Original Research

A prospective clinical trial to compare the performance of dried blood spots prenatal screening for Down's syndrome with conventional non-invasive testing technology

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Impact statement

Children born with Down's syndrome display a wide range of mental and physical disability. Currently, there is no effective treatment to ease the burden and anxiety of the Down's syndrome family and the surrounding society. This study is to evaluate the efficiency of dried blood spots against serum screening for Down's syndrome and to construct a two-tier strategy by topping up the fetal cell-free DNA (cfDNA) secondary screening over the high-risk women marked by the primary blood testing to build a practical screening tactic to identify fetal Down's syndrome. Results demonstrate that fetal cfDNA can significantly reduce false-positive rate close to none while distinguishing all true positives. Thus, we recommend that fetal cfDNA analysis to be utilized as a secondary screening tool atop of the primary blood protein screening to further minimize the capacity of undesirable invasive diagnostic operations.

Abstract

To evaluate, side by side, the efficiency of dried blood spots (DBSs) against serum screening for Down's syndrome, and then, to construct a two-tier strategy by topping up the fetal cell-free DNA (cfDNA) secondary screening over the high-risk women marked by the primary blood testing to build a practical screening tactic to identify fetal Down's syndrome. One thousand eight hundred and thirty-seven low-risk Chinese women, with singleton pregnancy, were enrolled for the study. Alpha-fetoprotein and free beta human chorionic gonadotropin were measured for the serum as well as for the parallel DBS samples. Partial high-risk pregnant women identified by primary blood testing ($n = 38$) were also subject to the secondary cfDNA screening. Diagnostic amniocentesis was utilized to confirm the screening results. The true positive rate for Down's syndrome detection was 100% for both blood screening methods; however, the false-positive rate was 3.0% for DBS and 4.0% for serum screening, respectively. DBS correlated well with serum screening on Down's syndrome detection. Three out of 38 primary high-risk women displayed chromosomal abnormalities by cfDNA analysis, which were confirmed by amniocentesis. Either the true detection rate or the false-positive rate for Down's syndrome between DBS and the serum test is comparable. In addition, blood primary screening aligned with secondary cfDNA analysis, a ''before and after'' two-tier screening strategy, can massively decrease the false-positive rate, which, then, dramatically reduces the demand for invasive diagnostic operation.

Keywords: Down's syndrome, prenatal screening, dried blood spots, non-invasive prenatal testing

***Experimental Biology and Medicine* 2017; 242: 547–553. DOI: 10.1177/1535370216683837**

Introduction

Down's syndrome, usually resulted from triple, rather than double copies of human chromosomal 21, is the most common form of human chromosomal diseases, which affects 1/800 live-born infants.[1] Children born with Down's syndrome display a wide range of mental and physical disability, which, to various extents, limits their physical activity and social engagement,[1] and currently, there is no effective treatment to ease the burden and anxiety of the Down's syndrome family and the surrounding society. Therefore, it is of critical clinical significance to develop a simple, affordable, and reliable method to screen the pregnant women in order to reduce the birth rate of Down's syndrome.[2]

Initially, the screening for Down's syndrome was solely determined by maternal age since it was known that older women had a greater chance to carry a Down's syndrome baby.[3] From early 1980s, several maternal serum proteins have emerged as significant biological markers to screen Down's syndrome. First, a significant relationship between lowered maternal serum alpha-fetoprotein (AFP) and

Down's syndrome was identified,[4,5] followed by detection of an association of a high human chorionic gonadotrophin (HCG, total-, or beta subunit, βHCG) level with Down's syndrome.[5,6] Later work suggested that second trimester unconjugated oestriol (uE3) was decreased as an important indicator for Down's syndrome.[7,8] More recent advance in serum screening has led to the discovery of two more altered serum proteins, specifically, depressed pregnancy-associated plasma protein A[9] and elevated inhibin A[10,11] correlated with Down's syndrome. Nowadays, serum test involving two biological markers in combination with maternal age has been globally employed as an initial Down's syndrome screening method since it has been documented that the efficiency and sensitivity of testing more than two serum markers have not been significantly improved.[12] Thus, the maternal AFP and HCG utilized as Down's syndrome biological markers were assayed in dried blood spot (DBS) and the correspondent serum samples in our current study.

