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Functional analysis of cell-free RNA using mid-trimester amniotic fluid supernatant in pregnancy with the fetal growth restriction

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Abstract

The prediction and monitoring of fetal growth restriction (FGR) fetuses has become with the use of ultrasound. However, these tools lack the fundamental evidence for the growth of fetus with FGR excluding pathogenic factors.

Amniotic fluid samples were obtained from pregnant women for fetal karyotyping and genetic diagnosis at 16 to 19 weeks of gestation. For this study, 15 FGR and 9 control samples were selected, and cell-free fetal RNA was isolated from each supernatant of the amniotic fluid for microarray analysis.

In this study, 411 genes were differentially expressed between the FGR and control group. Of these genes, 316 genes were up-regulated, while 95 genes were down-regulated. In terms of gene ontology, the up-regulated genes were highly related to metabolic process as well as protein synthesis, while the down-regulated genes were related to receptor activity and biological adhesion. In terms of tissue-specific expression, the up-regulated genes were involved in various organs while down-regulated genes were involved only in the brain. In terms of organ-specific expression, many genes were enriched for B-cell lymphoma, pancreas, eye, placenta, epithelium, skin, and muscle. In the functional significance of gene, low-density lipoprotein receptor-related protein 10 (LRP10) was significantly increased (6-fold) and insulin-like growth factor (IGF-2) was dramatically increased (17-fold) in the FGR cases.

The results show that the important brain-related genes are predominantly down-regulated in the intrauterine growth restriction fetuses during the second trimester of pregnancy. This study also suggested possible genes related to fetal development such as B-cell lymphoma, LRP10, and IGF-2. To monitor the fetal development, further study may be needed to elucidate the role of the genes identified.

Abbreviations: AF = amniotic fluid, AFS = amniotic fluid supernatant, bcl-2 = B-cell lymphoma, DEG = differentially expressed gene, FGR = fetal growth restriction, GO = gene ontology, HIF = hypoxic inducible factor, IGF-2 = insulin-like growth factor, LDLR = low-density lipoprotein receptor, LRP10 = low-density lipoprotein receptor-related protein 10, mUtA PI = mean uterine arteries pulsatility index, PCR = polymerase chain reaction, SGA = small for gestational age.

Keywords: amniotic fluid supernatant, cell-free fetal RNA, fetal growth restriction, transcriptome analysis

1. Introduction

Fetal growth restriction (FGR) is commonly defined as failure of fetal growth potential and the estimated fetal weight below the 10 percentile for its gestational age. Small for gestational age (SGA) refers to weight <10 percentile for that particular gestational age without in-utero growth in consideration. The terms FGR and SGA are often used interchangeably though there are subtle differences between the 2.[1] The major risk factors for FGR are fetal, environmental, and maternal factors during pregnancy, including abnormal maternal nutrition, placental insufficiency, fetal aneuploidy, fetal infection, and multiple gestations.[2] FGR fetuses have greater risks of morbidity and mortality, such as stillbirth, birth hypoxia, neonatal complications, impaired neurodevelopment, and possibly type 2 diabetes mellitus, and hypertension in adult life.[3] Because earlier FGR leads to greater risks of fetal prenatal growth and severe FGR may even have negative effects on long-term development during childhood and adulthood,[4,5] it is important to monitor the fetus with FGR consistently. Recently, prediction of FGR fetuses has become possible through the use of ultrasound biometry, ultrasound-estimated fetal weight, and ultrasound Doppler flow velocimetry. However, these tools lack the fundamental evidence for the growth of fetus with FGR excluding pathogenic factors.

The amniotic fluid (AF) is a dynamic environment according to the progression of the fetus during pregnancy. AF includes nutrients and growth factors for fetal development and is used as a source for clinical diagnoses, such as karyotyping, genetic diagnosis, lung maturation, and fetal infection.[6,7] In general,

Editor: Daryle Wane.

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The authors have no funding and conflicts of interest to disclose.

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Medicine (2018) 97:2(e9572)

Received: 21 June 2017 / Received in final form: 6 December 2017 / Accepted: 7 December 2017

http://dx.doi.org/10.1097/MD.0000000000009572

amniocytes are separated from the amniotic fluid supernatant (AFS) using centrifuge, and then are used for fetal karyotyping or DNA extraction for molecular genetic diagnosis. Even though large amounts of AFS are discarded, it contains abundant cell-free fetal nucleic acids that are different from those present in the maternal plasma. The AFS originate from the fetus, and are not contaminated by maternal nucleic acids even if the AF contains a small amount of fetal stem cells, due to the unidirectionality of the fetal-maternal circulation.[8–10] Bianchi et al[10] first reported that there was 100- to 200-fold more fetal DNA per milliliter of AF than in the maternal plasma. They also published the successful isolation of cell-free fetal RNA from AF, and the outcomes of genomic analysis have resulted in the discovery of new information on human development in pregnancy.[11] In addition, unlike amniocytes, cell-free RNAs in AFS are expressed according to gestational age and tissue.[12,13] Recent studies using the AF transcriptome have provided insights into prenatal pathophysiology and the treatment of genetic, developmental, and environmental diseases.[14–16] During pregnancy, transcriptome analysis at early gestational age has supported that cell-free RNAs originated from diverse tissues as well as placenta as fetal development progresses.[17] Global gene expression analysis of cell-free RNA in AFS has been performed in detail in aneuploidy fetuses, such as in cases of the Turner syndrome, Down syndrome, and Edward syndrome.[14–16] These studies have provided insights into the phenotypes of these syndromes, and identified many genes essential for fetal neurodevelopment as potential biomarkers. Therefore, studies of cell-free fetal RNA in AFS are valuable in order to understand fetal maturation during each stage of pregnancy. As research into fetal growth using cell-free RNA is still incomplete, cell-free transcripts might provide important information on the gene expression that occurs during fetal development.

