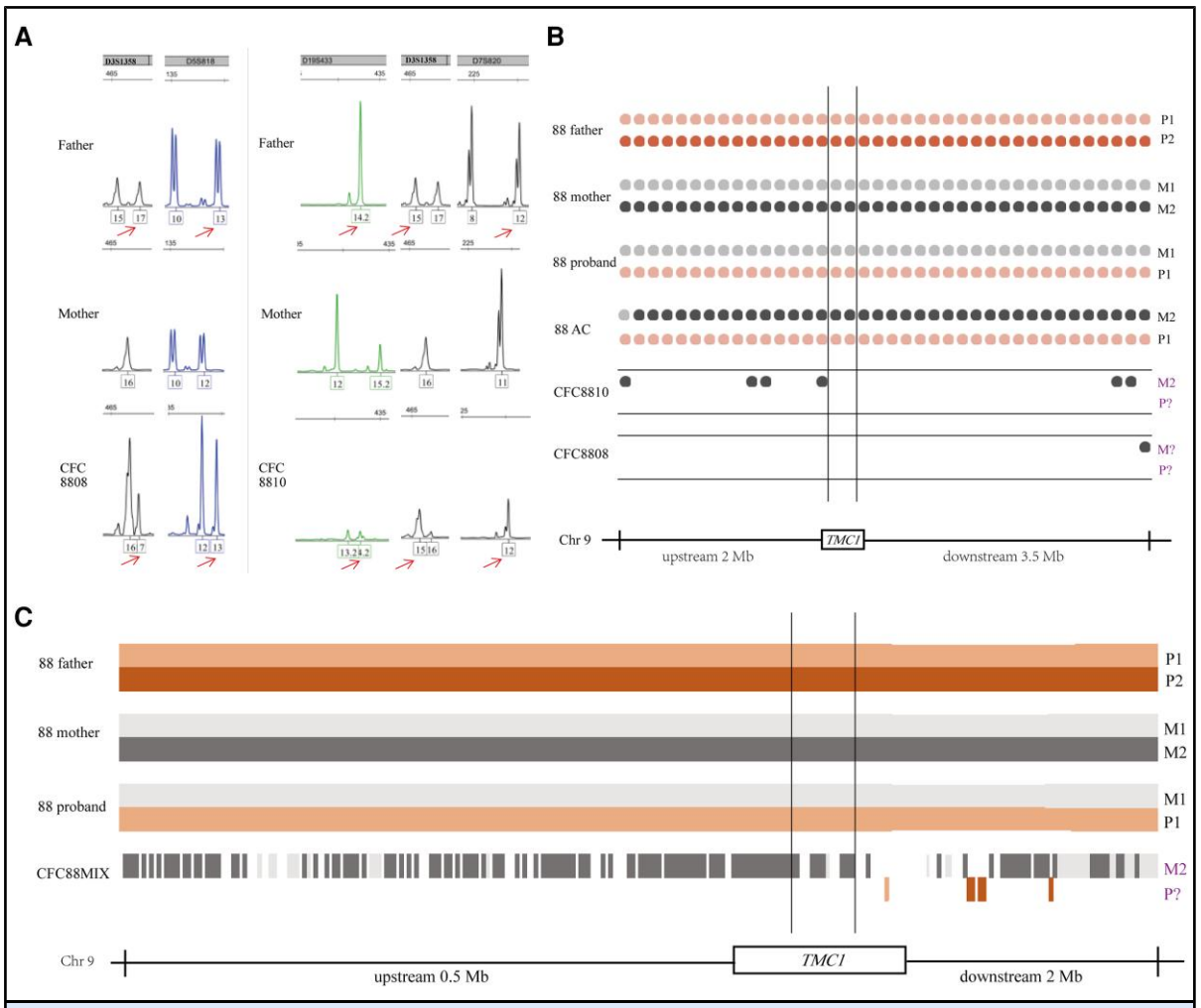

Fig. 3. cb-NIPT results from P88. (A), STR analysis. Red arrows indicate the paternal specific STR loci; (B), WES haplotype analysis. Each dot represents an informative SNP. Red indicates P2, pink indicates P1, black indicates M2, grey indicates M1, and purple indicates the inferred results from key SNPs, and vertical lines represent the location of target gene loci; (C), WGS haplotype analysis. Each small rectangular block represents an informative SNP. Light orange indicates P1, brown indicates P2, grey indicates M1, black indicates M2, and purple represents the inferred results for the key SNPs.

multicellular capture strategy and switched to a single-cell micromanipulation to achieve 100% purity. Through these methods, we demonstrated feasibility of NIPT-based single cell analysis.

