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Profiling of Cytokines Associated with Fetal Growth Restriction in Amniotic Fluid from Women with Uncontrolled Preeclampsia

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이 논문을 박사학위 논문으로 제출함

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1. Introduction

Preeclampsia (PE) is a hypertensive disorder, with or without proteinuria, that generally occurs after 20 weeks of pregnancy (1). PE is associated with maternal and fetal morbidity, and mortality (2); it affects maternal organs such as the liver, heart, lungs, and kidneys (3) and is a major cause of fetal growth restriction (FGR) and preterm birth (4).

The incidence of PE, which affects 3 to 8% of all pregnancies, is increasing worldwide, especially in developing countries (5, 6). Abnormal placental implantation is a known cause of PE (7). During placental implantation in normal pregnancy, trophoblasts of anchoring villi penetrate deep into the inner third of the myometrium and the spiral arteries of the maternal body, and these structural changes are associated with the functional modification of lowering spiral artery resistance (8). However, abnormal placental implantation leads to increased maternal arterial resistance, placental ischemia, and fetal complications (9). Recent studies have suggested that alterations in molecular factors play a pathogenic role in PE (10 - 12). The molecular mechanisms underlying the pathophysiology of PE are still not fully understood. However, elucidation of the molecular features of PE may contribute to a better understanding of its diagnosis, prevention, and treatment.

FGR is traditionally defined as an estimated fetal weight of less than the 10th percentile for a given gestational age, with conditions associated with uteroplacental insufficiency, fetal malnutrition, or restriction of the intrauterine space (13). FGR is identified in about 3 - 7% of all pregnancies (14). Clinically, FGR can be categorized into

placental, fetal, and maternal origin. Genetic anomalies and congenital infections are the fetal causes of FGR, while various maternal morbidities can cause FGR. Structural anomalies in the placenta can also cause FGR (15, 16). PE, which overlaps both maternal and placental causes, is one of the major causes of FGR and the main cause of perinatal mortality and morbidity (17). To investigate the molecular effect of PE on FGR, I analyzed the molecular profile of cytokines in the amniotic fluid (AF) of women with PE according to the presence of FGR.

2. Materials and Methods

2.1. Patient and Sample Collection:

Forty-six women with PE who delivered at less than 34 weeks' gestation due to uncontrolled hypertension were enrolled in the study, between January 2017 and May 2022 at the Keimyung University Dongsan Hospital, Daegu, Korea.

AF was collected at the time of delivery, and AF extracted during cesarean section in high-risk pregnant women has been stored in the Keimyung Human Resource Bank since 2017. All patients were educated about the study purpose and informed consent was obtained from each individual before their participation in the study. The study protocol was approved by the Institutional Review Board of Keimyung University Dongsan Medical Center.

AF and maternal hospital registration numbers were provided by the Keimyung Human Resource Bank and analyzed. The clinical characteristics of the pregnant women and their infants were retrospectively reviewed. Among the 343 cases investigated, 107 were excluded because they had multiple chorionicities; for example, twins or triplets were at a high risk of FGR due to other mechanisms.

Ninety-five pregnant women had PE and 27 cases were excluded: chorioamnionitis (n=6), placenta abruption (n=11), premature rupture of membrane (n=4), polyhydramnios (n=1), fetal anomaly (omphalocele, n=1), 1/5 min Apgar score <5 (n=2), and fetal cytomegalovirus infection (n=2).

Considering the inflammatory response, pregnant women with preterm

premature rupture of membrane or histologic chorioamnionitis were excluded from this study because the effect of inflammatory response on amniotic fluid analysis was not clear.

The FGR group was observed in 21 women with PE, whose birth weight was less than 10th percentile of the gestational age-specific birth weight. As a control group without FGR, 25 women with PE who had birth weights between the 25th and 75th pecercentile of the gestational age-specific birth weight were selected.

To further analyze the clinical factors associated with FGR, I retrospectively investigated clinical characteristics, including maternal age, maternal body mass index (BMI), parity, gestational age at delivery, birth weight, appearance, pulse, grimace, activity and respiration (APGAR) scores as well as history of assisted reproductive technology and pregestational hypertension, the presence or absence of hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome, gestational diabetes mellitus (GDM).

