ORIGINAL ARTICLE

Noninvasive prenatal diagnosis based on cell-free DNA for tuberous sclerosis: A pilot study

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Abstract

Background: Noninvasive prenatal diagnosis (NIPD) based on cell-free DNA (cfDNA) has been introduced into the clinical application for some monogenic disorders but not for tuberous sclerosis (TSC) yet, which is an autosomal dominant disease caused by various variations in *TSC1* or *TSC2* gene. We aimed to explore the feasibility of NIPD on TSC.

Methods: We recruited singleton pregnancies at risk of TSC from 14 families with a proband child. Definitive NIPD for TSC was performed using targeted next-generation sequencing of cfDNA in parallel with maternal white blood cell DNA (wbcDNA). The NIPD results were validated by amniocentesis or postnatal gene testing and follow-up of the born children.

Results: Missense mutations, nonsense mutations, frameshift mutations, and splice-site variants which were obtained through de-novo, maternal, or paternal inheritance were included. The mean and minimum gestational weeks of NIPD were 17.18 ± 5.83 and 8 weeks, respectively. The NIPD results were 100% consistent with the amniocentesis or postnatal gene testing and follow-up of the born children.

Conclusion: This study demonstrates that NIPD based on cfDNA is feasible for TSC, but required to be confirmed with more samples. Studies on TSC can contribute to the application and promotion of NIPD for monogenic disorders.

KEYWORDS
autosomal dominant disease, cell-free DNA, monogenic disorder, noninvasive prenatal diagnosis, tuberous sclerosis

Xiao-Yan Yang and Yan Meng should be considered co-first author, they contributed equally to this work.

1 | INTRODUCTION

As one of the main causes of birth defects, monogenic disease is reported to be with an overall high rate of morbidity and mortality with no effective treatment (Kingsmore et al., 2020). Therefore, to prevent the birth of children with monogenic genetic disease by prenatal diagnostic is essential. However, at present, prenatal diagnostic mainly depends on conventional invasive procedures, such as amniocentesis, chorionic villus sampling (CVS), and cordocentesis. While many pregnant women resist the procedure out of concern despite the lower procedure-related risk (Salomon et al., 2019).

In recent years, the great leap in genomic technology makes it possible to prenatally diagnose genetic diseases noninvasively. Especially since the discovery of cell-free fetal DNA (cffDNA) in maternal plasma in 1997 (Lo et al., 1997), noninvasive prenatal diagnosis (NIPD) for monogenic disorders has made considerable progress (Scotchman, Chandler, et al., 2020). Circulating cffDNA is released from the placenta at approximately 4 weeks' gestation and forms a mixture with maternal cell-free DNA (cfDNA). The proportion of cffDNA is only 5%–20% and is called fetal fraction (FF) which increases with gestational age and is influenced by factors such as smoking, maternal weight, and pregnancy complications, including preeclampsia (Drury, Hill, & Chitty, 2016; Scotchman, Chandler, et al., 2020). A major obstacle of NIPD based on cfDNA is the identification of the fetal disease-related genotype under the high background of the maternal cfDNA, especially when the mother carries the variant (Scotchman, Shawa, et al., 2020). Research in this field generally focuses on the technological advancements required to solve this issue. As the maternal component of circulating cfDNA is mainly derived from the hematopoietic system, maternal white blood cell genomic DNA (wbcDNA) can reflect the maternal basic status of cfDNA prior to pregnancy and, therefore, can represent the mothers' genetic background (Lui et al., 2002).

The clinical practice of NIPD based on cfDNA has been approved for some monogenic disorders, such as achondroplasia, thanatophoric dysplasia, Apert syndrome, and paternal mutation exclusion of cystic fibrosis (Drury, Mason, et al., 2016). All these diseases are with either hotspot mutations or specific mutation types. However, in clinical practice, many other monogenic disorders meet neither. One example is tuberous sclerosis (TSC), an autosomal dominant monogenic neurocutaneous disease caused by heterozygous pathogenic variants in either the *TSC1* (OMIM #191100) gene located at chromosome 9q34.13 or the *TSC2* (OMIM #613254) gene located at chromosome 16p13.3 (Cotter, 2020; Samanta, 2020). Until December 2021, a total of 1352 unique variants of *TSC1* and 4047 unique variants of *TSC2* were included in the Leiden Open Variation Database (LOVD, https://www.lovd.nl/3.0/home), and the mutation types included point mutations, frameshift mutations, large fragment deletions, and splicing site mutations, etc. The inactivating variants of the two genes cause hyperactivation of the mechanistic target of rapamycin (mTOR) pathway and consequently result in hamartomas in multi-organs including brain, skin, heart, lungs, kidneys, and eyes (Peron et al., 2018; Roach, 2016).