Although ''second trimester serum test'' is commonly administered in clinic to screen Down's syndrome in mainland China, collected serum samples often encountered delay or mismanagement during shipment because of huge population and vast territory with underdeveloped transportation facility in some rural area. Eighty percent of pregnant women in China reside in rural areas, such as central-western remote region and northeast farmlands, which are disadvantaged by limited supply of modern medical device and instrumentation. Strenuous transportation and shortage of contemporary prenatal care have challenged prenatal care to be distributed into rural China. Up to 2011, China's pregnancy serological screening coverage rate is only 22.7%,[13] hence it is necessary to explore a simple and practical screening method to diagnose prenatal defects resulting from chromosomal abnormalities. DBS offers a desired solution to overcome the difficulties of sample transportation and storage, which can significantly increase the coverage of prenatal screening in rural China; however, the blood screening, either DBS or classical serum test, is associated with a high false-positive rate, a challenge to be overcome yet.

Newly invented non-invasive prenatal testing (NIPT), also named as cell-free DNA (cfDNA) analysis, is, indeed, a more advanced assay on Down's syndrome screening with an almost perfect detection efficiency (>99.5%) and a low false-positive rate (< 0.1%).[13] Yet, its costive and refined experimentation involving massive sequencing has made it unrealistic to be utilized as a primary screening application in general prenatal clinic. However, the combination of initial maternal blood screening followed by more sophisticated NIPT reassurance can provide a non-invasive approach to largely identify prenatal Down's syndrome before subjecting to invasive diagnostic operation, which still carries a likelihood of traumatic abortion even in the contemporary clinic in developed countries.[12,14] Therefore, the main goal of our current study was to compare the performance of DBS against classical serum test for its sensitivity and specificity in an attempt to develop a field-friendly and cost-effective assay for primary screening on Down's syndrome; next, NIPT, as a secondary screening tool, was utilized to differentiate the true positive Down's syndrome from the false positives tagged by primary blood screening to further increase the specificity of Down's syndrome screening and to avoid the unnecessary invasive confirmation.

Methods

Patients

This clinical trial was carried out at Beijing Pinggu Hospital in the Pinggu District. The ages of all enrollees are between 19 and 43. Within 1946 individual prenatal visitors, 1837 pregnant women at second trimester between August 2013 and March 2014 were recruited for the trial with prior consents. Still, 62 patients from 1837 enrollee were spontaneously aborted from the trial; at last there were 1775 patients who completed the entire course of the trial. All medical personnel and staff were fully instructed and trained to comply with the regulation and disciplines relevant to the project before they were inscribed into the research team. DBS and maternal serum screening (MSS) sampling were performed in Beijing Pinggu Hospital. DBS, MMS, amniocentesis, and karyotyping were performed in Peking Union Medical College Hospital. NIPT sample detection was conducted by company of Berry Genomics. Single nucleotide polymorphism (SNP) array sample detection was conducted by company of Beikang. All enrollee are required to meet the following criteria: (a) Chinese natives, (b) singleton pregnancy, (c) 15–20 weeks of gestation, (d) accessible prenatal and postnatal follow-ups, (e) without major or chronic diseases.

Methods

MMS and DBS samples were collected simultaneously and labeled with the following personal and medical information: blood sampling dates, registration ID, telephone number, date of birth, weight, the last menstrual period, gestational age, and previous adverse pregnancy and medical history.

Plasma and serum collection: A total of 5 mL of whole blood was drawn from each patient and then separated them into plasma or serum before storing them in −20°C freezer.

DBS collection: Triple separated DBS samples, each with a diameter of 1.5 cm, on a fully soaked filter paper, were collected by DIFF-SAFE device (Alpha Scientific Corporation, USA) from each patient. DBS samples on filter papers were dried for at least 30 min to a maxima of 4 h before sealed in a plastic bag and then stored at room temperature for later assay.