In this study, we hypothesized that the transcriptome in the AFS of FGR fetuses might be different to that of the normal growth fetuses. This study will be helpful in understanding the prenatal development of FGR fetuses. In addition, it may suggest potential fetal biomarkers that can be predicted short- and long-term complications due to FGR in the future. To demonstrate this hypothesis, we performed a comparative analysis based on Affymetrix microarray using cell-free fetal RNA isolated from AF of fetuses with FGR and controls. Then, we analyzed these results using different tools such as PANTHER database[18] and DAVID database.[19]

2. Methods

2.1. Subjects

Women who presented at the Department of Obstetrics and Gynecology, CHA Gangnam Medical Center, CHA Medical University, Seoul, Korea from January 2013 to December 2014 were enrolled in the present study. All the samples in this study belong to a wider cohort of women enrolled in a prospective research study still in progress, aimed to molecularly monitor the fetal development using cell-free fetal RNA from the mid-trimester AFS.

The hand-written informed consent, which contains the information about this study, was given by all of the pregnant women. Ethics committee approval from the CHA Gangnam Medical Center (GCI-14-11) was obtained before the start of the data collection. AF samples were obtained from pregnant women for routine fetal karyotyping and genetic diagnosis. The gestational ages at the time of AF sampling ranged from 16^{+0} weeks to 18^{+6} weeks. For all subjects, gestational age was confirmed during early gestation using the crown-rump length measurement by transvaginal ultrasonography. Each fluid was immediately stored at −80°C until it was used in the study.

The American Congress of Obstetricians and Gynecologists defines FGR as an estimated fetal weight below the 10th percentile for its gestational age.[20] For this study, FGR in these singleton pregnancies was defined as a sonographic estimation of fetal weight below the 10th centile by using the Hadlock formula, with characteristic Doppler flow studies and abnormal placental pathology. We exclude all pregnancies complicated by pre-eclampsia, major fetal anomalies, abnormal karyotypes, gestational diabetes mellitus, intrauterine infection, or chronic maternal infection. Finally, we selected 15 cases of FGR from the cohort. All 15 cases had abnormal Doppler findings at mean uterine arteries pulsatility index (mUtA PI) (expressed as mUtA PI >95th centile) at the time of diagnosis.[21] We defined control subjects as those who showed no clinical abnormal signs and normal growth. A normal control set was selected and matched for similar gestational ages.

2.2. RNA extraction

Cell-free fetal nucleic acids were extracted from 5 to 10 mL of AFS and purified using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). Purified nucleic acids were used to

Table 1

Functional annotation of FGR-dependent DEGs using DAVID analysis.

Categories	Term	No. of genes	P^*	Fold enrichment	Benjamini–Hochberg
Up-regulated genes					
GOTERM_BP_DIRECT	GO:0006414 translational elongation	78	1.03E−130	59.2	9.74E−128
GOTERM_BP_DIRECT	GO:0000184 nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	76	1.19E−114	47.4	2.25E−112
KEGG_PATHWAY	hsa03010 Ribosome	76	7.04E−97	26.1	8.17E−95
GOTERM_BP_DIRECT	GO:0010467 gene expression	112	2.02E−77	8.6	1.74E−75
GOTERM_BP_DIRECT	GO:0044281 small molecule metabolic process	93	5.70E−63	4.0	4.11E−32
Down-regulated genes					
GOTERM_BP_DIRECT	GO:0007156 homophilic cell adhesion via plasma membrane adhesion molecules	22	5.22E−26	31.5	2.19E−23
GOTERM_BP_DIRECT	GO:0050808 synapse organization	3	.009	20.8	0.84
GOTERM_BP_DIRECT	GO:0060627 regulation of vesicle-mediated transport	2	.039	49.3	0.99

DEG = differentially expressed genes, FGR = fetal growth restriction.

* EASE score.

Table 2

Clinical characteristics of the study group.

	Control	FGR
Mothers		
Gestational age (weeks^{+days})	17^{+4} (16^{+0}–18^{+6})	17^{+5} (16^{+0}–18^{+6})
Maternal age, y	35.6 ± 3	36.4 ± 4
Delivery weeks (weeks^{+days})	39^{+1} (37^{+0}–40^{+6})	37^{+6} (35^{+0}–40^{+6})
Neonates		
Gender, male, n (%)	2/9 (22.2)	4/15 (26.7)
Birth weight, g	3339.3 ± 230	2243.7 ± 415
Birthweight percentile (%)	26–75	<10
APGAR score at 1 min*	8 (5–9)	8 (5–9)
APGAR score at 5 min*	9 (6–9)	9 (6–9)

Mean ± standard deviation, median (min–max).

APGAR = appearance, pulse, grimace, activity, respiration, FGR = fetal growth restriction.

* 0 to 3: Critically low, 4 to 6: fairly low, 7 to 10: generally normal at 1 and 5 min.

isolate genomic DNA using an on-column DNase digestion step according to the manufacturer's instructions. Next, cell-free fetal RNA was eluted and purified using the RNeasy MinElute Cleanup kit (Qiagen). The concentrations of the RNA samples were measured using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE) as an A260/A280 ratio; samples with ratios >1.8 were stored at −70°C until further analyses.

