

DNA Methylation and gene expression patterns are widely altered in fetal growth restriction and associated with FGR development

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ABSTRACT

Fetal growth restriction (FGR) is the failure of the fetus to achieve its genetically determined growth potential, which increases risks for a variety of genetic diseases, such as type 2 diabetes mellitus, coronary artery disease, and stroke, during the lifetime. The dysregulation of DNA methylation is known to interact with environmental fluctuations, affect gene expressions comprehensively, and be fatal to fetus development in specific cases. Therefore, we set out to find out epigenetic and transcriptomic alterations associated with FGR development. We found a set of differentially expressed genes associated with differentially methylated regions in placenta and cord blood samples. Using dimensional reduction analysis, the expression and methylation variables of the epigenetically altered genes classified the FGR samples from the controls. These genes were also enriched in the biological pathways such as metabolism and developmental processes related to FGR. Furthermore, three genes of *INS*, *MEG3*, and *ZFP36L2* are implicated in epigenetic imprinting, which has been associated with FGR. These results strongly suggest that DNA methylation is highly dysregulated during FGR development, and abnormal DNA methylation patterns are likely to alter gene expression.

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Introduction

Fetal growth restriction (FGR) is the failure of the fetus to achieve its genetically determined growth potential and is defined as estimated fetal weight less than the 10th percentile (Minior and Divon 1998; American College of Obstetricians 2013). Small-for-gestational-age (SGA) fetuses are not all the result of pathology and can develop as a result of normal biologic conditions; however, various factors, such as maternal disorders, infectious disease, teratogen exposure, multiple gestation, genetic disorders, and placental conditions, may contribute to the development of FGR (Gaccioli and Lager 2016). Growth-restricted fetuses have increased risks of stillbirth, neonatal mortality, and morbidity (Jaddoe et al. 2014; Burton et al. 2016). Furthermore, growth-restricted fetuses are more likely to suffer from diseases, such as obesity, type 2 diabetes mellitus, coronary artery disease, and stroke, during adulthood under the long-term influence of restricted growth and altered metabolism in the fetal, neonatal, and adult periods (Barker 2006; Sailasree et al.


2017). Meanwhile, epigenetic regulation is critical for appropriate gene expression for normal functions during fetus development (Gicquel et al. 2008). A variety of epigenetic factors are known to interact with environmental fluctuations and regulate gene expression comprehensively (Perez and Lehner 2019). The problem with epigenetic regulation is fatal to fetus development, and it seriously affects quality of life throughout a person's lifetime (Waterland 2009). In this study, we tried to discover epigenetic and transcriptomic alterations associated with FGR development by analyzing paired DNA methylation and the transcriptome of placenta and cord blood from FGR samples and normal controls.

Results

Differentially methylated regions were identified in placenta and cord blood of FGR samples

To unravel the epigenetic alterations underlying the FGR samples, we profiled DMRs for 23,049 gene

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promoters between the FGR and control samples (see Materials and Methods). We analyzed placenta and cord blood from which 3,255 (14.12% of all genes) and 430 (1.87% of all genes) genes were identified as significantly hyper- or hypo-methylated, respectively (Figure 1A). The methylation status of these DMRs were tested to determine whether it had potential for classifying FGR samples from normal controls. Principle component analysis (PCA) revealed that the methylation signals of significantly methylated promoters marginally clustered FGR samples, although the methylation signals of all promoters could not classify the two groups in both placenta and cord blood (Figure 1B). Interestingly, we also discovered significantly methylated DMRs of gene bodies and performed PCA, but the results were not grouped better than those based on the promoters (Supplementary Figure 1).