Both AFP and free βHCG were measured in serum as well as in DBS samples by a time-resolved immunofluorescence kit (PerkinElmer Life and Analytical Science, Turku, Finland). Meanwhile fetal cfDNAs were extracted from the maternal plasma for later NIPT analysis to reassert serum and/or DBS-positive results before further invasive examination. All assays were performed according to the protocols provided by the manufacturers. Miscarriages and abortions resulted from either fetal or maternal

medical problems were analyzed by SNP array. Amniocentesis followed by karyotyping was performed at Peking Union Medical College Hospital as a decisive procedure employed in the current study to confirm fetal Down's syndrome diagnosis.

Statistical analysis

A database was established by using Visual Foxpro 5.0 and analyzed by SAS 9.1 statistical software. Sensitivity and specificity for DBS or MSS were evaluated individually and then in parallel to compare and contrast the efficiency of two methods on detection of Down's syndrome. In addition, Youden's index, positive predictive value (%), negative predictive value (%), positive likelihood ratio, and negative likelihood ratio were also utilized to reaffirm the reliability of the two screening methods. Finally, Kappa coefficient analysis followed by McNemar test was applied to measure the statistical agreement on sensitivity and specificity between two DBS and MSS screening methods.

Results

1. **Enrollment and the dropout rate:** Figure 1 illustrates that, from 1946 prenatal visitors, 1837 patients with former consents were administered for this prospective clinical trial. And then, 1775 out of 1837 patients completed the trial as presented in Table 1; however, there were 62 patients who spontaneously dropped out through the course of the trial, which brought the dropout rate to 3.4%.
2. **Pregnancy outcomes:** Among 1775 enrollee, 1737 of them delivered live infants, wrapped with 25 premature babies and 22 babies with birth defects (1.2%) categorized as follows: nine cases of heart defects, seven cases of multifinger/toe deformity, three cases of ear deformities, two cases of cleft lip and palate deformity, two cases of gastrointestinal malformations, one case with multiple organ abnormalities (Table 1). On the other hand, 16 out of 1775 subjects prematurely ended the trial because of the adverse pregnancy consequences (Table 1), specifically, abortion (seven cases) which was further divided into social cause for three cases and maternal cause for four cases (one case of infection, one case of bleeding, two cases of the premature cervical dilation), fetal death (two cases due to umbilical cord twisting), and fetal chromosomal abnormalities (two cases).
3. **Comparison of the predictive value and correlation between two primary screening methods:** Data displayed on Tables 2 and 3 implicate that both screening methods share similar sensitivity and specificity to

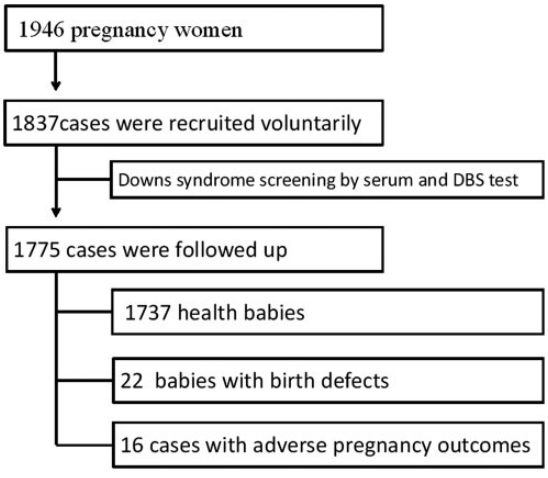

Figure 1 Enrollment and outcomes of the primary series of 1946 low-risk pregnant women

Table 1 Percentages of new-born babies with various defects of 1775 enrollers

Pregnancy outcome	Frequency, n = 1775(%)
Healthy babies	1737 (99.97)
Babies with birth defects*	22 (1.25)
Heart defects	*9 (0.51)*
Multifinger/toe deformity	*7 (0.40)*
Ear deformities	*3 (0.17)*
Cleft lip and palate	*2 (0.11)*
Gastrointestinal malformations	*2 (0.11)*
Adverse pregnancy outcomes	16 (0.91)
Abortion by social factors	*3 (0.17)*
Induction for heart and spina bifida deformities	*3 (0.17)*
Termination of pregnancy by the parent factors	*4 (0.23)*
Fetal death by umbilical cord twisting	*2 (0.11)*
Fetal chromosomal abnormalities	*3 (0.17)*

*One of these defects is multiple organ abnormalities.