2.2. Proteomic Analysis:

For proteomic analysis, AF was collected from 46 PE women at the time of delivery and stored at -80℃. The samples were sent to Olink (Watertown, MA, USA) and analyzed using the Olink Explore 384 Inflammation Panel. This panel was selected because of its unique inclusion of various inflammatory cytokines. The samples were processed using the Olink Exlore platform, which comprises a Proximity Extension Assay (PEA) with next generation sequencing (NGS). Potential protein biomarkers were evaluated using PEA as described on

the manufacturer's website (https://www.olink.com). The manufacturer's website (https://www.olink.com) provides an extensive list of potential protein biomarkers, comprising a total of 368 entries. Additionally, the website offers comprehensive assay validation data, including details on the limit of detection (LOD), lower and upper limits of quantification, and within- and between-run precision coefficients of variation. The assays' ultimate protein concentration output is presented in terms of Normalized Protein Expression (NPX) values. NPX is a logarithmic unit on a base 2 scale, where elevated NPX values correspond to higher protein concentrations. Protein expression data were obtained from 46 patients. 35 potential protein biomarkers have been identified. Based on an adjusted p value of less than 0.05, values greater than 1 or less than -1 were considered as differentially expressed proteins (DEPs), regardless of whether the $\log_2$ fold change was positive or negative.

2.3. Gene ontology (GO) Analysis of DEPs:

The biological implications of the DEPs were assessed using the web-based tool Database for Annotation, Visualization, and Integrated Discovery (DAVID) 2021 (DAVID, http://david.ncifcrf.gov/, accessed on April 10, 2023). The Gene Ontology (GO) terms were categorized into three distinct groups: biological processes (BP), cellular components (CC), and molecular functions (MF). A p values < 0.05 were considered significant.

2.4. Protein-Protein Interaction (PPI) Network analysis:

To analyze and visually represent the functional PPI network of the identified DEPs, we utilized Cytoscape software (version 3.9.1) and Search Tool for the Retrieval of Interacting Genes version 11.5 (STRING, http://string-db.org, date last accessed on 10 April 2023). The criteria for selecting interactions were a maximum number of interactors equal to 0, and a confidence score of $\geq$ 0.9.

The Cytoscape plugin Molecular Complex Detection (MCODE) was used to identify clustered modules within the PPI network using the following parameters; degree cutoff = 2, node score cutoff = 0.2, k117 score = 2, and maximal depth = 100.

2.5. Enrichment Analysis of Hub Genes:

The Metascape Database (Metascape, https://metascape.org, date last accessed on April 11, 2023) was used to perform enrichment analysis of hub genes. The Metascape Database consolidates various authoritative data resources, including GO, Kyoto encyclopedia of genes and genomes (KEGG), UniProt, and DrugBank. This integration significantly enhances the pathway enrichment and annotation of biological processes, providing comprehensive and reliable insights into the functional significance of genes and proteins.

2.6. Statistical Analysis:

The data analysis was performed using SPSS version 26.0 for Windows, developed by SPSS Inc. in Chicago, IL, USA. Differences in clinical information between the groups were analyzed using Pearson's chi-square test for categorical variables. Student's t-test were used to analyze continuous variables. Statistical significance was defined as a p value less than 0.05.

3. Results

3.1. Patients and Demographic Chracteristics:

Among a total of 46 pregnant women with PE, the FGR group consisted of 21 women and the control group consisted of 25 women. There were no differences in age, BMI, parity, history of assisted reproductive technology, chronic hypertension, HELLP syndrome, or GDM. Regarding fetal factors, the mean birth weight and 1-min APGAR score of the FGR group were lower than those of the control group. (945.2 ± 302.3 vs. 1590.0 ± 393.2, $p < 0.001$, 5.8 ± 1.3 vs. 6.7 ± 1.3, $p = 0.019$, respectively). Gestational age at birth, 5-min APGAR score, and mortality rate did not differ significantly among the groups (Table 1).

