

MS-HRM to Detect Serum DNA Methylation of Intrauterine Growth Retardation Children

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ABSTRACT

Intrauterine growth retardation (IUGR) is also called fetal growth restriction (FGR), which is the major complications in obstetrics and one of the most important causation from high morbidity and mortality in perinatal. In this study, MS-HRM (Methylation-Sensitive High Resolution Melting Curve Analysis) was used to detect the methylation status of serum DNA. Gene of insulin-like growth factor binding protein 3 (IGFBP-3) was detected in this study. Results showed that the serum DNA methylation level of FGR fetus were lower than that of the control group; different methylation levels were also found between male and female fetus; and the methylation level was increased with the birth weight of the newborn. Our results showed that the IGFBP-3 gene methylation level of serum DNA of newborn could be semi-quantitative detected which guide the early prevention and treatment of IUGR. It also indicated that the methylation status of serum DNA can be conveniently identified and quantified by inspection of the melting curves.

Keywords: MS-HRM; IUGR; IGFBP-3; Serum DNA

1. Introduction

Intrauterine growth retardation (IUGR) is also called fetal growth restriction (FGR), which is the major complications in obstetrics and one of the most important causation from high morbidity and mortality in perinatal. Furthermore, it is also one of the important reasons for many adult diseases such as hypertension, type II diabetes, coronary heart disease, kidney disease and so on [1] in the future.

Insulin like growth factor 1 (IGF-1) is a single basic protein composed of 70 amino acids; and known as one of the key factors to cellular proliferation and metabolism which plays an important role in fetal organ maturation and growth and development of children. The infant health status especially endocrine status could be indicated by serum IGF-1 level, for which it is produced by fetus itself because it cannot transfer through placental from maternal serum. Previous studies showed that the birth weight of IGF-1 knockout mice were only 60% of the normal mice [2].

Insulin like growth factor binding protein 3 (IGFBP-3) is also an important protein in human serum could form a compound by binding to IGF-1 which increase the bioactivity of IGF-1. The compound could prolong the half-life of IGF-1 in serum. IGFBP3 can also promote the cell division as well as involves placenta formation and growth and development of the infant directly. It was reported that umbilical cord blood IGFBP-3 level of IUGR fetus reduced about 50% than that in normal newborn [3]. Carter *et al* demonstrated that the serum IGFBP3 level decreased remarkable in IUGR animal model [4].

Therefore, the growth and development of infants was affected by IGFBP3 directly or indirectly.

Researches have shown that adverse intrauterine environment might lead to epigenetic changes which regulating the gene expression [5], such as passive smoking, unreasonable diet, and uterine artery blood supply deficiency. The methylation status of maternal IGFBP3 had been shown to be related with IUGR, while few reports revealing the potential relationship between IUGR and the methylation status of IGFBP3. The previous studies showed that hyper methylation of the promoter of IGFBP3 gene was highly associated with tumor [6], and the gene methylation alteration might be used as a potential diagnosis marker in colorectal cancer [7]. It could be inferred that the methylation status of IGFBP3 promoter has certain relation with growth and development. However, no report clearly indicated whether the changes of methylation status would affect the development of fetus, infants and even adult. Investigation of the methylation status especially the methylation level of IGFBP3 is significantly important to IUGR.

The most popular approaches of methylation detection rely on treatment of DNA samples with sodium bisulfate and subsequent amplification by PCR [8,9]. However, MSP (Methylation Specific PCR) is a non-quantitative measurement, which limited its field of application. MS-HRM (Methylation-Sensitive High Resolution Melting Curve Analysis), has been shown a simple and cost-effective post-PCR technique for routine clinical diagnosis, which is capable of analyzing methylation in a semi-quantitative manner [10]. All of the CpGs flanked by the primers binding to the target sequence can

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be scanned by HRM regardless of the methylation status of CpGs in the primer-binding site, with no post-PCR handling and no separation step, characteristics that improve analysis time [11]. HRM might be a good choice for the detection of IGFBP3 methylation status in this study.

The aim of this study was to detect the serum DNA methylation level of IUGR infants. Results generated by HRM were also validated with traditional MSP assays. The serum IGFBP-3 was measured with ELISA.

2. Materials and Methods

2.1. Participants

Serum samples were obtained from new-born baby between June, 2009 to July, 2011 in Maternity and Children's Health Care Centers in Wuxi. All the baby was born between 37 weeks to 40 weeks of gestation ages and the birth weight were ten percent lower than the newborn at the same gestation age. Mothers were at the age of 21-35 years old in this study, no basic disease, the weight gain during pregnancy is normal, and the gestational hypertension, diabetes, preeclampsia, polyhydramnios and pregnancy bile acid increased disease were all excluded. Fifty IUGR infants (thirty male and twenty female) and thirty normal controls (fourteen male and sixteen female) were selected in this experiment. This project was approved by the Ethics Committee of Wuxi Maternity and Children's Health Care Centers, Southeast University and Nanjing Medical University Clinical Research Ethics Committee, Nanjing, China.