A set of DNA methylation alterations was associated with differentially expressed genes in FGR samples

DNA methylation in promoters is principally known to have a negative correlation with gene expression. Therefore, we discovered overexpressed and underexpressed genes in FGR samples accordingly using the hypermethylated and hypomethylated promoters, respectively. In the placenta and cord blood, respectively, 250 and 14 differentially expressed genes (DEGs) were identified with DMRs, which had 126 (50.40%) and 8 (57.14%) DEGs that could be explained by DMRs on the relevant promoters that showed a negative correlation with each other (Supplementary Table 1). In contrast to the promoter, DNA methylation on gene bodies remains to be elucidated for its regulatory functionality in transcriptional regulation (Jjingo et al. 2012). We analyzed DEGs with the status of gene body methylations in FGR samples and found that fewer DEGs were negatively associated with methylation alteration than those of promoter methylations in placenta, although there were only negative correlations in cord blood for a small number of cases (Figure 2A). PCA was also performed for the expression of all genes, and they were not clustered between FGR samples and normal controls (Figure 2B). However, the expression of DEGs with DMRs on their promoters specifically grouped FGR samples in the PCA result when the alteration of methylations was negatively correlated with the direction of DEGs (Figure 2C). On the contrary, the PCA result for the expression of DEGs having positively correlated DMRs showed worse grouping in cord blood but successful grouping in placenta (Figure 2D). We profiled eigen values for the genes in PCA by eigen decomposition

analysis to calculate how much each gene contributed to the grouping of FGR samples. The genes with an eigen value over 0 were considered as contributing to the grouping in PCA. Then, we performed the biological pathway enrichment test for the two cases that were positively and negatively epigenetically regulated genes. This analysis could not be performed for the samples from cord blood because they had too few genes that could be profiled. As a result, we found that only cases having a negative correlation between DMRs and DEGs showed significant enrichment in the pathways related to metabolism and development that were known to be associated with FGR occurrence (Supplementary Figure 2). These results imply that DMRs on gene promoters would have biological relevance in FGR occurrence by perturbing gene expressions negatively.

Transcriptionally altered genes by DNA methylation were enriched in specific pathways relevant to FGR

We analyzed how the genes that we discovered were involved in biological pathways relevant to FGR occurrence (see Materials and Methods). Genes repressed by DNA hypermethylation in placenta were enriched in the pathways that were implicated with FGR, which were the ERK1 and ERK2 cascade (Ozmen et al. 2011), the negative regulation of BMP signaling pathway (Reid et al. 2012), and the regulation of angiogenesis (Ahmed and Perkins 2000) (Figure 3A). We also discovered that genes overexpressed by DNA hypomethylation in placenta were enriched in the pathways related to a set of metabolism pathways, such as the ChREBP activation of metabolic gene expression and the PI3K-Akt signaling pathway (Figure 3B). These metabolic pathways are known to have functional implications with FGR occurrence (Zinkhan et al. 2018; Xing et al. 2019). In the case of genes underexpressed by DNA hypermethylation in cord blood, pathways related to lipid metabolism, such as the glycerolipid biosynthetic process, glycerophospholipid metabolic process, phospholipid biosynthetic process, and the regulation of the lipid metabolic process, were found to be affected. There were no significantly enriched pathways that were related to the genes overexpressed by DNA hypomethylation in cord blood. We also could not discover concordant pathways of differentially epigenetically regulated genes between placenta and cord blood. Although there was a discrepancy in the pathway enrichment between placenta and cord blood, our results indicate that the differentially epigenetically regulated genes in FGR samples are associated with a set of metabolism pathways,