Table 2 Results of MMS, DBS, and follow-up outcome for Down's syndrome

		Follow-up outcome		
Method	**Result**	**Positive**	**Negative**	**Total**
MMS	Positive	2	70	72
	Negative	0	1703	1703
DBS	Positive	2	52	53
	Negative	0	1722	1722
	Total	2	1773	1775

Table 3 Screening result between MMS and DBS method for Down's syndrome

	DBS				Kappa coefficient
MMS	**Positive**	**Negative**	**Total**	***P****	**(95%*CI*)**
Positive	32	40	72	0.015	0.495 (0.385–0.604)
Negative	21	1682	1703		
Total	53	1722	1775		

DBS: dried blood spot; MMS: maternal serum screening.
*McNemar test.

detect Down's syndrome in low-risk pregnant women. Lastly, the statistical agreement between two primary screening methods evaluated by Kappa coefficient displayed on Table 4 demonstrates that DBS and MMS correlate very well on their reliability to capture initial high-risk pregnant women. However, the unique and simple way of blood sampling by DBS method highlights its realistic application in places which lacks advanced prenatal care clinic, such as rural China.

4. **Secondary screening by NIPT for fetal cfDNA:** In order to reduce the false-positive rate associated with primary blood screenings on fetal Down's syndrome, 38 high-risk women identified by DBS or MMS primary screening voluntarily underwent secondary screening by sequencing fetal cfDNA obtained from maternal plasma when blood samples were taken (Figure 2 and Table 5). Only three positives from 38 high-risk patients were marked by cfDNA analysis and the rest of 35 high-risk patients were negative on cfDNA analysis. Among those three positives detected by cfDNA, two were trisomy 21 Down's syndrome later confirmed by amniocentesis and one was false positive, presumably placental mosaicism (chrX+/T18 (mos), because the karyotyping result from amniocentesis showed a normal fetal chromosomal arrangement. Our result grounds that fetal cfDNA analysis can significantly cut down the false-positive rate from the high-risk population produced by primary screening and represents a valuable

Table 4 Diagnosis index of MMS and DBS method for Down's syndrome

Index	MMS	DBS
Sensitivity (%)	100	100
Specificity (%)	96.05	97.12
Youden's index	0.961	0.971
Positive predictive value (%)	2.778	3.774
Negative predictive value (%)	100	100
Positive likelihood ratio	25.32	34.72
Negative likelihood ratio	0	0

DBS: dried blood spot; MMS: maternal serum screening.

Table 5 Summary of the efficiency between MMS and DBS screening methods

Methods	MMS	DBS
Primary screening (n)	1775	1775
Positive screening (n)	76	56
Amniocentesis confirmation (n)	2	2
Karyotype confirmation (n)	2	2

DBS: dried blood spot; MMS: maternal serum screening.