Research on NIPD for TSC is rare: only two cases have been tested at the North East Thames Regional Genetics Laboratory at Great Ormond Street Hospital (Drury, Hill, & Chitty, 2016) but without detail. Although the strategy is straightforward for autosomal dominant disorders, the application of NIPD to TSC remains challenging. One of the most significant obstacles is that bespoke tests are needed for each family because no mutation hotspots exist in TSC patients. But fortunately, this also allows us to extend our research to other autosomal dominant diseases. In this study, we aimed to evaluate the feasibility and accuracy of NIPD based on cfDNA and maternal wbcDNA for TSC.

2 | METHODS

2.1 | Patient recruitment

The overall study design is presented in Figure 1. We recruited 14 families with a proband from 973 families with TSC at the Pediatric Department of the Chinese People's Liberation Army General Hospital (PLAGH) from 2016 to 2020. The inclusion criteria were (a) singleton pregnancies with gestational ages greater than 7 weeks and (b) the pregnant woman was not a patient with chromosomal aneuploidy and had not received allogeneic blood transfusion within 1 year of the study, immunotherapy with the introduction of exogenous DNA within 4 weeks, or transplant surgery or stem cell therapy. Since NIPD based on cfDNA has not achieved technological breakthrough in the detection of large deletions and duplications, patients with large deletions were not included in this study. Blood samples were collected from each pregnant woman, her husband, and the proband child.

2.2 | Study design of NIPD

Prior to NIPD, if the pathogenic variants of the probands and whether the parents carried the same variants had been determined, Sanger sequencing was conducted to verify the genotypes of the children and their parents. If not, targeted next-generation sequencing (NGS) was first performed to determine the probands' pathogenic variants of the *TSC1* or *TSC2* gene; Sanger sequencing for
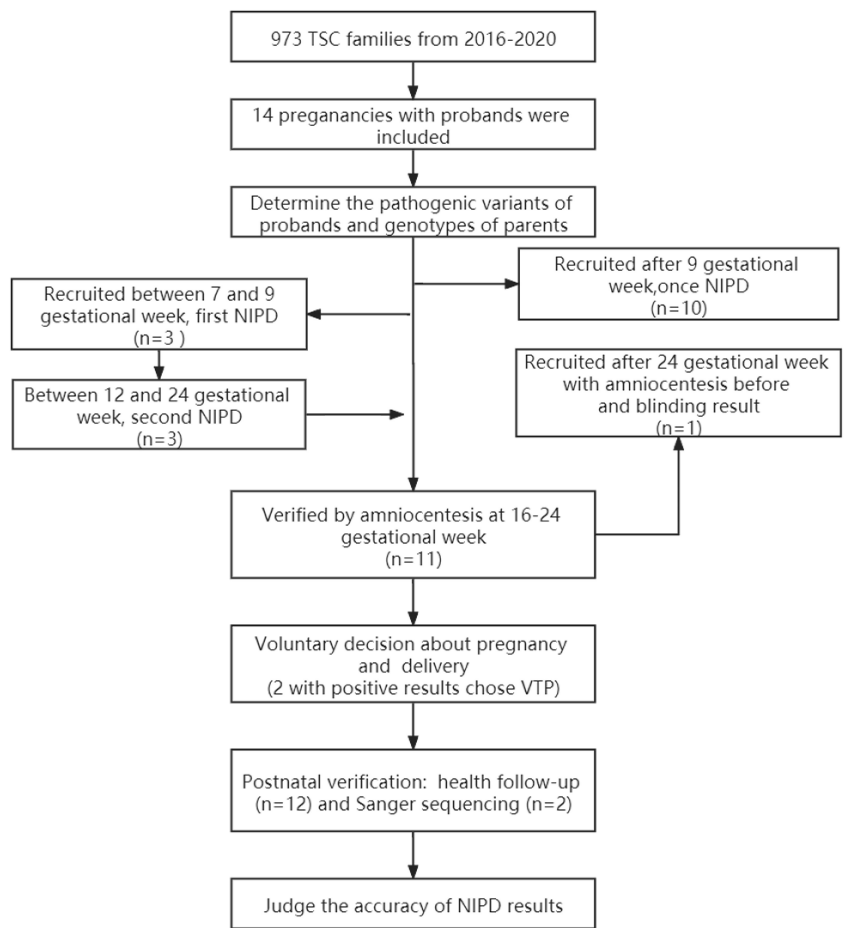
parents was then conducted to verify the result. All genetic variants of probands in this study had been classified as "pathogenic" or "likely pathogenic" according to the American College of Medical Genetics and Genomics (ACMG) guidelines for interpretation of genetic variants.