2.3. Microarray analysis

Before the microarray expression analysis, the extracted RNA was amplified using an in vitro transcription technology known as the Eberwine or reverse transcription-in vitro transcription method to obtain an appropriate concentration of RNA. First-strand cDNA was reversely transcribed using T7 oligo (dT), and the single-stranded cDNA was then converted into double-stranded cDNA by transcription. RNA derived from the double-stranded cDNA templates was biotinylated and amplified using the IVT Labeling Master Mix. Subsequently, biotin-modified aRNA was purified and fragmented for hybridization to microarrays. Gene expression profiles were identified using a GeneChip Prim View array (Affymeterix, Santa Clara, CA). Fragments of biotinylated aRNA were hybridized for 16 h at 45°C on the GeneChip Human array. After washing, GeneChips were stained in the Affymetrix Fluidics Station 450 and scanned using the Affymetrix GeneChip Scanner 3000 7G.

2.4. Data analysis

All data were analyzed by Affymetrix default analysis settings and global scaling was used as the normalization method. The normalized and the log-transformed intensity values were analyzed using GeneSpring GX 7.3 (Agilent Technologies, Santa Clara, CA). Hierarchical clustering was performed using the Cluster 3.0 and TreeView software programs, developed at Stanford University. The clustering algorithm was set to complete linkage clustering using a Pearson correlation. For data preprocessing, we converted the probe-level data into expression measures if the signal was considered to be "detected," by selecting the value larger than the median value of the control probe signal. Detected genes were calculated by *t* test. Significant changes in gene expression for the Affymetrix data were identified by selecting genes that satisfied the significance threshold criteria of *P* values using analysis of variance for the variance of the mean values between groups, followed by Benjamini–Hochberg multiple testing corrections as $P \leq .05$ with a fold change ≥ 1.5. The list of genes identified as differentially expressed genes (DEGs) between FGR and control fetuses is shown in Table 1.

The gene ontology (GO) analysis and tissue-term analysis were performed using DAVID, an ontology-based web tool (http:// david.abcc.ncifcrf.gov/)[22,23] and PANTHER analysis tool (http://www.pantherdb.org/).[18] The analysis was performed for separate lists of positive or negative DEGs, filtered for ≥1.5-fold differences in expression with respect to the control group. Each list of genes with ≥1.5-fold change with respect to the controls was used for interpretation analysis of DEGs using Excel software by considering the result significant if the EASE score (a modified Fisher exact test) had a $P < .05$

Table 3

Clinical information of the fetus with FGR.

No.	Maternal age	Indication of amniocentesis	Gestational age at amniocentesis	Gestational age at delivery	Gestational weeks at first scan	Estimated weight at first scan, g	Percentile at first scan, %	mUtA PI at first scan	Karyotype	Birth weight, g	Percentile at delivery, %	Maternal complication
1	34	High NTD*	16^{+6}	35^{+6}	21^{+2}	305	<5	1.32	46, XY	1510	<1	None
2	30	Screening (+)†	17^{+2}	35^{+3}	24^{+2}	510	<5	1.27	46, XX	1940	5	None
3	35	Screening (+)	17^{+3}	37^{+0}	23^{+2}	452	5–10	1.29	46, XY	2350	5	None
4	41	Screening (+)	17^{+4}	37^{+4}	21^{+5}	300	<5	1.31	46, XX	2090	1	None
5	36	Screening (+)	17^{+1}	37^{+4}	23^{+0}	460	5–10	1.3	46, XX	2300	4	None
6	45	AMA	17^{+0}	40^{+6}	21^{+3}	320	5–10	1.32	46, XX	2790	5	None
7	34	Screening (+)	17^{+1}	36^{+4}	21^{+6}	330	5–10	1.31	46, XX	2020	3	None
8	41	Screening (+)	17^{+1}	40^{+5}	21^{+4}	320	5–10	1.31	46, XY	2860	8	None
9	39	Screening (+)	18^{+1}	40^{+6}	21^{+6}	325	5–10	1.31	46, XX	2750	5	None
10	31	Screening (+)	18^{+2}	40^{+5}	23^{+1}	465	5–10	1.3	46, XX	2785	6	None
11	33	Infertility workup	17^{+6}	38^{+3}	22^{+4}	360	<5	1.3	46, XX	1680	1	None
12	34	Screening (+)	18^{+1}	35^{+0}	23^{+2}	430	<5	1.29	46, XX	1970	5	None
13	38	Screening (+)	17^{+4}	38^{+6}	20^{+2}	255	5–10	1.33	46, XX	2390	2	None
14	38	AMA	16^{+5}	35^{+1}	20^{+4}	270	5–10	1.33	46, XX	2000	6	None
15	37	Screening (+)	17^{+4}	36^{+5}	20^{+3}	265	5–10	1.33	46, XY	2220	7	None

AMA = advanced maternal age, FGR = fetal growth restriction, mUtA PI = mean uterine arteries pulsatility index.

* High-risk group for neural tube defect.

† Serum screening positive.

in DAVID. To characterize the interesting gene list, we explored the tissue localization of proteins encoded by these genes using DAVID based on Uniprot tissue list and Human protein Atlas.

2.5. Real-time polymerase chain reaction

Cell-free fetal RNA isolated from the AFS went through transverse transcription using the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). Real-time polymerase chain reaction (PCR) conditions were as follows: 40 cycles of 20 s at 94°C, 20 s at 60°C, and 20 s at 72°C. All reactions were normalized using 28S-rRNA as an internal control. Primers used in this study are as follows: hypoxic inducible factor (HIF) 1A 5′-TTCACCTGAGCCTAATAGTCC-3′ and 5′-CAAGTCTAAATCTGTGTCCTG-3′, FOXO4 5′-CCGTGAAGAAGCCGATATGT-3′ and 5′-ACCTCAGACTCTGGCCTCAA-3′.