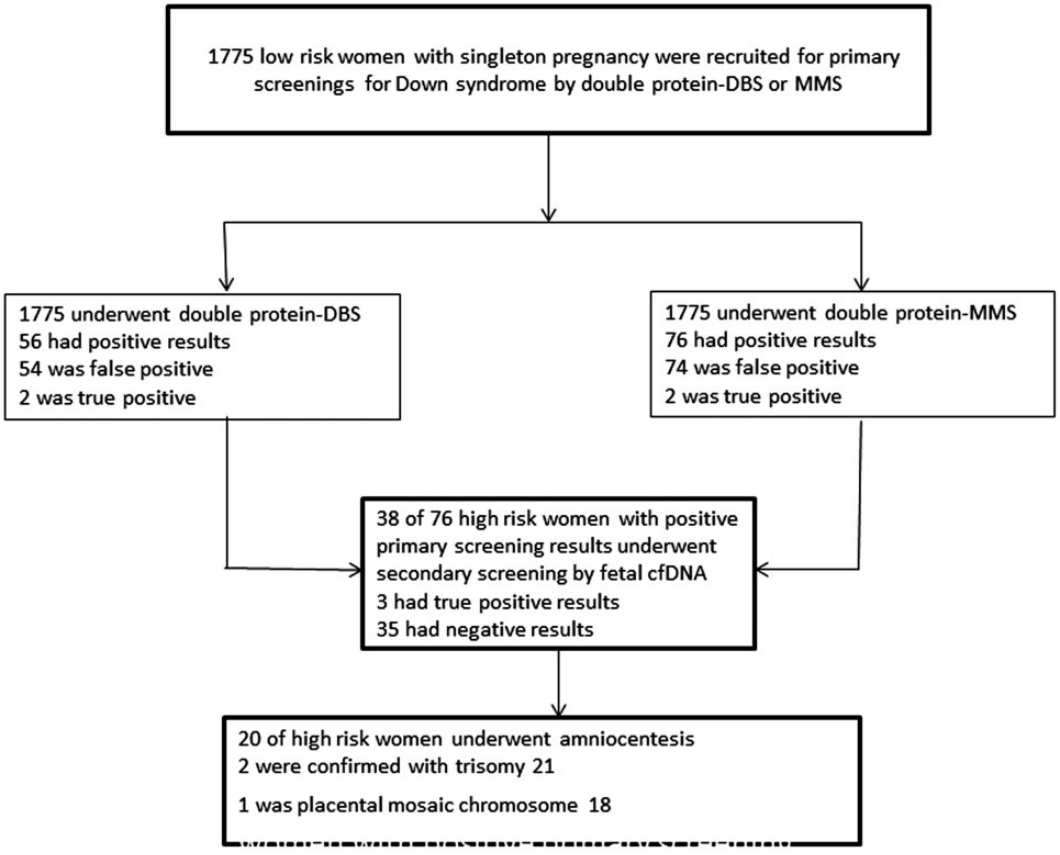

Figure 2 Schematic presentation of the allocations of the total enrollee in each primary, secondary, and conformational analysis on Down's syndrome

supplement to the primary screening, which can dramatically minimize the number of patients to be operated by amniocentesis or other invasive diagnostic technology. On the other hand, our data also imply that results from cfDNA analysis cannot be treated as definitive diagnosis, for example, the mismatch of placental mosaicism with a normal fetal chromosomal arrangement in this study, and in other word, cfDNA examination is still a *screening* technology. However, its low false-positive rate together with its non-invasive nature has dramatically widened its acceptance and advanced the efficiency of Down's syndrome screening in prenatal clinic.

5. **Confirmation of Down's syndrome by amniocentesis and fetal karyotyping:** Table 5 summarizes the results of this clinical trial. Within 1775 enrollee, 76 patients in total were identified as the high-risk pregnancy screened by either primary MMS or DBS, from which 38 patients underwent secondary screening by cfDNA analysis. From three positive cases labeled by cfDNA examination, two were trisomy 21 Down's syndrome and one was a false positive resulted from placental mosaicism. All 56 high-risk cases measured by DBS were coincided with MMS positive population without a single case exception. Amniocentesis was operated in 19 of 76 high-risk pregnant women followed by karyotyping confirmation, two of which were diagnosed as fetal trisomy 21 Down's syndrome, and then the pregnancy was medically terminated for those two women. SNP array showed that miscarriages and abortions resulted from either fetal or maternal medical problems were without chromosome abnormalities.