2.2. Extraction of DNA and Sodium Bisulfate Modification

DNA from serum was extracted with a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA was extracted from 2 mL of serum and eluted in 50 μ l of TE buffer. About One microgram of DNA was subjected to bisulfite conversion with the EZ DNA methylation kit (Zymo Research, USA). DNA quantity was assessed spectrophotometrically. The eluted DNA was used for the HRM analysis and MSP validation.

2.3. HRM Analysis

PCR amplification and HRM were performed on the ABI 7500 fast (Life Technology, USA) as adapted from the published protocol [10]. The primers were designed as outlined, not more than 1 to 2 CpG sites and were placed at or adjacent to the 5'-end. The sequences of primers for IGFBP3 were as follows: forward: GGGTTAAGGTTAGAGGTGGTGTTAT; reverse: AAAAAAATTTACAATTTACAAAACTC. PCR was performed in a 20 μ l volume containing buffer, 2 U HotstartTaq DNA polymerase (Takara), 250 nM of each primer, 2.5 mM SYTO-9, 10 ng bisulfite treated DNA template, and 3 mM MgCl₂.

The cycling conditions were as follows: 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 30 s; followed by an HRM step of 95°C for 1 min, 40°C for 1 min, 70°C for 15 s, and continuous acquisition to 95°C at 1 acquisition per 0.1°C. A standard curve with known methylation ratios was

included in each assay and was used to deduce the methylation ratio of each fetal and maternal sample. Differences between and among groups were compared using Pearson's chi-square test for qualitative variables and using Student's t test or analysis of variance for continuous variables.

Methylation-sensitive PCR was performed through BioRad PCR system as described previously [12]. 2.5 μ L (approximately 50 ng) of bisulfite-treated DNA was amplified using 2 pmol of forward primer and reverse primer. PCR conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 50 s and 1 cycle at 72 °C for 8 min.

3. Results

3.1. HRM Assay and Dilution Matrix

We validated the correlation between T_m value and methylation level by analyzing different sets of methylation level standards (0%, 1%, 10%, 25%, 50%, 75%, 100%) firstly. All HRM assays were able to detect reproducibly 1% methylated DNA in a background of unmethylated DNA. Linear regression analysis (Figure 1) revealed that methylation levels were highly correlated with T_m value, that T_m value can be used as the standards of methylation.

3.2. Methylation Level of IGFBP3 in Serum of IUGR Infants

In this present study, the serum DNA methylation level of IGFBP-3 was measured by MS-HRM. The analysis of all samples was repeated twice by HRM and part sample randomly selected were measured by MSP. We also tested whether varying amounts of bisulfite treated target DNA would influence HRM results. We found no differences in methylation ratios when using two different DNA amounts (50 ng versus 10 ng).

Results showed that lower methylation level of found in IUGR infants compared with normal control baby (P < 0.01, T-test). It could be inferred that certain relation might exist between the IGFBP-3 methylation and the development of IUGR. Different methylation level was also found in different gender of newborn. It can be seen that the methylation level of

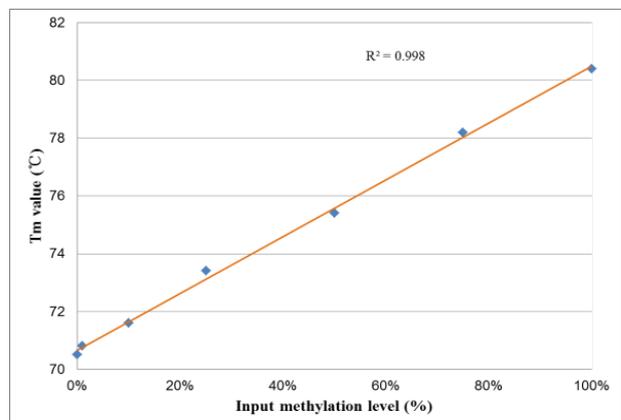


Figure 1. Correlation between T_m value and methylation level measured by MS-HRM.

IGFBP-3 in female new-born baby was lower than that male ones ($P < 0.01$, T-test) (Figure 2). The detection results by MSP in part sample was consistent with that of MS-HRM (data not shown).

Lower birth weight is one of the important indicators in IUGR infant's diagnosis. The IGFBP-3 methylation level was also assayed according to the different birth weight of new-born baby (Figure 3A). The results showed that the IGFBP-3 methylation level was increased with the birth weight (Figure 3B).