3. Results

To examine the important transcriptome change in fetus with FGR, we performed comparative analysis. We compared baseline characteristics between FGR and control group (Table 2). In

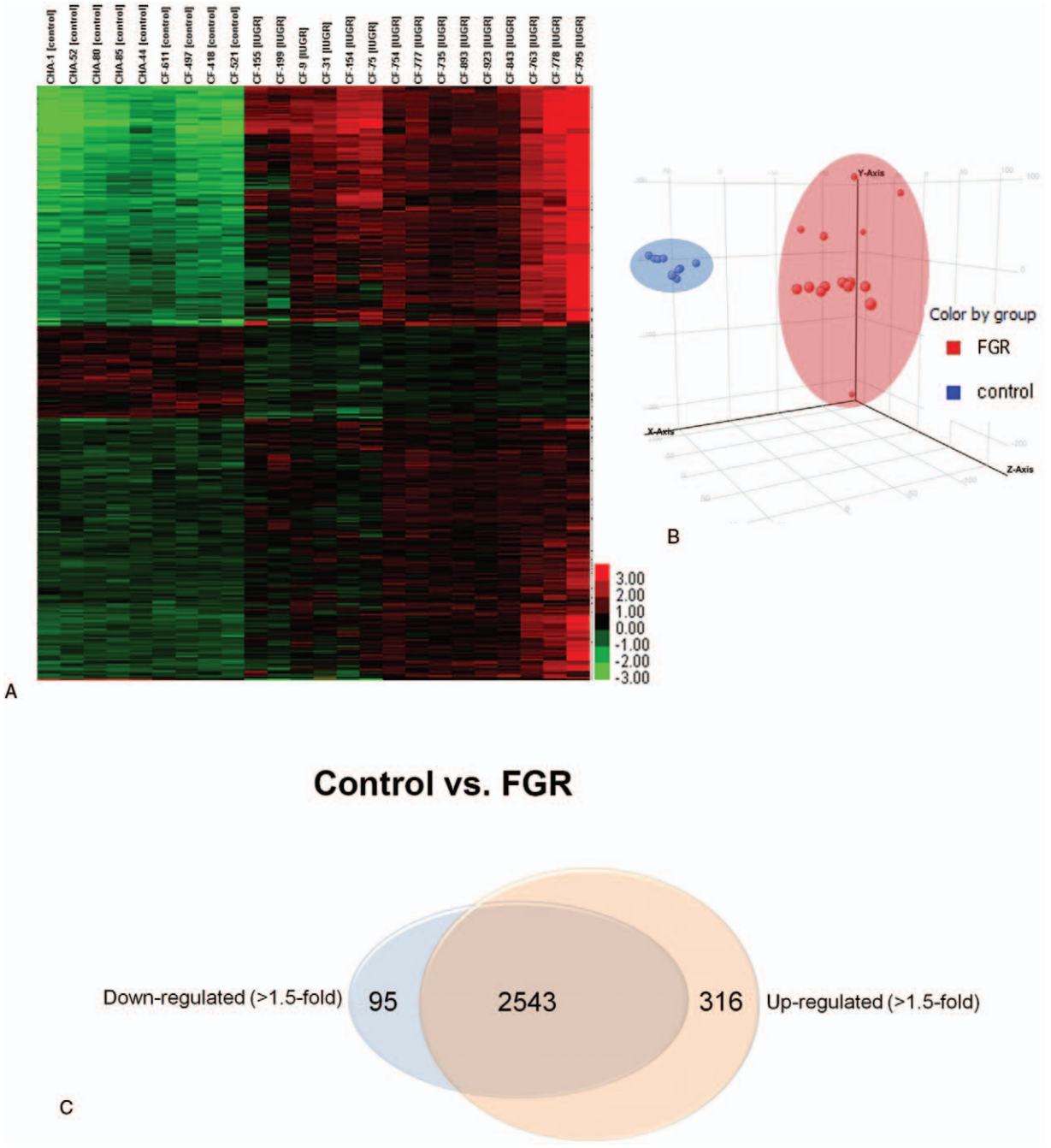

Figure 1. Hierarchical clustering and Venn diagrams comparing FGR and normal growth fetuses. (A and B) Clustering was generated by normalization of log2 intensity values from GeneSpring GX7.3 to display the relative transcription levels of genes differentially expressed (red = relatively up-regulated; green = relatively down-regulated) in both samples. (C) Venn diagrams illustrate the proportion of DEGs in FGR and control microarray datasets. DEG = differentially expressed gene, FGR = fetal growth restriction.

addition, the detailed clinical information of the FGR samples is reported in Table 3.

We isolated cell-free fetal RNA from the AFS obtained from second trimester pregnancies and compared the genome-wide differential RNA expression patterns between the control and the FGR groups using the microarray analysis. And we generated a heat map of the DEGs for FGR samples with respect to the control group using the Affymetrix PrimeView Human Gene Expression Array (Fig. 1A and B). This array consists of more than 530,000 probes covering more than 36,000 transcripts and variants, representing over 20,000 genes mapped through RefSeq or by UniGene annotation. Clustering showed the contrast expressed patterns in FGR compared with the control group. It was defined that 411 genes were differentially expressed in the compared groups, and the up-regulated genes and down-regulated genes were subdivided according to the calculated fold changes and *P* values. Venn diagrams showed that there were 316 up-regulated genes and 95 down-regulated genes when the lists were filtered to include only $P < .05$ and a >1.5-fold change in gene expression between the 2 groups (Fig. 1C).