Discussion

Since fetal trisomy 21, a cause for Down's syndrome, is the most common chromosomal aneuploidy, which can lead to ill health on acquired children, it has long been sought to establish an acceptable and reliable screening method to effectively identify fetal Down's syndrome in order to reduce its birth rate.[15] In developed countries, a serum double or triple protein test[14,16] in conjunction with cfDNA analysis[14,16] has offered a nearly perfect score by identifying >99.5% patients.[1,14] However, cfDNA analysis involves massive sequencing,[14,17] which results in intensive labor and generates unacceptable cost, thereby, inhibiting its application as a primary screening technology. Thus, the serum protein test is still the most popular primary screening tool in prenatal clinic practice. Yet, this scenario cannot be imaged in the developing countries, where contemporary prenatal care is restrained by limited medical personnel and substantial supplies. In the current study, we first focused on validation of DBS method accompanied by time-resolved immunofluorescence analysis,[18] which can be affordably applied in the medically disadvantaged areas, such as rural China.

Indeed, DBS method has been used as a screening technology for a wide range of diseases in clinic since Guthrie first introduced it to screen neonatal disease in 1963[19] followed by its application in hepatitis B[20] and Down's syndrome screening.[21,22] However, the strength and the uniqueness of our current study reside in: first, we conducted both screening methods, i.e. DBS and MMS, in parallel on the exact same low-risk population; second, there was a reasonable sample size with 1775 enrollments for this type of labor-intensive prospective clinical trial; third, protein assays of AFP and HCG for both methods were performed in the same facility to reduce procedural bias; fourth, we added depth in the experimental design, i.e. a two-tier screening strategy, in which high-risk women labeled by primary blood screening were again tested by secondary advanced cfDNA analysis to expeditiously narrow down the number of patients subjecting to invasive confirmation; finally, the trial with almost all enrollment was followed up and the true positive cases were affirmed by amniocentesis along with karyotyping.

The incidence of Down's syndrome from our study was matched with existing database[23] with a rate of 1/800 screened by either primary method, which implicates that efficiency of DBS is equivalent to that of classical serum protein tests or MMS. In contrast to MMS, the way of blood sampling, handling, and storage in DBS provides a realistic circumstance to cover a larger area with dense, but medically deprived population. In addition, it is still under debating[12,23] whether double protein assay in screening Down's syndrome can be as sensitive and accurate as the triple protein one. Clinically and statistically, our data presented a solid evidence in favor of two protein assays because all babies born with negative screening test were unaffected by Down's syndrome, which was of clinic relevance in terms of the summed cost for each extra protein assay.

In this study, we have utilized different groups of statistic methods, i.e. Youden's index, positive and predictive value, positive and negative likelihood ratio, to precisely quantify the efficiency of each primary assay and then to measure the agreement on sensitivity and specificity between DBS and MMS by Kappa coefficient analysis. Certainly, a significant correlation was reached between two primary blood screening assays in detecting high-risk fetal Down's syndrome women. Likewise, the false-positive rate calculated by DBS (3%) screening method was similar to MMS (4%) value, also in agreement with others' previous studies.[12,14,21,22] Thus, our data show that DBS stands as a valid primary screening alternative with its efficiency comparable to the widely accepted MMS screening, and the combination of two methods in screening Down's syndrome did not provide additive value because both methods identified the overlapped high-risk women and the true positive cases.

The quality of DBS samples has been evaluated previously by Palomaki *et al.* who reported that AFP and HCG proteins were well preserved on the filter paper for more than nine days at 37°C with 53–67% elution rate.[21] A similar study found that biological characters of AFP protein sampled by DBS were highly maintained with an elution

rate up to 86.36–90.1%.[24] Another study conducted by Canick *et al.*[7] described that the DBS AFP on a filter paper sent by mail could even be stable for as much as four weeks at 4, 25, or 37°C temperature. Together, all of the above studies suggest that DBS sampling is dependable for a large-scale screening program and the samples can be sent from various distance, but analyzed at an equipped facility.