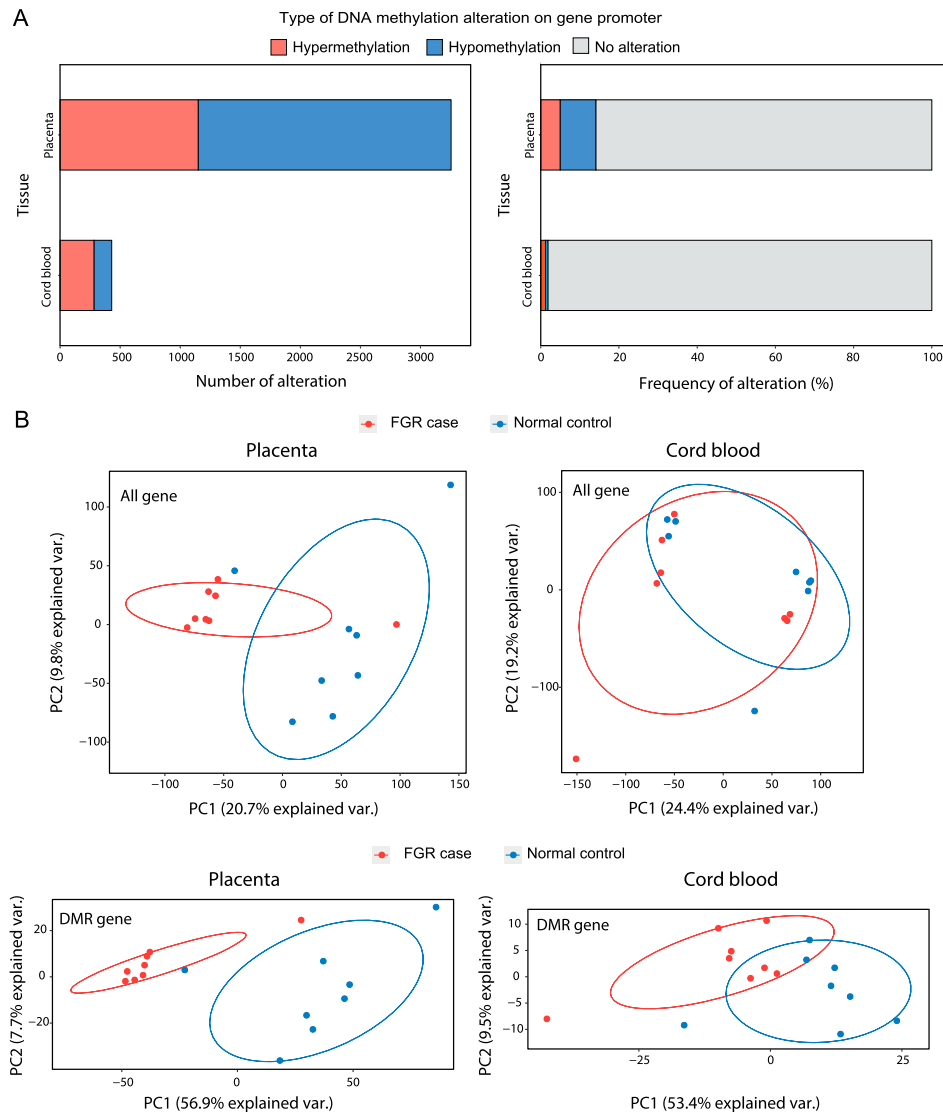


Figure 1. Epigenetic alteration in placenta and cord blood of FGR samples. (A) The number (left) and frequency (right) of hypermethylated and hypomethylated genes that had differentially methylated regions on the relevant promoters. (B) Principle component analysis (PCA) with DNA methylation variables were performed for all gene promoters found in placenta (top left) and cord blood (top right). PCA was also performed for genes with differentially methylated region (DMRs) on their promoters found in placenta (bottom left) and cord blood (bottom right).

including the known pathways related to FGR occurrence.

Discussion

The fetus in a placenta interacts with a variety of environments via cord blood. Several environmental signals could cause epigenetic variations that might be critical to the development and growth of fetus. In this study, we discovered a set of DMRs on the gene promoters of placenta and cord blood in FGR samples. Additionally, relevant expression perturbations were identified by changes in DNA methylation. We found out that FGR and normal control samples were classified

in the PCA profiles when their gene expressions were measured for the DEGs having DMRs on their promoters. The gene expressions of all genes of the FGR samples were not classified from control samples, indicating that the genes regulated by DNA methylation are involved in FGR development. However, only a subset of genes was significantly differentially expressed among those having DMRs, implying that the regulation of promoter methylation is greatly complicated. We also discovered the pathways for differentially epigenetically regulated genes in FGR samples. A set of metabolism pathways and specific signaling pathways were identified as enriched for FGR occurrence, although they were not completely coincident between placenta