4. Discussion and Conclusion

Several reported studies have investigated that IUGR is correlation with mother's nutrition, placenta, umbilical cord dysplasia, intrauterine hypoxia ischemia, disease and so on. Recently, the function of IGF-1 and IGFBP-3 in regulation of growth and development was more and more concerned in the study of IUGR. Epigenetic changes such as DNA methylation play an important role in gene expression and development of many diseases. The aim of this study is to investigate whether the methylation status of IGFBP-3 gene promoter was related with IUGR. The results indicated that the serum DNA methylation of IGFBP-3 promoter was strongly correlated with IUGR. It is consistent with the finding of previous study that gene mutation of IGFBP-3 resulted in Silver-Russell syndrome (SRS), a severe IUGR, and also lead to hypo-methylation[13].

It is said that DNA methylation play key roles in X-inactivation which may lead to methylation difference between male and female[14]. Just as the result obtained in this study, some research also indicated that DNA methylation level was different between male and female ones. While inconsistent results were found in other studies. In this study, higher methylation level was found in male fetus may indicate that the development of IUGR was different between male and female fetus.

Previous studies reported that the promoter methylation of certain gene was related with birth weight of newborn [15,16]. In this study, we found that DNA methylation of IGFBP-3 was positive correlation to birth weight of newborn, the other possible factor such as the age of mother and nutrition, twin pregnancy etc. excluded. It is consistent with the fact that IUGR will more likely result in low birth weight of newborn.

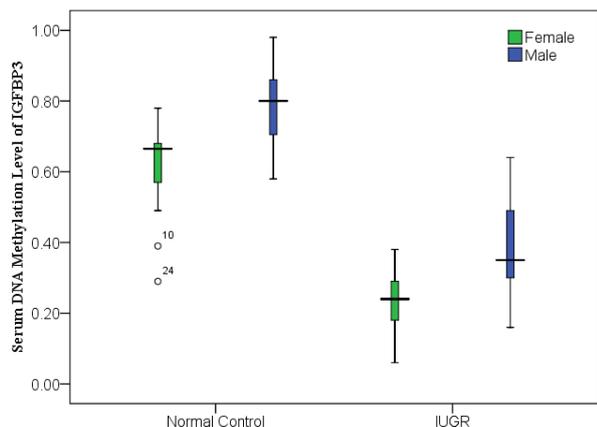
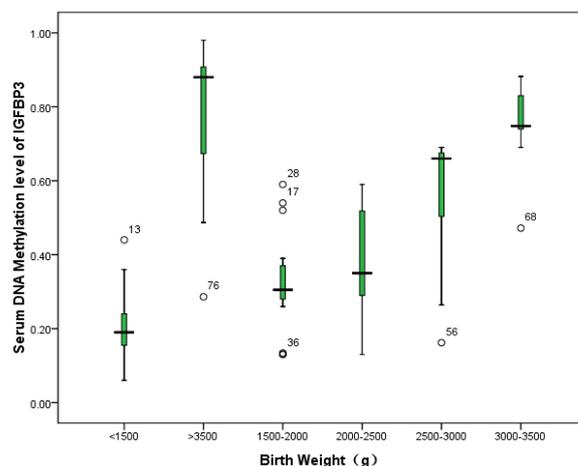
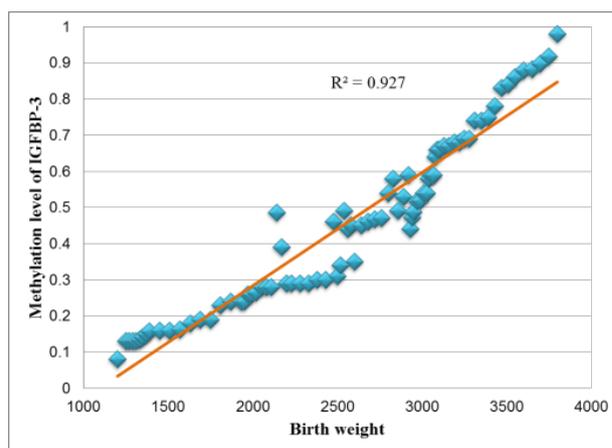


Figure 2. Methylation level of IGFBP-3 in serum of IUGR infants.



A



B

Figure 3. Correlation between methylation level and birth weight of IUGR infants.

In this present study, cell free DNA separated from serum was used to study the gene methylation level in IUGR infants and controls, and hypo-methylation of IGFBP-3 promoter was detected in IUGR infants comparing with the controls. It is suggested that DNA methylation affect the development of IUGR which provides the theoretical basis and reference for the prevention and treatment of IUGR.

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