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Quantitative detection of cell-free fetal DNA in peripheral blood of pregnant women during early pregnancy

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Summary

Objective: This study aimed for the quantitative detection of cell-free fetal DNA (cffDNA) in peripheral plasma of pregnant women, which provides basic data for clinical non-invasive prenatal screening in early pregnancy. *Materials and Methods:* A total of 243 individuals with gestational age of 5-10^{+6} weeks were selected for this study, who required abortion.mDNA was extracted from villi, and Y-chromosome specific SRY gene was detected by nested PCR, which was regarded as standard for quantitative detection of accuracy. CffDNA was extracted from peripheral blood and SRY gene's expression was detected by real-time fluorescent quantitative PCR. *Results:* Among 243 cases, nested PCR detected 163 individuals with SRY gene positive, and 80 were negative. Out of 163, in 140 cases SRY was detected in cffDNA, but 23 cases with gestational age 5-6 $^{+6}$ weeks were negative. However at 7th week of pregnancy, it was also detected in those 23 cases. The average concentration of cff DNA in 10th week of pregnancy was found significantly higher than in 7th, 8th, and 9th week of pregnancy. *Conclusion:* Thus, this study indicates the efficiency and reliability of cffDNA in peripheral blood of 7-10th week of gestational period for the detection of early pregnancy.

Key words: Cell-free fetal DNA; SRY gene; Nested PCR; Fluorescence quantitative PCR; Gestational period.

Introduction

The previous prenatal diagnosis was based on the extraction of fetal DNA through invasive operation, such as chorionic villus sampling, amniocentesis, and cord blood collection, etc., however these methods have risks for both pregnant women and the fetus, and generally they require at least 11 weeks of gestational [1-3]. With the high risk, the choice of termination of pregnancy is often very painful for the pregnant women and their families. The development of molecular diagnostics aided the use of cell-free fetal DNA (cffDNA), which circulates freely in mother's blood as a non-invasive method for prenatal diagnosis. The real-time quantitative PCR assay based method to measure the concentration of fetal DNA in maternal plasma and serum in early pregnancy (as well as 7th week of pregnancy) was developed two decades ago [4].

Fetal DNA is usually fragmented (into approximately 200 bp) when placental microparticles are shed into the maternal blood circulation [5, 6]. CffDNA can be detected in maternal peripheral blood as early as 5th week of pregnancy, and the amount of cffDNA is increased with the increase of gestational weeks [7-9]. Thus, the detection of cffDNA can not only achieve non-invasive prenatal screening, but can also advance the detection of gestational age, and abnormal pregnancy as early as possible, reducing the mental and economic burden of pregnant women and their families.

The male-specific SRY gene on Y chromosome is popularly used as target for prenatal diagnosis using cffDNA, as this specific gene on this chromosome can be easily detected in 7th week of pregnancy, as earlier studies reported [7, 10]. The present study investigated the efficacy and accuracy of diagnosis of pregnancy by using cffDNA from peripheral blood of pregnant women with 5-10^{+6} weeks of gestational ages, with an objective to establish this molecular diagnostic methods for clinical application throughout the world.

Materials and Methods

A total of 243 singleton pregnant individuals (with gestational age 5-10^{+6} weeks) who required artificial abortion for family planning in Zigong Maternal and Child Health-Care Hospital during 2016-2017 were selected for this study. Patients with anemia, heart, liver, and kidney diseases were excluded. In addition, one healthy man was selected as the positive control, and a healthy non-pregnant woman was included as negative control. All the subjects signed the informed consent. The research protocol was approved by the ethical committee of Zigong Maternal and Child Health-Care Hospital.

The cell-free DNA BCT STRECK was used to collect blood from the individuals. Ten mL of peripheral blood with anticoagulant was taken from pregnant women, and centrifuged at 3,000 rpm at 4°C for ten minutes, then the supernatant was collected in new sterile Eppendorf tubes and centrifuged again at 16,000 rpm at 4°C for ten minutes. The supernatant was collected and cffDNA

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was then extracted using the QIAamp circulating nucleic acid kit. Tissue DNA extraction kit was used to extract the DNA of the villi. The cffDNA concentration and fragment distribution of maternal peripheral blood were analyzed by a agilent 2100 bioanalyzer and high Sensitivity DNA analysis kit.

Internal and external primers were used for the design of nested PCR, based on the SRY gene on the Y chromosome. The sensitivity and specificity of nested PCR were detected with healthy male and healthy female's genomic DNA. PCR reaction system was as follows: template 1 μL, dNTP 0.5 μL, Taq enzyme 0.2 μL, primer (10 μM) 1 μL, buffer 2.5 μL, and dd H_2O was added to make final volume of 25 μL.The first round reaction condition was as follows: 95°C for five minutes, 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds for 30 cycles, 72°C for 5 minutes, and hold on at 4°C. The product was of 138 bp size. The second round of reaction condition was as follows: 95°C for 5 minutes, 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds for 30 cycles, 72°C for 5 minutes, and hold on at 4°C. The product was of 116 bp size. 2% agarose gel electrophoresis was carried out after the end of PCR reaction.