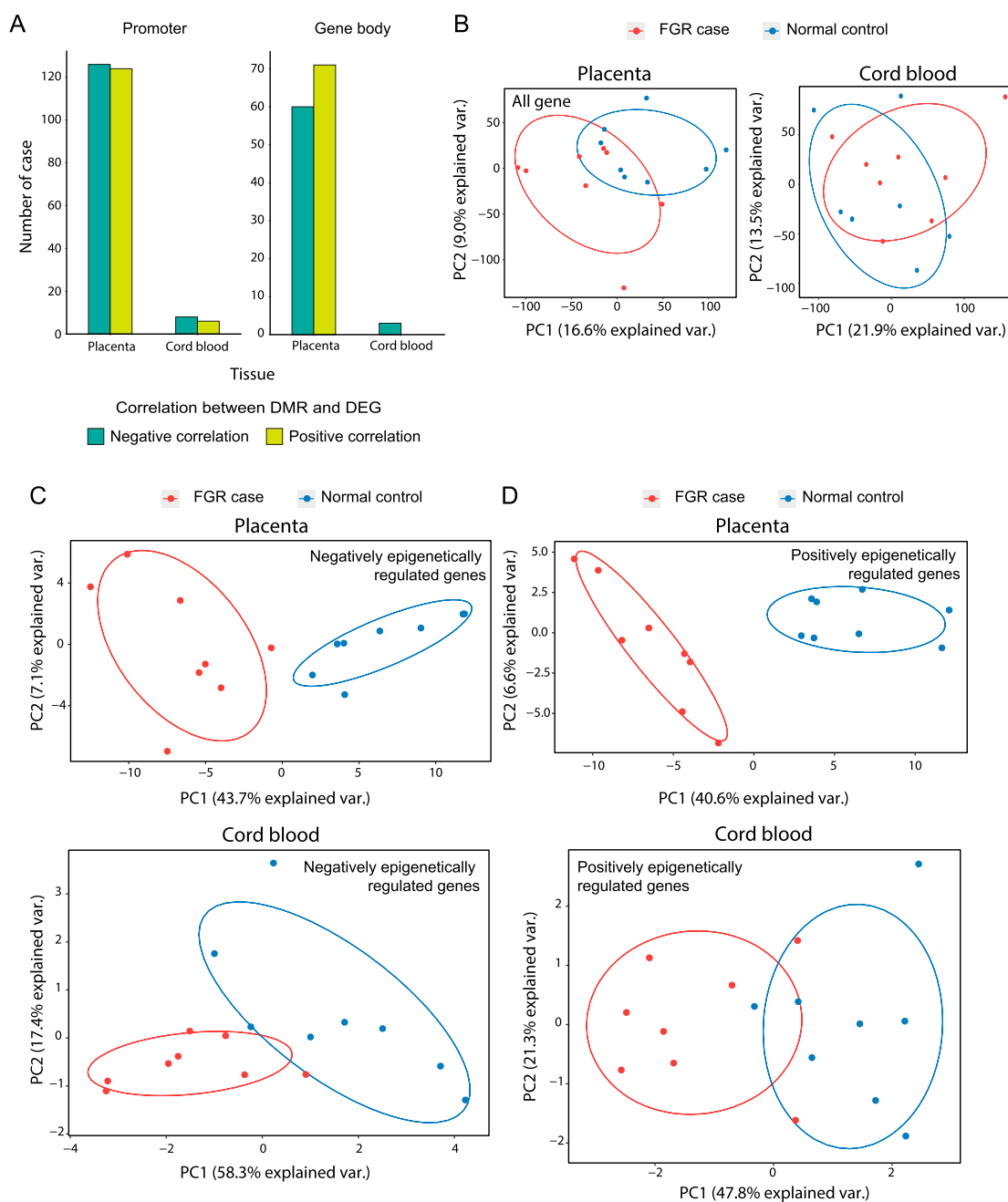


Figure 2. Transcriptional perturbations associated with epigenetic dysregulation in placentae and cord blood of FGR samples. (A) The ratio of differentially expressed genes (DEGs) from genes with differentially methylated region (DMRs) on their relevant promoters and gene bodies. The ratio was calculated based on negative and positive correlations between DEGs and DMRs. (B) Principle component analysis (PCA) with expression variables were performed for all genes found in placentae (left) and cord blood (right). PCA was also performed for DEGs with DMRs that correlated negatively (C) or positively (D) with each other.