An initial NIPD test was performed when the recruited pregnant women were of 7–9 weeks' gestation. When the women reached weeks 12–24 of gestation, a second NIPD was conducted for confirmation. Amniocentesis verification was performed at 16–24 weeks' gestation. If the gestational age exceeded 9 weeks at the time of recruitment, NIPD was performed only once, and amniocentesis was performed for verification. If the optimal time for amniocentesis had passed at the time of recruitment, NIPD was conducted only once, and cordocentesis was suggested. For the born children who did not get amniocentesis or cordocentesis validation during pregnancy, postnatal Sanger sequencing would be performed to confirm whether the target locus of *TSC1* or *TSC2* gene was normal. In addition, we conducted telephone follow-up of children who were born and collected the related information, including the child's date of birth, gender, general health, and more importantly, whether TSC-related manifestations, such as skin lesions, epilepsy, developmental retardation, autism spectrum disorders, and heart/kidney hamartoma, had developed.

2.3 | Sample processing and DNA extraction

Exactly 10 ml of maternal blood was collected into EDTA-containing tubes and centrifuged at 1600× *g* at 4°C for 10 min. The plasma obtained was centrifuged once more under the above conditions, and the middle white layer of white blood cells was separated; both fractions were stored at −80°C. Referring to the protocols, plasma DNA was extracted by a MagPure Circulating DNA Kit (Magen, Guangzhou, Guangdong, China), and wbcDNA was extracted by a MagPure Tissue DNA LQ Kit (Magen, Guangzhou, Guangdong, China).

2.4 | Analytical strategy of NIPD

We applied gene-amplification and gene-trapping technologies to explore the feasibility and accuracy of these strategies in NIPD for TSC. And the transcripts we referred for *TSC1* gene were NM_000368.5 and NM_000548.5 for *TSC2* gene. The protocols were done following corresponding manufacturer instructions.

For gene amplification, we designed the probes of the targeted mutation region of the proband and then

amplified the targeted region using cfDNA and wb-cDNA as templates with TransStart® FastPfu DNA Polymerase (TransGen, Beijing, China). The amplified product was purified, and the corresponding library was constructed using a KAPA Hyper Prep Kit (Roche Sequencing Solutions, Pleasanton, CA, USA) and sequenced using the SE75 or PE75 sequencing strategy on the NextSEQ500 platform (Illumina, San Diego, CA, USA). Reads from the map to the target position of the GRCH37/HG19 human reference genome were counted, and the mutation ratio of the target site was calculated using the mutation reads number divided by total reads number within the same region. Read heterozygosity at single-nucleotide polymorphisms (SNPs) was used to infer FF (Dang et al., 2019).

For gene trapping, a DNA probe was designed to capture the exon regions of *TSC1*, *TSC2*, and *FGFR3*(OMIM *134934), *GJB2*(OMIM *121011), *MITF*(OMIM *156845), *MYO15A*(OMIM *602666), *PAX3*(OMIM *606597), *SLC26A4*(OMIM *605646), *SOX10*(OMIM *602229), and *TMC1* (OMIM *606706) genes. The next-generation sequencing library was prepared using a KAPA Hyper Prep Kit (Roche Sequencing Solutions, Pleasanton, CA, USA) from the extracted plasma cfDNA and wbcDNA fragments of 150–200 base pairs (bp) processed by ultrasound. The target region was trapped using an xGen Hybridization and Wash Kit (Integrated DNA Technologies, Coralville, Iowa, USA), enriched by PCR, and then sequenced using the PE75 strategy on the NextSeQ500 platform. After deduplication and decoupling, the data were compared with the GRCH37/HG19 human reference genome using BWA-MEM (v0.7.17-R1188), and the mutation information was detected by GATK (v4.1.2.0). The SNP data detected by xGen Human ID Research Panel (Integrated DNA Technologies, Coralville, Iowa, USA) were used to calculate FF (Zhang et al., 2019).