To examine the functional significance of FGR-dependent genes, we analyzed the molecular function and biological process of genes by the web-based PANTHER annotation database. In terms of molecular function, DEGs were associated with 9 categories based on GO terms. Among these categories, up-regulated genes were mainly associated with "Binding (GO:0005488)" and "Structural molecular activity (GO:0005198)," whereas the down-regulated genes were associated with "Receptor activity (GO:0004872)" as well as "Binding (GO:0005488)" (Fig. 2A). In terms of the biological process, DEGs were associated with 13 categories based on GO terms. Up-regulated genes were mainly associated with "Metabolic process (GO:0008152)," whereas the down-regulated genes were associated with "Developmental process (GO: 0032502)" and "Biological adhesion (GO:0022610)" (Fig. 2B). In terms of GO and KEGG, up-regulated genes were highly related to protein synthesis including translational elongation and ribosome biogenesis (Table 1).

Meanwhile, 22 genes out of the 95 down-regulated genes were enriched for "cell adhesion" similar to the terms of the biological process. These results suggested the possibility that FGR might be caused by decreased function of genes involved in signal transduction or cell-to-cell interaction.

To consider the spatial comparison between the control and the FGR fetuses, we used the DAVID database to determine whether the DEGs were organ specific. Of the 411 DEGs, 298 up-regulated genes were associated with various organs, while the 86 down-regulated genes were associated with a few organs in AFS from the FGR fetuses compared with the control fetuses (Table 4).

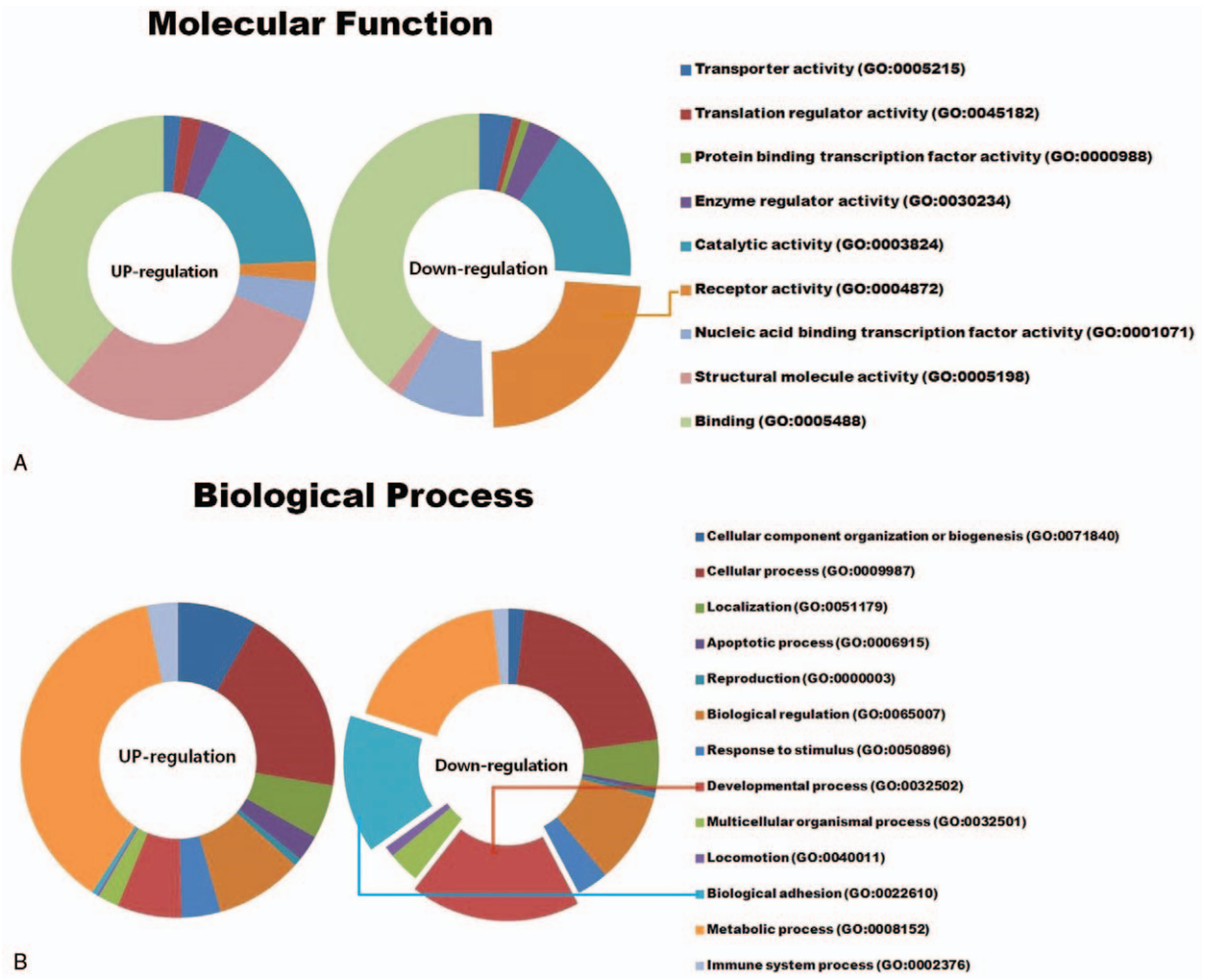

Figure 2. Molecular function (A) and biological process (B) of fetal growth restriction-dependent differentially expressed genes using PANTHER analysis.

Table 4

Organ-specific genes of FGR-dependent DEGs using DAVID analysis.