3.2. Identification of DEPs:

Sixteen DEPs were identified. All 16 genes were upregulated in the FGR group compared to the control group. The gene symbols, description, $\log_2$ fold change, and p values are listed in Table 2, and a heatmap showing the identified DEPs is shown in Figure 1.

3.3. DEPs Enrichment Analysis:

To analyze the functional profiles of the DEPs, GO term analysis was

performed using the DAVID software. In the biological processes, the DEPs were mainly involved in the positive regulation of the ERK1 and ERK2 cascade, chemokine-mediated signaling pathway, neutrophil chemotaxis, cellular response to interleukin-1, inflammatory response, chemotaxis, cellular response to tumor necrosis factor, immune response, induction of positive chemotaxis, cell-cell signaling, signal transduction, calcium-mediated signaling using an intracellular calcium source, lymphocyte chemotaxis, monocyte chemotaxis, negative regulation of neuronal death, and negative regulation of cell proliferation. In the cellular components, these genes were mainly distributed in the extracellular region, space, and matrix. In the molecular function analysis, DEPs were mainly involved in cytokine activity, chemokine activity, heparin binding, metalloendopeptidase inhibitor activity, and CCR chemokine receptor binding (Table 3).

3.4. PPI Network and Hub Gene Analysis:

I obtained a PPI network composed of 16 nodes and 19 edges, with an average node degree of 2.38 ($p = 2.36E-10$) (Fig. 2). Further analysis was performed using the MCODE plugin, and six hub genes were identified: C-C motif chemokine (CCL20), granulocyte colony-stimulating factor (CSF) 3, erythropoietin (EPO), vascular endothelial growth factor A (VEGFA), Interleukin (IL)-17C, and Granzyme (GZM) B (Figure. 3).

3.5. Enrichment Analysis of the Hub Genes:

CCL20, CSF3, EPO, VEGFA, IL17C, and *GZMB* were subjected to additional enrichment analysis using the Metascape database, which combines GO function and KEGG pathway analyses. These genes were predominantly enriched in processes related to the positive regulation of peptidyl-tyrosine phosphorylation, cytokine-mediated signaling pathways, and positive regulation of the mitogen-activated protein kinase cascades.

KEGG pathway enrichment analysis showed that hub genes were significantly enriched in cytokine-cytokine receptor interactions (Fig. 4).

Table 1. Maternal and Fetal Characteristics

	FGR group (n = 21)	Control group (n = 25)	*p* value
Maternal factors			
Age (year)	32.8 ± 3.4	33.9 ± 3.8	0.284
BMI	28.2 ± 7.3	30.4 ± 5.0	0.228
Parity, n (%)	7 (33.3)	9 (36.0)	0.850
ART, n (%)	3 (14.3)	2 (8.0)	0.495
Chronic hypertension, n (%)	1 (4.8)	1 (4.0)	0.900
HELLP syndrome, n (%)	5 (23.8)	5 (20)	0.516
GDM, n (%)	1 (4.8)	2 (8.0)	0.658
Fetal factors			
Gestational age (week)	212.1 ± 18.0	219.5 ± 13.2	0.112
Birth weight (g)	945.2 ± 302.3	1590.0 ± 393.2	< 0.001
APGAR score, 1 min	5.8 ± 1.3	6.7 ± 1.3	0.019
APGAR score, 5 min	7.9 ± 0.8	8.2 ± 0.7	0.135

Data are presented as the mean ± SD unless otherwise indicated.

Abberviations: APGAR, appearance, pulse, grimace, activity and respiration; ART, assisted reproductive technology; BMI, body mass index; FGR, fetal growth restriction; GDM, gestational diabetes mellitus; HELLP, hemolysis, elevated liver enzyme and low platelet; PE, preeclampsia.