Fetal genotype was deduced based on deviations from maternally derived alleles and maternal backgrounds, fetal allelic fraction is equal to half of the FF (Figure 2 showed examples). We applied a well-received statistical algorithm based on a binomial model to determine the combined genotypes and likelihoods in the maternal plasma (Jiang et al., 2012). Formulas using this method were listed as follows:
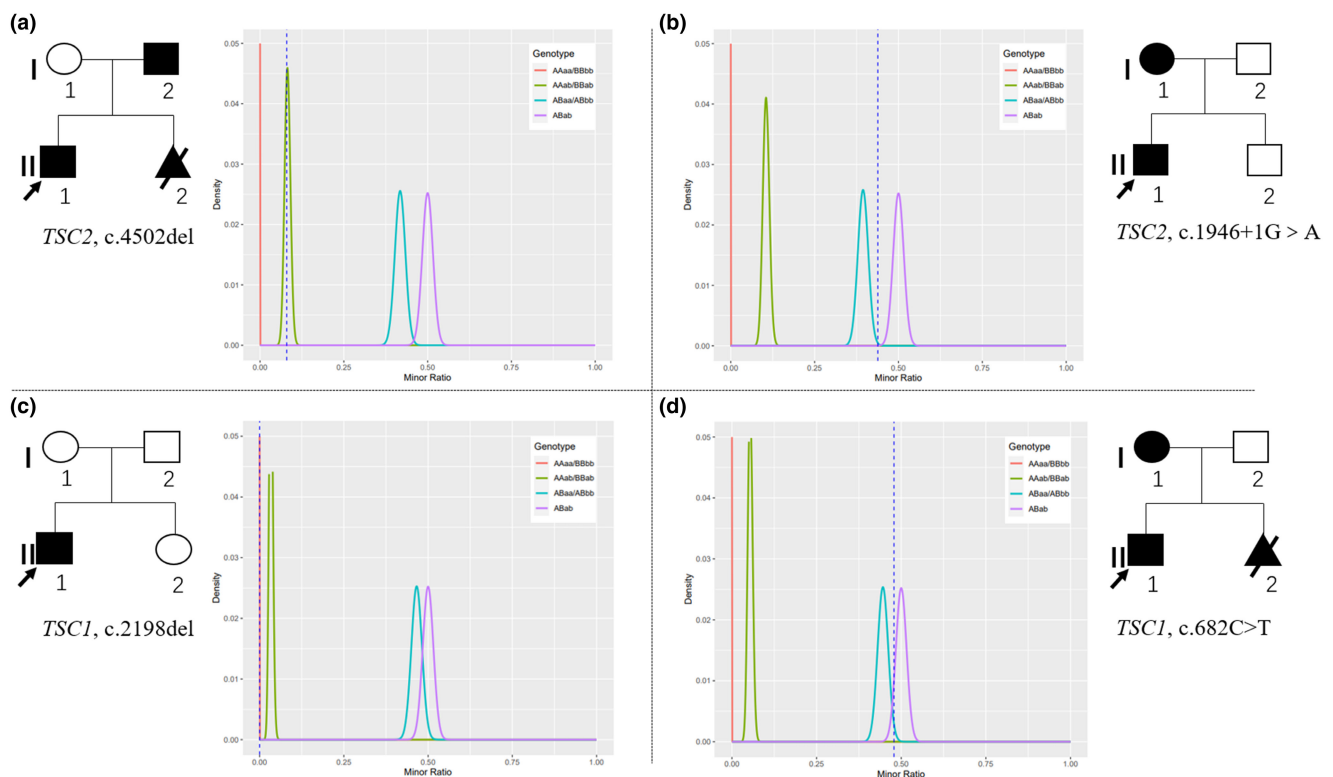