Expression	Categories	Number of genes	Fold enrichment	Benjamini–Hochberg
Up	B-cell lymphoma	28	13.0	6.48E−20
	Pancreas	58	4.2	8.63E−19
	Esophagus	14	36.6	6.30E−16
	Lung	89	2.5	1.46E−15
	Eye	52	3.6	5.10E−14
	Pituitary	21	9.4	2.31E−12
	Placenta	95	2.1	2.52E−12
	Corpus callosum	14	18.9	5.47E−12
	Prostate	36	4.1	3.62E−11
	Epithelium	79	2.2	6.34E−11
	Muscle	41	3.5	8.55E−11
	Skin	54	2.2	9.33E−07
	Fetal brain cortex	17	4.9	4.33E−06
	Liver	56	2.0	7.40E−06
	Bone marrow	30	2.8	1.09E−05
	Ovary	31	2.7	1.32E−05
	Uterus	46	2.0	9.45E−05
	Tongue	19	3.4	1.02E−04
	Colon	36	2.2	1.13E−04
	Cervix	21	3.0	2.04E−04
	Primary B-cells	6	9.6	0.003
	Keratinocyte	9	5.0	0.003
	Hepatocyte	6	8.6	0.004
	Cajal-Retzius cell	11	3.7	0.005
	Platelet	18	2.4	0.007
	B-cell	12	3.1	0.009
Down	Brain	51	1.42	0.05
	Bone marrow	8	2.18	0.93

DEGs = differentially expressed genes, FGR = fetal growth restriction.
*EASE score.

Of the up-regulated genes, many genes were enriched for B-cell lymphoma, pancreas, eye, placenta, epithelium, skin, and muscle. Among others, in particular, these genes are highly associated with secreted organs such as the pancreas and the organs that are tangent to the AF such as the placenta and epithelium. However, down-regulated genes were enriched only for the brain and bone marrow. These results indicated that neurodevelopment, which is an active process during the entire pregnancy from early-term to full-term, might be delayed although the overall development was active in fetus with FGR. Furthermore, we characterized placenta-specific up-regulated genes according to the protein class because placental factors are one of the representative causes of FGR (Fig. 3).

Although proteins encoded by the up-regulated genes belong to various classes such as protease, storage protein, cytoskeletal protein, hydrolase, etc., most of them belong to "Nucleic acid binding." Down-regulated genes involved in the brain mainly belong to "Receptor" and "Cell adhesion molecules" (Fig. 3). In this result, we expected that the roles of signal transduction such as nutrient signaling or metabolic signaling might be different in the FGR group compared with the control.

To evaluate the functional significance of the FGR-related expression profiles in detail, the interesting DEGs were summarized (Table 5). Ubiquitously expressed low-density lipoprotein receptor-related protein 10 (LRP10) was significantly increased (6-fold) in the FGR cases. Unexpectedly, fold change of insulin-like growth factor (IGF-2) was dramatically increased (17-fold) in the FGR cases compared with the control. Among the down-regulated genes, several interesting genes were cerebral-cortex-specific and down regulated (2-fold) in the FGR cases compared with the control. And PDGFB, vital growth factor for the completion of prenatal development, was down-regulated (1.9-fold). To figure out whether common molecular levels of FGR such as up-regulated IGF-2 transcription and down-regulated PDGFB transcription are related to hypoxia stress or placenta condition, we examined the expression levels of HIF-1,[24,25] a master regulator of cellular and systemic homeostatic response to hypoxia, and FOXO4,[26] transcription factor enriched placenta, using real-time PCR (Fig. 4). As shown in this result, transcription of HIF-1α was increased but FOXO4 was slightly reduced in the FGR cases.

4. Discussion

To our knowledge, this is the first study to evaluate the comparing gene expression of FGR and normal growth fetuses. This study investigated differences in the transcription levels of cell-free fetal RNAs isolated from AFS of FGR and control fetuses in the second trimester of gestation using transcriptome analysis. This information regarding the changes occurring at the molecular level will be very useful and may lead to improved antenatal recognition, which will then reduce the morbidity and mortality associated with FGR.

In this study, we examined the AFS of FGR fetuses with abnormal Doppler findings and the mothers and the fetuses were free of any other complications during pregnancy. Therefore, by comparing the transcriptional gene expression of the FGR and the normal growth fetuses, we are able to figure out how FGR is recognized or how it affects fetal development in the second trimester.

In the result, 411 DEGs were identified in the FGR cases compared with the control. In particular, up-regulated DEGs were highly associated with various organs, which reflect the vigorous molecular activities occurring for growth during this period.

We found that cfRNA related to the endocrine organs and blood components including the pancreas, pituitary, prostate, ovary, platelet, and B-cells was increased in AF of FGR fetuses. This corresponds to the result of the cell-free AF transcriptome analysis in euploid pregnancies.[17] The proteins related to B-cell lymphoma are genes in charge of apoptosis and apoptosis is important in the physiological placental development.[27] B-cell lymphoma (bcl-2) can either be proapoptotic or antiapoptotic and one of the causes of FGR is the abnormal apoptotic activity of trophoblasts.[28] According to recent studies, inadequate or disrupted oxygenation plays an important role in abnormal apoptosis. Hypoxia generates free oxygen radicals and eventually increases oxidative stress and thus causes imbalance in the apoptotic gene activity.[29] The genes related to bcl-2 also increased in this study. In order to show a correlation with hypoxia, HIF-1α level was compared in the control and the FGR group and the results in the FGR group were significantly high.

