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Hypermethylated ERG as a cell-free fetal DNA biomarker for non-invasive prenatal testing of Down syndrome

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ABSTRACT

Background: Previous reports have shown that the ERG gene is hypermethylated in the placenta and hypomethylated in maternal blood cells. In this study, we explore the feasibility of hypermethylated ERG as a cell-free fetal (cff) DNA biomarker for non-invasive prenatal testing (NIPT) of Down syndrome.
Methods: We randomly selected 90 healthy pregnant women, including 30 cases at each trimester of pregnancy. In addition, 15 pregnant women were recruited as the case group whose fetuses had been confirmed to have trisomy 21 by amniotic fluid analysis at 18th to 26th week gestation. Using HpaII, Msp to digest cell-free maternal plasma DNA, we performed SYBR Green PCR to detect methylated sites of ERG sequences, and analyzed the concentrations of cff DNA in maternal plasma in different gestational trimesters and the case group.
Results: The ERG median concentrations of the maternal plasma after Hpa II digestion (LG copies/ml) in first, second and third-trimesters were 5.38, 6.10, and 7.04, respectively (Kruskal–Wallis, $P < 0.01$); and that in the trisomy 21 case group was 6.85, which was higher than the second-trimester (Mann–Whitney, $P < 0.01$).
Conclusions: The study demonstrated that ERG gene is hypermethylated in cff DNA but hypomethylated in maternal DNA; and the median concentration of ERG gene in the trisomy 21 case group is higher than that in the gestational trimester matched normal group. ERG gene, as a fetal DNA biomarker, may be useful for NIPT of Down syndrome.

1. Introduction

Down syndrome is the most common genetic chromosomal disorder, and intervention largely depends on prenatal screening and diagnosis. To reduce the risk of prenatal diagnosis, more and more efforts have been made to establish practical non-invasive prenatal testing (NIPT) methods. With the development of molecular biology, the gene detection technology provides a theoretical basis and technical methods for NIPT. The circulating cell-free DNA identified in plasma was originally considered as a marker for cancer detection, which drew attention on the potential clinical value of circulating cell-free DNA in peripheral blood for NIPT. In 1997, Lo reported the presence of cell-free fetal DNA (cff-DNA) in maternal plasma [1]. Considering that maternal plasma includes both maternal and fetal cell-free DNA, we hoped to find universal DNA biomarker to distinguish fetal DNA from maternal DNA for NIPT for Down syndrome.

DNA methylation is a biochemical process involving the addition of a methyl group to the C-5 position of cytosine nucleotides, and is catalyzed by DNA methyltransferases which use S-adenosyl-L-methionine (SAM) as a methyl donor. In mammals, DNA methylation mainly occurs at cytosine of the CpG dinucleotide sequences; these CpG accumulation regions are called CpG islands. Many studies suggested that the methylation of CpG islands is closely related to numerous human diseases, especially cancers and genetic diseases [2–4]. With the discovery and in-depth research of fetal nucleic acids in maternal blood [5], it has been established that the transfer of nucleic acids between the mother and the fetus is an interactive process. Therefore, the epigenetic approaches examining differential methylation patterns between maternal blood cells and cell-free fetal (cff) DNA could be explored.

ERG gene located at human chromosome 21q22.3 is a member of the ETS family of transcription factors [6], and plays a crucial role in the regulation of cell proliferation, differentiation, and apoptosis. Old et al. [7] recently proved that the promoter of ERG gene is hypermethylated in the placenta, but hypomethylated in maternal blood cells. This methylation pattern raises the possibility of using placental-derived methylated ERG as a fetal DNA marker for NIPT of Down syndrome. In this study, we could potentially remove the background maternally derived hypomethylated ERG sequences by methylation-sensitive restriction enzyme (MSRE) [8] digestion, whereas the hypermethylated cff DNA ERG sequences were expected to be resistant to MSRE digestion. Then we could use SYBR Green PCR for detecting and quantifying ERG gene methylated CCGG sites to analyze the concentrations of cff DNA in the maternal plasma at different trimesters of pregnancy. We also

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compared the concentrations between the trisomy 21 case group and the gestational trimester matched normal group.

2. Materials and methods

2.1. Sample collection

Ninety healthy pregnant women with singleton pregnancies who had no gestational hypertension [9], cancer or other diseases were recruited from Bao'an Maternal and Child Health Hospital, Shenzhen, China, from April to June 2011. These patients were divided into First-trimester group (30 cases), ranging from 10th to 13th week; Second-trimester group (30 cases), ranging from 18th to 26th week; and Third-trimester group (30 cases), ranging from 30th to 37th week. All fetuses were later confirmed to be karyotypically normal. Another 15 pregnant women were recruited whose fetuses had been diagnosed with trisomy 21 by prenatal amniotic fluid karyotyping at 18th to 26th week gestation as the case group from Shenzhen People's Hospital, China, during 2011–2012. We also collected blood samples from 2 women who had never had children, and 2 chorionic villus samples from the induced abortion room of Bao'an Maternal and Child Health Hospital, as control specimens respectively.

All of these subjects had signed informed consent form to participate in this study. The study was approved by Bao'an Maternal and Child Health Hospital Ethics Committee.