FIGURE 2 Representative results of NIPD. Fetal genotypes were deduced based on the deviations from maternal genetic alleles and maternal background. When $p > 70\%$, the results were reliable. The letters 'A' and 'B' referred to the maternal wild and mutant alleles, respectively, and letters 'a' and 'b' referred to the fetal wild and mutant alleles, respectively. (a) Family 1. The maternal-fetal genotype was AAab, the mutation ratio was 0.080 and $p = 100.0\%$. (b) Family 2. The maternal-fetal genotype was ABaa, the mutation ratio was 0.438 and $p = 97.8\%$. (c) the result of first NIPD of family 5. The maternal-fetal genotype was AAaa, the mutation ratio of cfDNA was 0 and $p = 100.0\%$. (d) Family 10. The maternal-fetal genotype was ABab, the mutation ratio was 0.479 and $p = 78.8\%$. *TSC1* accession: NM_000368.5, *TSC2* accession: NM_000548.5

$$D_i = \binom{N}{b} \mu_k^b (1 - \mu_k)^{N-b}$$

$$G = G_i \{ D_i = Max(D_1, D_2, \dots, D_n) \}$$

$$P = \frac{Max(D_1, D_2, \dots, D_n)}{\sum (D_1, D_2, \dots, D_n)}$$

Where N and b represent the total and mutant allelic count at target SNP locus, respectively, D is to describe allele's binomial distribution, k is maternal-fetal genotype combination such as AAaa, AAab, ABaa, or ABab (according to the standard genotype nomenclature, the letters 'A' and 'a' refer to maternal and fetal wild-type alleles, respectively, and letters 'B' and 'b' refer to maternal and fetal mutant alleles, respectively). Theoretically, if we assume the FF is f, μ is expected mutant allele fractions respected to each genotype combination, which expected to fluctuate around 0, f/2, 0.5, and 0.5-f/2, respectively. Finally, G is the most likely genotype and P is the corresponding likelihood. For quality control, the cut-off to call a valid genotype required total allele count (N) > 1000 and a likelihood (*p* value) > 70%.

3 | RESULTS

3.1 | Baseline data of the recruited families

The clinical demographics of the studied families are shown in Table 1. The types of pathogenic variants identified among the 14 probands included five missense mutations, four frameshift mutations, three nonsense mutations, and two splice-site variants. Among the variants detected, 12 were *TSC2* genes and two were *TSC1* genes. In terms of the source of variants, 11 arose de novo, two were inherited from the mother, and one was inherited from the father. Three of the 14 pregnant women received their first NIPD before 9 weeks' gestation, and the mean gestational age of all NIPDs was 17.18 ± 5.83 weeks (range: 8 to 29 weeks).

3.2 | NIPD results and validation

Among the 14 families recruited for this study, two fetuses were identified to have the same pathogenic variants as the probands, including a paternal variant detected at 22 weeks' gestation (family 1) and a maternal variant at 14 weeks' gestation (family 10) (Figure 2a,d). Both families chose voluntary termination of the pregnancy (VTP) after amniocentesis validation.

Twelve fetuses were diagnosed as normal by NIPD, and their siblings' variant source included de novo, paternal, and maternal inheritance. Nine of the cases were validated by amniocentesis, and the results were consistent with those of NIPD. Among the cases who were not verified by amniocentesis, one (family 3) had just passed the optimal gestational age for amniocentesis at the time of recruitment and received postnatal sequencing validation, one (family 5) completed second NIPD and postnatal sequencing validation, and one (family 7) had just accepted one NIPD without any experimental verification.

All 12 fetuses with normal NIPD results, including five males and seven females, were delivered. Till our last follow-up, their mean age was 2.74 ± 0.96 years. Apart from one child, all the other 11 were with no classic symptoms of TSC, especially cardiac hamartomas and hypopigmented macules, which present prenatal or early after birth. The only one (family 5) reported of a depigmentation plaque with diameter < 10 mm but was reported to be with negative result on postnatal Sanger sequencing. Also, only when there are three or more depigmentation plaques, can the symptoms be regarded as an item conforming major clinical diagnosis criteria of TSC (Northrup & Krueger, 2013).