Most of the up-regulated genes were related to protein synthesis, which provides evidence of an increase in developmental processes. In other studies, the genes related to protein synthesis in amniocyte also increase even in second trimester of normal growth fetuses but, down-regulated genes were related to cell adhesion. Kim et al[30] evaluated the placental tissue transcriptome such as the amnion, chorion, and decidua and showed an increase of the focal adhesion and integrin pathways. These results showed the important role of the adhesion and

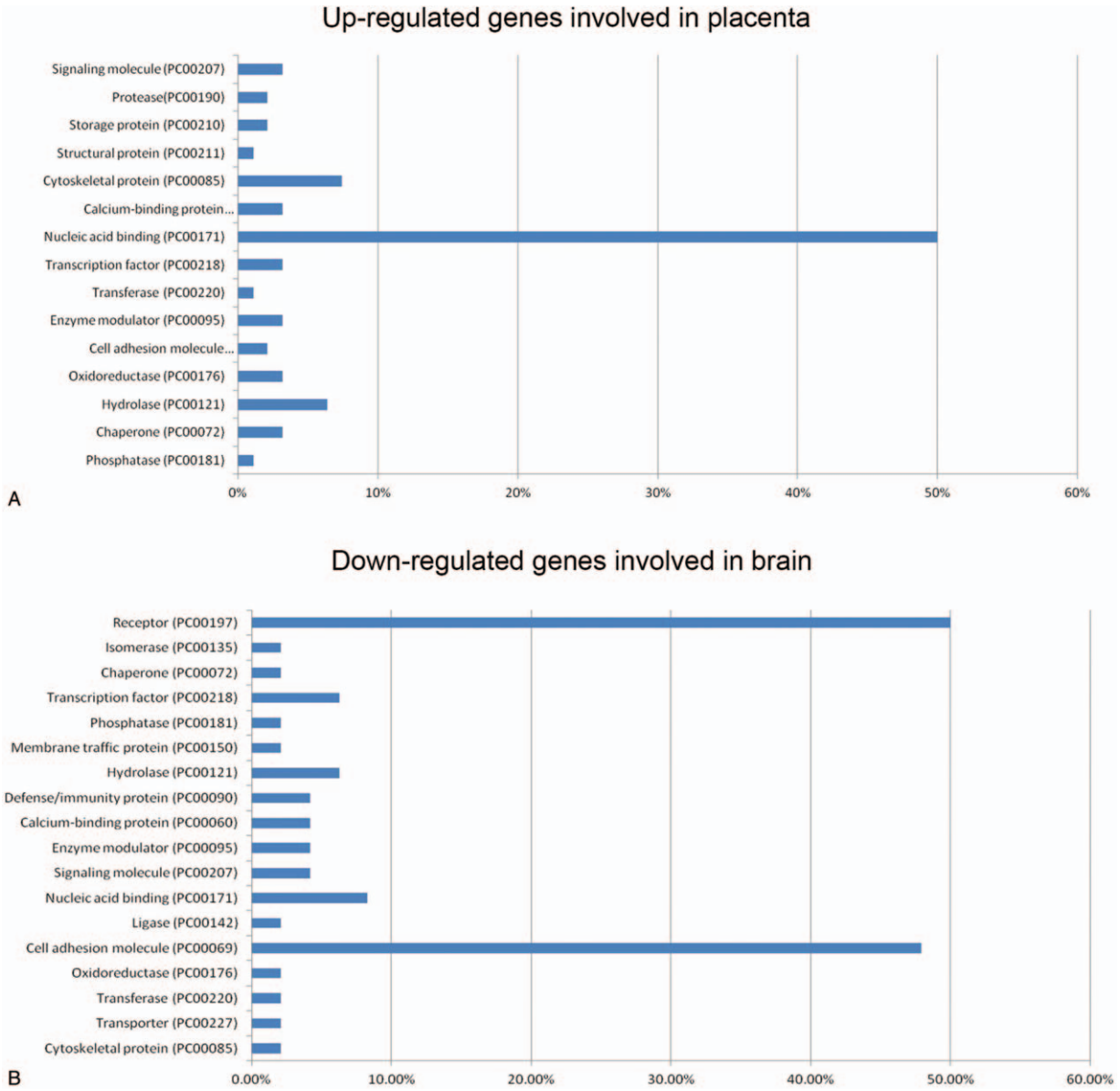

Figure 3. Protein class of fetal growth restriction-dependent differentially expressed genes using PANTHER analysis. The categories are organized according to differential expression; up-regulated genes enriched in placenta (A) and down-regulated genes enriched in brain (B).

integrin pathways in the normal development of the placenta. Thus, it can be assumed according to this study that the decrease in genes related to cell adhesion causes abnormal placental development and thus the FGR occurs. Impaired placental transport of amino acids, fatty acids, or glucose between maternal and fetal circulation has been associated with several conditions known to restrict growth of the fetus.[31]

In the analysis of tissue-specific enrichment, we observed that the genes that originate from various organs of the fetus and the placenta are differentially expressed in FGR. Interestingly, transcription of LRP10, low-density lipoprotein receptor (LDLR)-related protein 10, was significantly increased in FGR cases compared with the control. It had been reported that LRP10 is a negative regulator of canonical Wnt/β-catenin signaling pathway, which plays a crucial role in the development of the human lung.[32,33] In earlier studies, it was thought that the FGR fetuses may accelerate lung maturation and pulmonary surfactant secretion compared to appropriately growing fetuses of the same gestational age.[34] However, this theory lacks evidence and still remains controversial. The most recent study suggested that FGR leads to decreased pulmonary diffusing capacity and lung compliance due to fetal hypoxemia, nutrient restriction, or an altered fetal endocrine environment.