Table 2. Differentially Expressed Genes According to the Presence of Fetal Growth Restriction

Gene symbol	Description	Log_2 FC	Adjusted p value
EPO	Erythropoietin	1.98	0.037
WFIKKN2	Wap, Kazal, immunoglobulin, Kunitz and NTR domain-containing protein 2	1.67	0.006
CLSTN2	Calsyntenin-2	1.46	0.017
CSF3	Granulocyte colony-stimulating factor	1.44	0.017
COL9A1	Collagen alpha-1(IX) chain	1.33	0.031
SCG3	Secretogranin-3	1.30	0.028
CCL23	C-C motif chemokine 23	1.26	0.038
SKAP2	Src kinase-associated phosphoprotein 2	1.25	0.037
CCL20	C-C motif chemokine 20	1.15	0.006
GZMB	Granzyme B	1.14	0.047
TIMP3	Metalloproteinase inhibitor 3	1.14	0.029
FIS1	Mitochondrial fission 1 protein	1.06	0.028
IL17C	Interleukin-17C	1.03	0.017
PON3	Serum paraoxonase/lactonase 3	1.02	0.006
VEGFA	Vascular endothelial growth factor A	1.02	0.006
CXCL8	Interleukin-8	1.02	0.017

Abberviations: Log_2 FC, log_2 fold change.

Table 3A. Gene Ontology Terms of Differentially Expressed Genes According to the Presence of Fetal Growth Restriction

Category	Term	Gene names	*p* value
GO_BP	GO:0070374 Positive regulation of ERK1 and ERK2 cascade	*CCL20, CCL23, EPO, VEGFA*	< 0.05
	GO:0070098 Chemokine-mediated signaling pathway	*CCL20, CCL23, CXCL8*	< 0.05
	GO:0030593 Neutrophil chemotaxis	*CCL20, CCL23, CXCL8*	< 0.05
	GO:0071347 Cellular response to interleukin-1	*CCL20, CCL23, CXCL8*	< 0.05
	GO:0006954 Inflammatory response	*CCL20, CCL23, CXCL8, IL17C*	< 0.05
	GO:006935 Chemotaxis	*CCL20, CCL23, CXCL8*	< 0.05
	GO:0071356 Cellular response to tumor necrosis factor	*CCL20, CCL23, CXCL8*	< 0.05
	GO:0006955 Immune response	*CCL20, CCL23, CXCL8, CSF3*	< 0.05
	GO:0050930 Induction of positive chemotaxis	*CXCL8, VEGFA*	< 0.05
	GO:0007267 Cell-cell signaling	*CCL20, CCL23, IL17C*	< 0.05
	GO:0007165 Signal transduction	*CCL20, CCL23, CXCL8, EPO, SKAP2*	< 0.05
	GO:0035584 Calcium-mediated signaling using intracellular calcium source	*CCL20, FIS1*	< 0.05
	GO:0048247 Lymphocyte chemotaxis	*CCL20, CCL23*	< 0.05
	GO:0002548 Monocyte chemotaxis	*CCL20, CCL23*	< 0.05
	GO:1901215 Negative regulation of neuron death	*CSF3, EPO*	< 0.05

Table 3B. Gene Ontology Terms of Differentially Expressed Genes According to the Presence of Fetal Growth Restriction (continued)

Category	Term	Gene names	*p* value
GO_BP	GO:0008285 Negative regulation of cell proliferation	*CCL23, CXCL8, SKAP2*	< 0.05
GO_CC	GO:0005576 Extracellular region	*CCL20, CCL23, CXCL8, TIMP3, COL9A1, CSF3, EPO, GZMB, IL17C, PON3, SCG3, VEGFA*	< 0.05
	GO:0005615 Extracellular space	*CCL20, CCL23, CXCL8, TIMP3, WFIKKN2, COL9A1, CSF3, EPO, IL17C, PON3, VEGFA*	< 0.05
	GO:0031012 Extracellular matrix	*TIMP3, COL9A1, VEGFA*	< 0.05
GO_MF	GO:0005125 Cytokine activity	*CSF3, EPO, IL17C, VEGFA*	< 0.05
	GO:0008009 Chemokine activity	*CCL20, CCL23, CXCL8*	< 0.05
	GO:0008201 Heparin binding	*CCL23, CXCL8, VEGFA*	< 0.05
	GO:0008191 Metalloendopeptidase inhibitor activity	*TIMP3, WFIKKN2*	< 0.05
	GO:0048020 CCR chemokine receptor binding	*CCL20, CCL23*	< 0.05

Abberviations: BP, biological process; CC, cellular component; GO, gene ontology; MF, molecular function.