2.2. Sample preparation

5 ml of maternal peripheral blood sample was collected in EDTA tubes and processed within 2 h. The whole blood sample was initially centrifuged at 2100 ×*g* for 10 min at 4 °C, then the plasma portion was collected in a 1.5-ml Eppendorf tube and recentrifuged at 18,000 ×*g* for 10 min at 4 °C, and the supernatant was stored at −80 °C until processed for testing.

The peripheral blood samples from the non-pregnant women were collected in EDTA tubes at 4 °C, 2 ml each. The 2 chorionic villus samples were kept in saline at 4 °C.

2.3. DNA extraction

Circulating cell-free DNA was extracted from 1 ml of the maternal plasma sample with QIAamp DNA Blood Minikit (Qiagen), according to the manufacturer's instructions, and was eluted in 50 μl of buffer AE.

Blood cell DNA was isolated from 200 μl of the peripheral blood sample from the non-pregnant women using QIAamp DNA Blood Minikit (Qiagen), and was eluted in 200 μl of buffer AE.

Chorionic villus DNA was isolated from 25 mg of chorionic villus tissue using QIAamp DNA Minikit (Qiagen), and was eluted in 200 μl of buffer AE.

2.4. Enzyme digestion

MSRE Hpa II (NEB) and its isoenzyme Msp I (NEB) were separately used to digest the extracted DNA at 37 °C for 3 h respectively. The reaction mixture contained 10 U of enzyme, 2 μl of 10 × NEBuffer, and 10 μl of plasma DNA or 2 μl of whole blood cell DNA or 2 μl of chorionic villus DNA. Then ddH_2O was added to the system volume to 20 μl.

2.5. Primer design and synthesis

According to the ERG gene sequences containing methylated CCGG sites, we designed primers for SYBR Green PCR amplification: forward primer 5′-ACCTTTGCATGTGAGAGGCATT-3′, reverse primer 5′-TTTTGT CGAGCCAG CGTCTT-3′, synthesized by Takara Biotechnology Co., Ltd. The size of PCR product was 124 bp.

2.6. Standard plasmid preparation

The amplified fragment of ERG gene was cloned into pMD-18T for constructing a recombinant plasmid. The sequencing result of inserted DNA fragment was in accordance with the known ERG gene. The recombinant plasmid was serially diluted, and we used these 5 dilutions (10^4–10^8) to establish the standard curve for SYBR GREEN PCR.

2.7. SYBR Green PCR and agarose gel electrophoresis

SYBR Green PCR amplifications were performed using MJ Opticon 2™ Real-Time PCR Detection System (MJ Research). A volume of 25 μl of PCR mixture was prepared on ice, which contained 12.5 μl of SYBR Premix Ex Taq™ II (Takara), 1 μl of each primer (10 pmom/μl), 5 μl of enzyme-digested DNA template and 5.5 μl of ddH_2O. SYBR GREEN PCR amplifications were performed as follows: 95 °C for 30 s, (95 °C for 5 s, 60 °C for 30 s)for 40 cycles, 95 °C 15 s, 60 °C 30 s, and then fluorescence values were recorded every 0.5 °C as the temperature was rising slowly from 60 °C to 95 °C. Finally we obtained a melting curve of PCR product. PCR products were analyzed by agarose gel electrophoresis.

2.8. Statistical analysis

All the experimental data were analyzed with SPSS 17.0 software. Mann–Whitney test was used to compare the median concentrations in trisomy 21 case and normal groups. Kruskal–Wallis test was performed to compare the median concentrations of different gestational trimesters. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of the methylated sites in ERG gene

The MSRE HpaII can cleave CCGG sequence but is sensitive to the methylation at the internal C. When this site carries a methylated C, Hpa II cannot cleave CmCGG. Whereas, Msp I is the isoenzyme of HpaII, which cuts both unmethylated and methylated CCGG equally. Therefore, we can confirm that the DNA sample contains methylated CCGG target site if the DNA region can be amplified after HpaII digestion and cannot be amplified after MspI digestion.

The SYBR Green PCR products were identified by agarose gel electrophoresis (Fig. 1). All of the maternal plasma DNA, the chorionic villus DNA and the non-pregnant women DNA demonstrated bands of amplified product the same length as the expected target fragment length (124 bp) of ERG gene before enzyme digestion, but all the bands were absent after Msp I digestion. After Hpa II digestion, both the maternal plasma DNA samples and the chorionic villus DNA samples had these bands, but the sampled non-pregnant women DNA samples did not.

The results showed that methylation occurred in ERG gene of the maternal plasma DNA and the chorionic villus DNA. Thus methylated plasma cell-free DNA and cff DNA have the same origin as chorionic villus DNA (derived from fetal tissues or placenta).

3.2. Standard and melting curves

MJ Research Opticon Monitor 3.1 monitoring software was used to generate SYBR Green PCR kinetic curves, standard curve and melting curves. SYBR Green PCR reaction gave typical S-like amplification curves, which showed a good linear relationship between the concentrations and cycle threshold values of the standard templates. The correlation coefficient (r^2) of the standard curve was 0.997, and the equation for the linear relationship was $y = -0.256x + 12.76$. The melting curves showed that there was only one specific melting peak for EGR gene amplified fragment, which was clearly detected at uniform T_m value of 85.0 °C. Furthermore, there were no melt peaks of primer–