and cord blood. These pathway enrichment results imply that the perturbation of DNA methylation could cause FGR development through the alteration of gene expressions.

Meanwhile, genetic imprinting is an epigenetic process including DNA methylation that causes a gene to be expressed in a specific allele (Wood and Oakey 2006). A set of imprinted genes are known to be

important for normal development and placenta growth (Isles and Holland 2005; Plasschaert and Bartolomei 2014). Therefore, the inappropriate imprinting of developmental genes that perturb their expressions would be associated with FGR occurrence (Diplas et al. 2009). In this rationale, we profiled whether the genes perturbed by DNA methylation in FGR samples were matched to known imprinting genes. The curated lists

and ZFP36L2. INS, a gene coding insulin, is crucial in glucose metabolism and is known to be involved in the regulation of fetus development (McMinn et al. 2006). MEG3 was found to be repressed by DNA hypermethylation on its promoter in our FGR placenta samples, which is consistent with the results of a previous study that described the underexpression of MEG3 in FGR placentae (McMinn et al. 2006). ZFP36L2 is a member of the ZFP36 family and is a transcription factor regulating the response for the growth factor. It was reported that ZFP36L2 is associated with the regulation of the early developmental stage in a mouse model (Hacker et al. 2010). In our study, ZFP36L2 was down regulated by DNA hypermethylation in FGR placenta samples. These three genes are proposed as candidates for the perturbed imprinting genes related to FGR development. However, we did not have sequencing data to unravel allele-specific expression, so we could not explain which exact allele from the parents is involved in imprinting regulation.

Materials and Methods

IRB

This study was approved by the Institutional Review Board of Inje University Busan Paik Hospital (approval number: 18-0069)

Sample collection

This was case-control study that included 8 patients with FGR and 8 normal controls delivered between 24 and 42 weeks of gestation. The FGR group included women who delivered low-birth-weight infants below the 10th percentile. The control group was comprised of singleton pregnancies without FGR. All clinical samples were collected at the Department of Obstetrics and Gynecology, Inje University, Korea. Blood samples were collected from studied women before delivery. We evaluated maternal clinical characteristics, such as age, gestational age at delivery, parity, pre-pregnancy body mass index (BMI; kg/m²), mode of delivery, frequency of preterm delivery, gestational diabetes mellitus, preeclampsia, and fetal birth weight. Preterm delivery was defined as delivery that occurred between 24 + 0 and 36 + 7 weeks of gestation. Preeclampsia was defined as the presence of hypertension with proteinuria (urine protein content >300 mg/24 h or protein/creatinine ratio \geq 0.3) or a systemic symptom. We also evaluated the neonatal outcomes, including birth weight, neonatal death, neonatal intensive care unit (NICU) admission, Apgar score at 5 minutes after birth, mechanical

ventilation, respiratory distress syndrome (RDS), intra-ventricular hemorrhage (IVH), and necrotizing enterocolitis (NEC), of the FGR and control groups. Neonatal death was defined as the death of a live-born neonate within 28 days of delivery. RDS was diagnosed by compatible chest X-ray findings and arterial blood gas results. IVH was detected by bleeding inside or around the ventricles in the brain via postnatal cranial ultrasonography, whereas NEC was diagnosed via clinical and abdominal X-ray findings.