Numerous previous reports have examined the similarity and difference between DBS and MMS on their efficiency in assaying serum proteins, but most of the studies concentrated on the assay *per se*[18] to compare the assay's validity represented by intra- and inter-assay variability between two sampling methods. Almost all studies[18] agreed upon those analysts, such as AFP and HCG assayed from DBS samples, correlated well with the results produced by conventional serum assay. In addition, most assays examined retrospective blood samples[25] in contrast to our prospective clinical trial with advanced careful and matched experimental designs. Argumentatively, there was one study contradicted with existing database by Palomaki *et al.*,[21] who observed that DBS sampling produced a slightly lower Down's syndrome screening performance; however, the authors acknowledged that their DBS and serum samples were obtained from different cohorts represented by two studying population. Thus, our study was an addition to previous work by direct comparison of the performance of two primary screening assays with exactly matched blood samples from the same pregnant women. With the paired blood samples, DBS appeared to be as efficient as MMS in Down's syndrome screening. Our data strongly suggest that DBS can be a substitute, rather than a supplement, to be used as the primary screening assay in detecting Down's syndrome with equivalent efficiency to classical serum test. By weighing in its field-friendly and cost-effective nature, this assay can reasonably be considered as the first pick in primary Down's syndrome screening without sacrificing screening efficiency in general prenatal care.

Both primary screening methods produced a similar false-positive rate between 3 and 4% in our study, consistent with comparable published studies with low-risk population.[12,14] By counting on 1/800 Down's syndrome incidence rate, there would be on average one true positive case in every group of 30 invasive amniocentesis if the primary screening solely depends on the results of blood protein test, which represents the most significant setback associated with current blood protein tests on Down's syndrome detection. We, thus, sought a before and after, rather than head-to-head, two-tier secondary screening strategy to overcome this challenge with which fetal cfDNA test was only applied to high-risk patients tagged by primary blood protein test. In this regard, our study is a valuable extension of previous published work by effectively but not more costly distinguishing the true positives with fetal Down's syndrome, and indeed, this ''before and after'' Down's syndrome screening scheme dramatically short-listed the false-positive cases because there were only three positive subjects identified by fetal cfDNA analysis from 38 high-risk patients obtained from primary DBS or MMS screening. Of those three positives produced by fetal cfDNA, two cases were confirmed as fetal Down's syndrome and one was a placental chromosomal mosaicism.

Since Lo *et al.*[26] first described the presence of circulating fetal cfDNA in maternal plasma in 1997, its diagnostic value has come into clinic with amber applications including prenatal chromosomal aneuploidy detection.[14,27–29] Gradually, maternal plasma cfDNA scrutiny has integrated into routine prenatal care mostly in detecting chromosomal aneuploidy, after a transition of experimental and clinical validation of the methodology.[27] From the year of 2011, fetal cfDNA analysis has been approved to be incorporated into prenatal care clinic as an alternative to fetal screening technology in the United States[14] and elsewhere across the world.[28] The fetal cfDNA test, also named as NIPT, appears to be superior to all existing prenatal screening methods in its specificity, with an improvement factor of 10,[14] which, of course, significantly decreases the false-positive rate. However, there are two major weaknesses tied with this technique and inhibiting its wide clinical application: first, it demands tremendous sequencing resulting in intensive labor and high cost; second, it is still a screening tool, not a diagnostic method. Put differently, its positive results have to be confirmed by invasive diagnostic operation, such as amniocentesis with karyotyping.

Conclusions

Our study, by embracing a ''before and after'' two-tier strategy, demonstrates that fetal cfDNA, applied as a secondary screening tool after primary blood protein test, can significantly reduce false-positive rate close to none while distinguishing all true positives. Thus, by considering the ratio of its cost to efficiency, we recommend that fetal cfDNA analysis to be utilized as a secondary screening tool atop of the primary blood protein screening to further minimize the capacity of undesirable invasive diagnostic operations, which still carries a risk of abortion even in the most advanced prenatal clinic in developed countries.[12]

Authors' contribution: LM contributed to the concept of the study; HH and YJ collected the data. MZ and SL made the data analysis. NH, JZ, JL, and XZ prepared the manuscript.

ACKNOWLEDGEMENTS

This study was supported by the Scientific Research Foundation of Middle-aged and Young Scientist of Peking Union Medical College Hospital (pumch-2013-017).

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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(Received September 1, 2016, Accepted November 14, 2016)