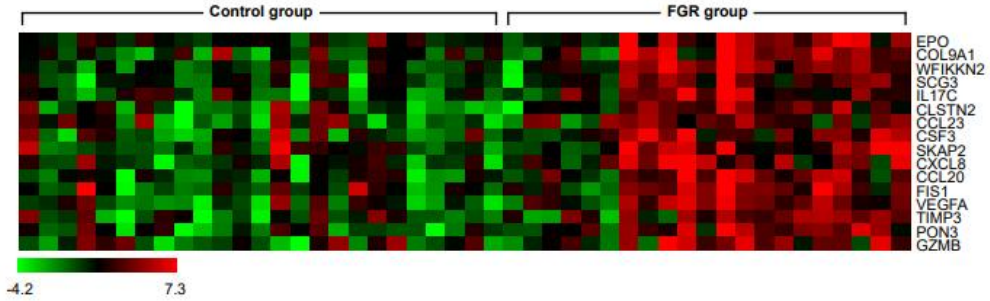

Figure 1. Heatmap showing the identified differentially expressed genes between the fetal growth restriction (FGR) and control groups in the amniotic fluid of women with preeclampsia.

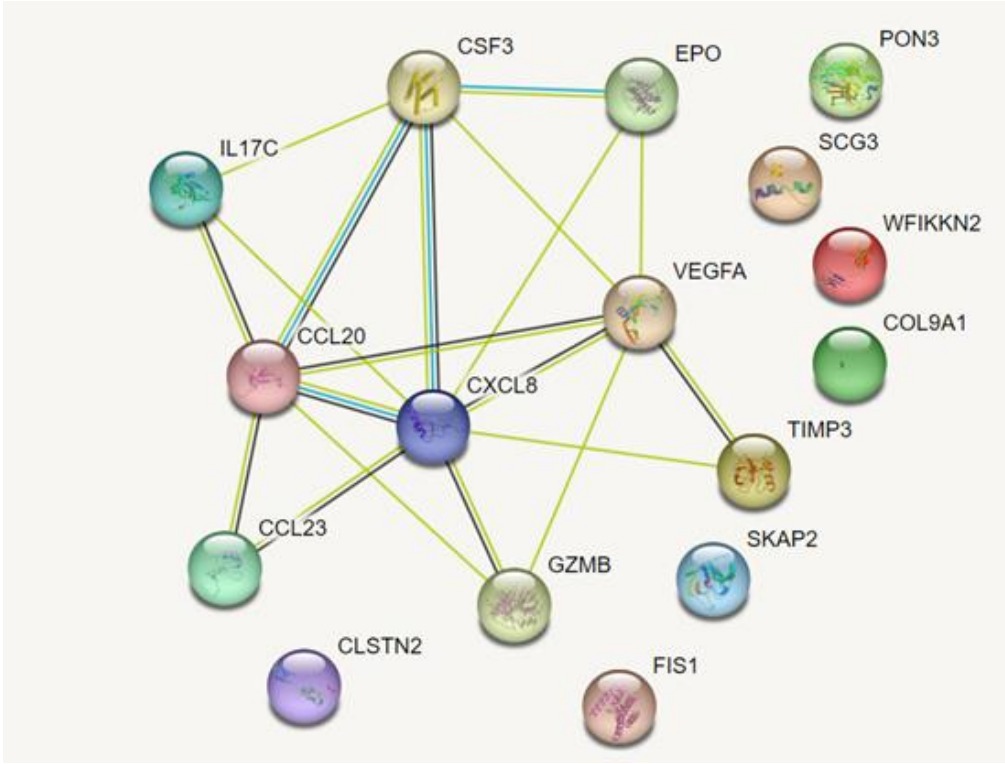

Figure 2. Protein-protein interaction network composed of 16 nodes.