Noticeably, there was a child (family 12) born with congenital hypertonia in her lower limbs. However, postanal whole-exome sequencing (WES) detected no responsible variants (neither *TSC1/2* gene aberrance nor other pathogenic variants), and her medical history provided no clues. Luckily, at the latest follow-up, she was able to walk with help at the age of 2 years after persistent rehabilitation.

4 | DISCUSSION

In this study, we explored the feasibility and accuracy of NIPD based on cfDNA and maternal wbcDNA in 14 families at risk for TSC. Despite the difference in sequencing methods and patients' phenotypes, all results were accurate.

The subjects we included can represent the majority of TSC patients. The mutational spectra of this study consisted of missense mutations, nonsense mutations, frameshift mutations, and splice-site variants, these represented the largest number of variants in TSC patients (Salussolia et al., 2019). The inheritances included 11 de-novo variants (78.57%), one paternal variant (7.14%), and two maternal variants (14.29%), consistent with the previously reported inheritance characteristics of TSC (i.e., 67%–75% of patients have spontaneous mutations and the rest inherit the mutation from their parents in an autosomal dominant manner) (Ehninger, 2013; Roach, 2016). Although the most common variants of the patients are de novo in

TABLE 1 Overview of studied families

	Variant of proband				NIPD							
Family	Gene	NA change	Variant type	Inheritance	Gestational week	Strategy	FF (%)	Mutation ratio	Probability value (%)	Result	Verification	Follow-up
1	TSC2	c.4502del	frameshift	paternal	22 w	amplification	16.40	0.080	100.00	+	A+	VTP
2	TSC2	c.1946+1G >A	splice-site	maternal	29 w	amplification	21.10	0.438	97.80	−	A-	4 y, M, healthy
3	TSC2	c.2713C>T	missense	de-novo	27 w	amplification	18.40	0.000	100.00	−	P-	4 y, F, healthy
4	TSC2	c.1903del	frameshift	de-novo	8 w / 19 w	amplification	19.70 / 24.80	0.000 / 0.000	100.00 / 100.00	−	A-	3 y 4 m, F, healthy
5	TSC1	c.2198del	frameshift	de-novo	8 w / 18 w	amplification	6.77 / 9.10	0.000 / 0.000	100.00 / 100.00	−	P-	3 y 4 m, M, healthy (1 white patch,<1 cm)
6	TSC2	c.1513C>T	nonsense	de-novo	9 w / 15 w	amplification	4.90 / 7.80	0.002 / 0.002	100.00 / 100.00	−	A-	3 y 3 m, F, healthy
7	TSC2	c.3080del	frameshift	de-novo	19 w	amplification	8.60	0.000	100.00	−	N	3 y, M, healthy
8	TSC2	c.4858C>T	missense	de-novo	18 w	trapping	16.00	0.000	100.00	−	A-	2 y 7 m, F, healthy
9	TSC2	c.5138G>A	missense	de-novo	20 w	trapping	5.40	0.000	100.00	−	A-	2 y 8 m, F, healthy
10	TSC1	c.682C>T	nonsense	maternal	14 w	trapping	10.80	0.479	78.80	+	A+	VTP
11	TSC2	c.3685C>T	nonsense	de-novo	17 w	amplification	17.00	0.000	100.00	−	A-	2 y 6 m, M, healthy
12	TSC2	c.5227C>T	missense	de-novo	13 w	amplification	10.90	0.002	100.00	−	A-	2 y 1 m, F, Not TSC, hypertonia in lower limbs
13	TSC2	c.5228G>A	missense	de-novo	18 w	amplification	12.00	0.003	100.00	−	A-	1 y 1 m, M, healthy
14	TSC2	c.5069-3_5069-2del	splice-site	de-novo	18 w	amplification	13.30	0.000	100.00	−	A-	1 y 1 m, F, healthy

Note: TSC1 Accession: NM_000368.5, *TSC2* Accession: NM_000548.5.

Abbreviations: NA, nucleic acid; FF, fetal fraction; No, not conducted; VTP, voluntary termination of pregnancy; M, male; F, female; N, no verification done. Symbol: +: positive result; −: negative result; A+: positive result by amniocentesis; A -: negative result by amniocentesis; P-: negative result by postnatal sequence.

nature, parents are likely to have another TSC child because of the possible mosaic or intronic germ line mutations (Rose et al., 1999; Verhoef et al., 1999). Thus, when equipped with higher accuracy, NIPD focusing on the culprit variants in affected families is necessary. However, the current NIPD based on cfDNA is not suitable for the direct detection of mosaic mutations because the low concentration of cffDNA in maternal peripheral blood can result in high rate of false negatives. As for the realization of intron mutation detection through NIPD, it depends on the further advancement of detection technology.