Primers and probes for fluorescent quantitative PCR were designed for the SRY gene in sex determination region of Y chromosome. The healthy male genomic DNA was diluted and a standard curve was drawn. The primers and probe sequences were synthesized. The sequences were as follows:

SRY forward: 5'-TACAGGCCATGCACAGAGAG-3'

SRY reverse: 5'-TGTTGTCCAGTTGCACTTCG-3'

Probe: 5' – (FAM)-CCATGCACAGAGAGAAATACCCGAA – (TAMRA)-3'

Fluorescence quantitative amplification system: was as follows: primer (10 μM) 1 μL, Light Cycler 480 Probes Master mix 10 μL, probe 0.4 μL, template 5 μL, and ddH_2O was added to 20 μL. Reaction conditions were as follows: 95°C for 5 minUTES, 95°C for 10 seconds, 57°C for 15 seconds, 45 cycle at 72°C for 1 second, and hold at 4°C.

Results are presented as mean ± standard deviation. Excel 365 was used for data analysis, and non-normal measurement data using rank-sum test ($\alpha = 0.05$) was the statistical basis.

Results

The authors selected healthy male genomic DNA samples for testing. The concentrations of male genomic DNA after dilution were 10 ng/μL, 1 ng/μL, 0.1 ng/μL, 0.05 ng/μL, 0.01 ng/μL, and 0.005 ng/μL. Healthy female genomic DNA was used as negative control. The results of agarose gel electrophoresis of PCR products showed that nested PCR can specifically detect male DNA. The sensitivity of the assay was 0.05 ng/μL, as shown in Figure 1a. A total of 243 cases of pregnant women's villi tissue samples were used for nested PCR test. The results showed that 163 samples are positive for SRY gene and 80 samples were negative. A representative electrophoresis result of PCR products is shown in Figure 1b.

The healthy male's genomic DNA was used for quantitative fluorescence detection after gradient dilution. The resulting amplification curves and standard curves are shown in Figures 2a, b. Amplification efficiency was found 1.983, in line with experimental requirements.

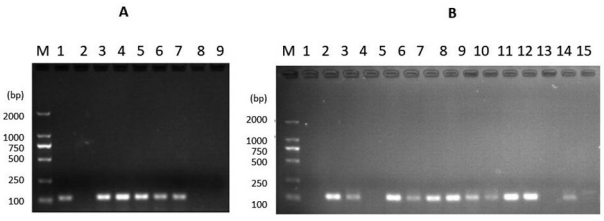

Figure 1. — Electrophoresis result of Nested PCR. A. Nested PCR detection of SRY gene. Lane M: maker; Lane 1, 3: positive control; Lane 2: negative control; Lane 4-9: different concentration DNA samples in positive control: 10 ng/μL, 1 ng/μL, 0.1 ng/μL, 0.05 ng/μL, 0.01 ng/μL, and 0.005 ng/μL. B. Nested PCR detection of villi tissue samples by agarose gel electrophoresis. Lane M: marker; Lane 1: negative control; Lane 2: positive control. Lane 3-15: tested samples.

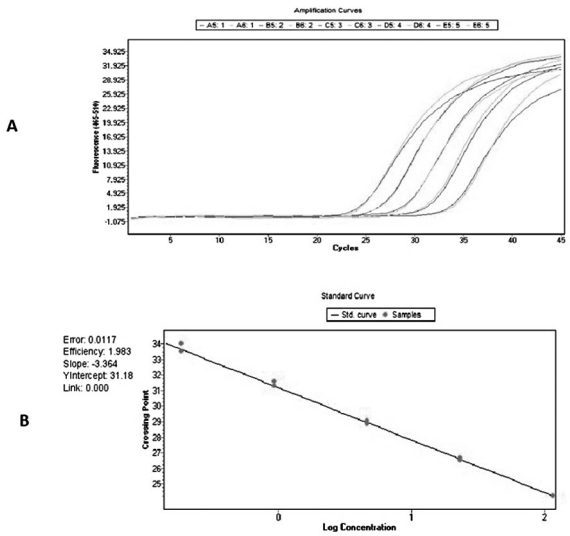

Figure 2. — Fluorescence quantitative PCR curve. A. Fluorescence quantitative PCR is used to detect the amplification curve of SRY gene in positive control samples. B. Fluorescence quantitate detection standard curve.

Nested PCR positive samples for fluorescence quantitative detection showed that cffDNA can be detected as early as 7th week of pregnancy. Among 163 nested PCR-positive samples, 140 samples could detect cffDNA at 5-6th week but fluorescence quantitative PCR test results were negative 23 cases at 5-6th gestational week. Eighty cases of nested PCR negative samples were also found negative for fluorescence quantitative PCR.