Moreover, impaired fetal lung development could affect the respiratory compromise during the postnatal period and reduce lung function and increase the risk for respiratory morbidity and mortality for adults.[35] One possible reason for impaired fetal lung development in FGR fetuses has been suggested in this study. Also, we found that IGF-2 was increased in FGR fetuses compared to that of the normal growth fetuses. Borzsonyi et al[36] reported that IGF-2 and insulin-like growth factor binding protein-3 genes were over-expressed in FGR complicated placentas. In 2010, Lee et al[37] reported that the expression of IGF-2 was increased and the expression of IGF-1 was decreased

Table 5

The top 5 interesting up- or down-regulated DEGs.

Name	Tissue expression	Protein localization	Fold change	Description
Up-regulated genes				
IGF2	Placenta	Extravillous placental trophoblasts	17	Insulin-like growth factor 2
KRT17	Breast, skin	Squamous epithelia, urinary bladder, salivary gland, breast, prostate, and respiratory tract	7.5	Keratin, type I cytoskeletal 17
LRP10	In all	Alveolar cell, trophoblastic cells of placenta	6	Low-density lipoprotein receptor-related protein 10
APOA1	Liver, small intestine	Kidney and liver	1.5	Apolipoprotein A-I
MEST	Placenta	—	1.5	Mesoderm-specific transcript
Down-regulated genes				
KIF5A	Cerebral cortex	Most abundant in central nervous system	−2.0	Kinesin family member 5A.
PDGFB	Placenta	Ubiquitous	−1.9	Platelet-derived growth factor subunit B
PCDHGA1	Cerebral cortex	—	−1.8	Protocadherin gamma subfamily A, 1
NLGN3	Cerebral cortex	Neuronal cells	−1.6	Neuroligin 3
NELL2	Cerebral cortex Hippocampus	—	−1.5	Neural EGFL like 2

DEGs = differentially expressed genes.

in FGR placentas. The results of this study are also in accordance with the results of the previous 2 studies, and showed a result of 17-fold up-regulation change of IGF-2 in FGR fetuses. The reason why IGF-2 is overexpressed in the placenta of FGR fetuses is due to the physiological change in order to optimize and compensate the energy distribution in a low-energy environment. Intrauterine hypoxia condition is one of the factors related to the development of FGR fetuses and may have a connection to highly expressed IGF-2 due to the fact that the increase of IGF-2 is associated with hypoxia-stress as tumor.[38]

In addition, we found that down-regulated genes such as PCDHGA1, KIF5A, and NLGN3 were cerebral-cortex-specific.[39,40] These results were encouraging because fetal brain development such as the neurulation, differentiation of cerebral vesicles, and neurogenesis occur in the second trimester.[41] PDGFB as a key component of the unique placental hematopoietic that protects hematopoietic stem/progenitor cells from premature differentiation, was also down-regulated in the FGR cases.[42] Because the placenta, a hematopoietic organ, generates differentiated blood cells for the fetus, loss of PDGFB signals altered the placental hematopoiesis. This finding could support PDGFB as potential biomarkers to predict FGR. However, it lacked validity to name these genes as biomarkers due to small amount of cell-free fetal RNA.

In this study, some of the DEGs are known to be critical for human development. In addition, we demonstrate that important brain-related genes are differentially expressed in FGR fetuses during the mid-trimester of pregnancy in addition placenta. FGR may result in delayed or impaired setting for brain development compared with the normal growth fetuses. However, it was limited to examine expression of more candidates because of small amount of cell-free fetal RNA. Therefore, this result is required for further study to understand different molecular levels in FGR and to elucidate how these genes participate in fetal development. To know whether these genes were constitutionally expressed after childbirth, we will examine expression analysis from newborn's umbilical cord blood.

5. Conclusions

It has been confirmed that genes related to protein synthesis are positively regulated, while cell signaling-mediated genes are negatively regulated in the AFS of FGR fetuses. Based on these results, we suggest that signal transduction pathways necessary for growth development may be more impaired in the FGR fetuses than in the control fetuses. This study has highlighted the differential expression of important genes at early stages of fetal development such as bcl-2, LRP10, and IGF-2 but this study is insufficient to understand how these genes may affect the underlying mechanisms of FGR. Therefore, further work is required to elucidate the role of the genes identified. Although this study has limitation for practical application, it provides evidence for valuable longitudinal studies at different stages of development, such as before birth, during early postnatal life, adulthood, and how individuals affected by FGR continue their growth and development after

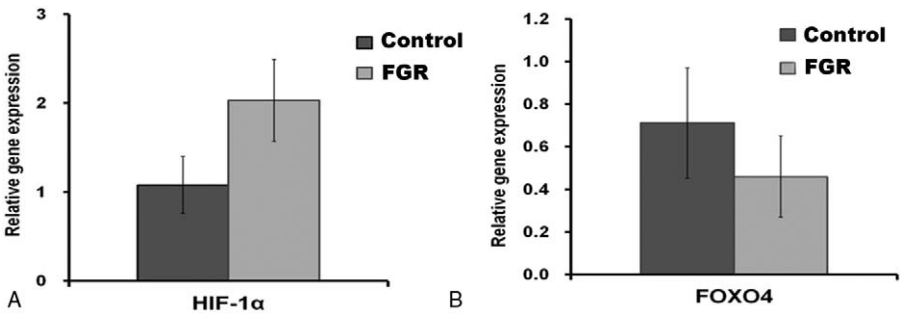

Figure 4. Comparison of expression levels of HIF-1α (A) and FOXO4 (B) between fetal growth restriction and control group.

birth in accordance with the different expression profiles of these potential marker genes.

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