Amniocentesis is the most commonly applied technique among the invasive prenatal diagnosis approaches, but the timeframe for this technique is limited to 16–24 weeks′ gestation. Chorionic villus sampling can be performed at an earlier gestational age of 10–13 weeks but presents higher associated risks (Akolekar et al., 2015; Alfirevic et al., 2017). NIPD based on cfDNA can be achieved in earlier gestational weeks without harming the fetus. Although the average gestational age we included was greater than the first trimester, we had two subjects whose NIPD was made at eight gestational weeks and one at nine gestational weeks. The results were verified to be reliable. At present, the earliest recommended application time of NIPD for monogenic diseases is 9 weeks′ gestation (Hill et al., 2014; Scotchman, Chandler, et al., 2020). In future work, we plan to include more participants with gestational age earlier than 9 weeks to identify the earliest feasible timing of NIPD because we believe that earlier prenatal diagnosis will reduce parental anxiety and allow more time for decision-making and planning.

As reported in previous studies, the major challenge of NIPD for autosomal dominant disease detection is the situation wherein the mother carries the pathogenic variant because detecting the inheritance of the maternal allele against the extensive contamination of maternal cfDNA requires an accurate quantitative strategy. The main methods employed to address this problem include relative mutation dosage (RMD) and relative haplotype dosage analysis (RHDO) (Lo et al., 2010; Lun et al., 2008). Although the latter one is reported to be more robust, it is also more complicated and expensive. In our study, we deduced the fetal genotypes based on deviations from maternally derived alleles and maternal backgrounds. As shown by the results, the likelihoods all reached 100% when the maternal genotypes were normal, while in addition, the mother carried the variant, the likelihoods were lower than 100%, especially when the fetus was affected by variants. However, as long as the likelihood is above 70%, the result is accurate.

Although our 14 results were all accurate, more samples were required to verify the accuracy of the method because cases classified to specific conditions were fewer. Besides, there are some biases that may affect the accuracy of the experiment. For example, during library construction and sequencing process, low level allele count bias and allele-specific copy number variations can be introduced that result in sequence background noise (Schirmer et al., 2015). In addition, gene amplification may produce allele dropout on account of highly fragmented cfDNA. Furthermore, we deduced fetal genotypes depended on SNP and SNP-based FF in this study, however, maternal inbreeding coefficient and sequencing error would affect the accuracy of inferring SNP and FF (Dang et al., 2019). To resolve this problem, we also detected wbcDNA besides cfDNA so that can improve the precision of FF estimation by inferring maternal inbreeding coefficient and combining reads from maternal wbcDNA and cfDNA. Nevertheless, SNP has some distribution biases in reality that may affect the accuracy of deducing, for example, transitions are more frequent than transversions among substitution mutations and CpG dinucleotides are dominated near transitions (Zhao & Boerwinkle, 2002). Therefore, with consideration of the probabilities of sequencing errors, alignment errors as well as stochastic variations, the likelihoods threshold of 70% in deducing fetal genotype is determined after experiments (Jiang et al., 2012).

The other limitation of our study was that our strategy was not applicable for large deletions, but this type variant only accounts for a small part (1.23%–5.96%) of TSC genotypes (Au et al., 2007; Ding et al., 2020). In fact, the detection of variants such as large deletions/duplications and triplet duplications is a great challenge for NIPD based on cfDNA. Therefore, although great progress has been made in NIPD, there is still a long way to go to promote the clinical application of NIPD for monogenic disorders because there remained technical challenges to overcome and the accuracy of the developed technologies needed to be confirmed by different diseases. Since TSC is a disorder caused by a variety of gene mutation types, it can contribute to the application and promotion of NIPD.

CONFLICT OF INTEREST
The authors report no conflict of interest.

ETHICAL COMPLIANCE
This study was approved by the Ethical Committee of Chinese People′s Liberation Army General Hospital (PLAGH) and was conducted after obtaining the written informed consents from all participating pregnant women and their husbands.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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