Fluorescence quantitative results showed that the average concentration of fetal free DNA in the peripheral blood of women of 7-10^{+6} week of pregnancy was 98.57 pg/mL. The average concentration of cff DNA in the 7th week of pregnancy was 47.22 pg/mL, the average concentration of cff DNA in 8th week of pregnancy was 57.89 pg/mL, 61.16 pg/mL in 9th week, and on 10th week, the average concentration of cff DNA was found at 205 pg/mL, which was sig-

Table 1. — *Cell-free fetal DNA concentrations in peripheral blood of different gestation weeks (x±s, pg/mL)*

7th week	8th week	9th week	10th week	square	*p*
47.22 ± 24.80[a]	57.89 ± 26.71[a]	61.16 ± 32.09[a]	205.00 ± 104.76[b]	70.422	0.000

a,b indicate that there is a statistically significant difference between each other.

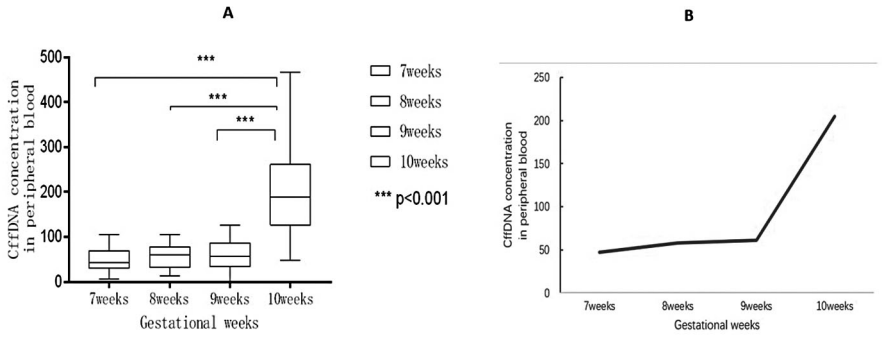

Figure 3. — CffDNA in different gestational periods. A. Differences in cffDNA content in different gestational weeks. B. Trends of cfDNA in different gestational weeks.

nificantly higher than 7-9th week (Table 1, Figures 3a, b).

Discussion

Non-invasive prenatal diagnosis has emerged as a highly accurate method of screening for genetic diseases without incurring procedural risks, which gives parents an opportunity to make decisions on reproductive and pregnancy management precise fetal genomic information [11, 12]. To ensure the safety of the mother and also avoid the damage of the fetus and risk of fetal diseases, non-invasive prenatal diagnosis is of great concern to the researchers, and the discovery of cffDNA has opened up the exciting possibility of non-invasive prenatal diagnosis [13-15].

Different studies have confirmed the presence of cffDNA in maternal plasma, of which placental trophoblast cells are the main source, and very few originate from fetal hematopoietic cells [16-18]. As fetal tissues and cells become apoptotic, fetal DNA is released into the blood through the fetal-placenta-maternal blood circulation into the pregnant woman's peripheral plasma. Fetal DNA is released directly from the fetal tissues through the fetal-placental-maternal blood circulation system into the pregnant woman's peripheral plasma. Finally, fetal cells go directly into the maternal plasma cycle, are destroyed by the maternal immune system, and release of fetal DNA left in the maternal plasma [16-18].

The present experimental results show that at 7th week of pregnancy, detection of SRY gene in cffDNA by real-time fluorescent quantitative PCR technique was highly sensitive and specific. Moreover, it can accurately quantify trace nucleic acid in plasma of pregnant women and the detection speed is rapid, as it can be finished within two to three hours. Under normal physiological conditions, the content of cffDNA in maternal peripheral blood was positively correlated with gestational week, but there were also individual differences. The earliest time to detect cffDNA in peripheral blood plasma of pregnant women was not uniformly standard. This might be due to the short gestational period, where the peripheral plasma of cffDNA content is too small, or due to the sample storage and transportation for a long time that caused the degradation of fetal DNA, or the cffDNA concentration is too low for amplification of the minimally required template quantity. However, this result of this study could detect cffDNA in pregnancy as early as previous studies reported [8, 9, 19]. As placental trophoblast cells are considered to be one of the sources of cffDNA, and trophoblast cells are mainly infiltrated through the endometrial stroma at nearly 10th week of pregnancy [20], cffDNA might release into maternal blood at 10th week significantly. Thus, it is suggested that the cffDNA test can better be detected after 10th week, which may have higher detection accuracy.

In many pathological gestations and pregnancy-related diseases, like in fetal chromosomal abnormalities (for example, 21-trisomy syndrome), cffDNA concentration increased abnormally, and also the cffDNA content in the peripheral blood plasma of patients with ectopic pregnancy is higher than that in normal pregnancy [16, 17]. Also, in early miscarriage, the amount of cffDNA in the peripheral blood is increased [15]. Although the reason is not fully understood, it has been postulated that the pathological pregnancy or pregnancy-related diseases may alter the structure of the maternal-fetal interface, and cause a significant increase in cffDNA concentration in the peripheral plasma of pregnant women.

Conclusion

Non-invasive prenatal diagnosis using cffDNA in peripheral blood of pregnant women has a great prospect. The results of this study show that cffDNA can be easily ex-

tracted and quantitatively analyzed during early pregnancy of pregnant women, and provide evidence for the clinical application of cffDNA. However, due to the limited sample number of this study, the average concentration range of the sample is likely to be biased, and the sample size should be increased in further studies.

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