Sample characteristics

The maternal and neonatal clinical characteristics of the study population are presented in Table 1. There were no significant differences in maternal age, gestational age at delivery, parity, BMI, mode of delivery, preterm delivery, or gestational diabetes between the FGR and control groups. Women in the FGR group had higher rates of preeclampsia than those in the control group (75.0% vs. 12.5%, $p=0.0147$). Birth weights were significantly lower in the FGR group than in the control group (1568.75 ± 795.60 vs. 2872.50 ± 702.86 gm, $p=0.0037$). There was no neonatal death in this studied group. The FGR neonates had higher rates of neonatal complications than the control group (87.5% vs. 37.5%, $p=0.0455$). For other neonatal complications, there were no significant differences between the two groups.

Statistics

Data were analyzed using MedCalc version 11.0 software (Frank Schoonjans, University of Gent, Belgium). Continuous variables were calculated using Student's *t*-tests and Mann-Whitney U tests. Categorical variables were compared using chi-squared or Fisher's exact tests.

Identification of differentially methylated genes

DNAs extracted from placentae and cord blood were analyzed by using the Infinium Human Methylation EPIC-BeadChip (Illumina, San Diego, CA, USA). A set of methylation signals on the common promoter and gene body were merged as a maximum value to determine the differentially methylated region (DMR), and the difference in methylation between 8 FGR and 8 control samples for a DMR was calculated by a difference of the mean value of each case and the control group. The Wilcoxon test was performed to calculate the statistical significance of the difference. The states of hypermethylation and hypomethylation were defined when the difference between the mean methylation value of

Table 1. Maternal and neonatal clinical characteristics.

Characteristics	FGR group (n = 8)	Control group (n = 8)	p-value
Maternal characteristics			
Maternal age (years)	34.50 ± 4.81	35.62 ± 3.77	0.6110
GA at delivery(weeks)	33.68 ± 4.60	36.62 ± 2.64	0.1399
Nulliparity	5(62.5%)	3(37.5%)	0.3329
Pre-pregnancy BMI (kg/m ²)	21.94 ± 3.53	24.23 ± 4.22	0.2578
Cesarean delivery	8(100%)	8(100%)	1.0000
Preterm delivery	5(62.5%)	3(37.5%)	0.3329
GDM	1(12.5%)	0	0.3173
Preeclampsia, mild or severe	6(75.0%)	1(12.5%)	0.0147
Neonatal outcomes			
Birth weight(grams)	1568.75 ± 795.60	2872.50 ± 702.86	0.0037
Neonatal death	0	0	1.0000
NICU admission	7(87.5%)	3(37.5%)	0.0455
5min Apgar score <7	3(37.5%)	0	0.0628
Mechanical ventilation	4(50.0%)	3(37.5%)	0.6256
Respiratory distress syndrome	4(50.0%)	3(37.5%)	0.6256
Intraventricular hemorrhage	0	0	1.0000
Necrotizing enterocolitis	1(12.5%)	0	0.3173

the cases and controls was greater than +0.05 and less than -0.05, respectively.

Identification of differentially expressed genes

For transcriptome analysis, mRNAs were extracted from placenta and cord blood and were analyzed by using the GeneChip Human Gene 2.0 ST Array (Affymetrix) as described by the manufacturer. Differentially expressed genes (DEGs) were analyzed using transcriptome profiles of 8 FGR samples and 8 controls who also had DNA methylome data. The DEGs that showed a significance in their expression difference were determined to be under a p value of 0.05 based on DEG analysis by Student's t-tests. We also defined overexpressed and underexpressed genes when the -log₂ transformed the fold change between mean expression value of cases, and controls was more than 1 and less than -1, respectively.

Principal component analysis for expression and methylation variables

Principal component analysis (PCA) was used to confirm whether the variables of gene expression or methylation of FGR samples and controls were adequate for classifying each group. All expression and methylation variables were normalized using the Python StandardScaler statistics library.

Pathway enrichment analysis

We performed pathway enrichment analysis using the EnrichR tools (Kuleshov et al. 2016), which contain a large collection of diverse gene set libraries available for pathway enrichment analysis. We used four

pathway databases from EnrichR: KEGG, Reactome, Panther, and Gene Ontology Biological Process.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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