Candidate cells need to be fixed with paraformaldehyde (PFA) before immunofluorescence staining. In previous studies, cells were usually treated with 4% or 2% PFA (19, 30). However, high concentrations of PFA can impair the uniform genome amplification due to the cross-linking effect between DNA and histone residues (31), while low concentration of PFA can affect the immunofluorescence staining of cells. We adjusted the cell fixation method by changing the 4% PFA incubation for 10 min to a 0.5% PFA incubation for 15 min based on the work of Chiara Carretta et al. (31) and achieved satisfactory cell staining results and NIPT results.

For single-cell whole genome amplification, we compared the performance of 4 WGA kits using lymphocytes, with the indices of SNP number, 1Mb bins CV, ADO ratio, and allele dropin ratio. We found that the PicoPLEX kit was the most suitable for monogenic diseases (Supplemental Fig. 10).

We performed WGA amplification and STR identification on single candidate cells. The WGA products of identified fetal cells were sequenced individually and

Table 3. Evaluation of consistency between gold standard and cb-NIPT.

	Gold standard (IPD results or mother's genotype)[a]		
cb-NIPT results	Het	No variation	Sum
Het	6	0	6
No variation	1[b]	10	11
Sum	7	10	17

[a]For CMC8001, CMC8205, CMC8608, and CMC8907, gold standard refers to the mother's genotype.
[b]*TMC1*:c.627 C was uncertain in P88, equivalent to a missed diagnosis of one site, therefore belonging to this grid.

after mixing. Sequencing and data analysis methods covered WES, WGS, haplotype analysis, and Sanger sequencing. By comparing several analysis strategies, we found that (*a*) the mixture of WGA products from multiple fetal cells identified by STR had a better success ratio in detecting the target gene loci than single-cell WGA products and the direct mixture of fetal cells; (*b*) WGS was significantly better than WES in haplotype analysis because it had more key informational SNPs in the upstream and downstream regions of the target gene loci; (*c*) for WES, WGS, SNP-haplotype analysis, and Sanger sequencing, any of the aforementioned sequencing protocols had a certain degree of diagnostic error rates and detection failure rates; by combining the previous analysis strategies and pooling multiple fetal cell's WGA products, the problem of insufficient genome information in a single-cell was remedied; (*d*) to ensure the success of cb-NIPT, it is necessary to test not just a sample (a fNRBC or a WGA mixture) but multiple samples. Our method showed a high accuracy for the detection of autosomal recessive/dominant hereditary hearing loss.

In summary, the aforementioned multilevel optimization, exploration, and innovation makes our method feasible for genetic variant detection in hereditary deafness using fNRBCs. The validity and accuracy of the method were verified by invasive prenatal diagnosis. To the best of our knowledge, this is the first study describing the diagnosis of fetal hereditary hearing loss based on fNRBCs. In addition, the potential feasibility of using this strategy to synchronously diagnose multiple monogenic diseases was demonstrated. Naturally, some limitations should be considered. First, our study only had a small sample size and a single disease. In the future, a larger sample size and wider variety of monogenic diseases are needed to verify the practicability. Second, our research was labor-intensive and costly, so directions for future development include automation, specific capture of fNRBCs, and more efficient testing.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: NIPT, noninvasive prenatal testing; cffDNA, cell-free fetal DNA; fNRBC, fetal nucleated red blood cell; WGA, whole-genome amplification; ADO, allele dropout; PCR, polymerase chain reaction; STR, short tandem repeat; NGS, next-generation sequencing; gDNA, genomic DNA; WES, whole-exome sequencing; WGS, whole-genome sequencing; CFC, circulating fetal cell; SNP, single-nucleotide polymorphism; cb-NIPT, cell-based-NIPT; IPD, invasive prenatal diagnosis; PFA, paraformaldehyde.

Human Genes: *SLC26A4*, solute carrier family 26 member 4; *GJB2*, gap junction protein beta 2; *TMC1*, transmembrane channel like 1; *MYO7A*, myosin VIIA.

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