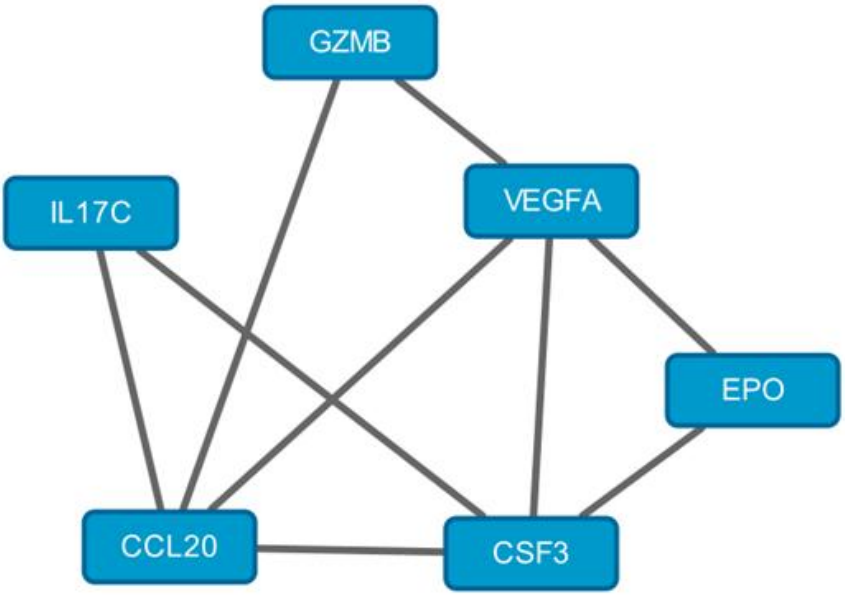

Figure 3. Identified 6 hub genes.

4. Discussion

PE is a major cause of both maternal and fetal morbidity and mortality and is traditionally considered a consequence of abnormal placental implantation (18). Inadequate remodeling of the spiral arteries during placental implantation results in failure to lower the resistance of the uterine circulation and leads to high maternal blood pressure and hypoperfusion of the placenta (19). Mechanical stress may result in maternal organ damage, including systemic endothelial damage and FGR (20). To date, PE treatment mainly focused on regulating maternal blood pressure (21). However, accumulating evidence reveals that PE is a metabolic rather than a mechanical disorder (22, 23). Similarly, recent studies have focused on aberrant cytokine production in PE, and the evidence supports the hypothesis that abnormal cytokine expression plays a key role in the development and manifestation of PE (24-27). However, the molecular mechanisms underlying PE remain unclear.

In the present study, we analyzed the AF of 21 PE women with FGR and 25 PE women without FGR, and identified six hub genes (*CCL20, CSF3, EPO, VEGFA, IL17C,* and *GZMB*) from 16 overexpressed proteins in the AF of PE women with FGR. The proteins encoded by these genes are mainly involved in cytokine-cytokine receptor interactions. CCL20 is a chemokine ligand (28) which regulates the inflammatory reaction by recruiting regulatory T cells and Th17 cells (29, 30). Wang et al. reported that CCL20 is upregulated in the late and early pregnancy plasma of patients with PE, and concluded that CCL20 might be a novel potential predictive and diagnostic biomarker of PE (31). EPO regulates red blood cell production (32). Hypoxic events

induce renal EPO-producing cells, leading to increased plasma EPO levels to promote erythropoiesis (33). There are studies reporting results consistent with findings that the EPO level is elevated in AF in PE or FGR (34, 35). IL-17 plays a key role in T-cell activation. IL-17 mainly regulates the innate immunity against pathogens by promoting neutrophilic inflammation (36). Excessive IL-17 activity leads to autoimmune and inflammatory diseases (37). Lu et al. reported that IL-17 levels were elevated in both the maternal serum and placenta in women with PE (38). VEGF is produced by various cells, and mainly involves in angiogenesis (39). Interestingly, Sahay et al. reported that VEGF levels were higher in the control group than in the PE group (40). However, there are reports that VEGF levels are increased in the maternal serum and AF of patients with PE (41, 42). CSF3 is known to play a key role in embryo implantation and placental development; however, to the best of our knowledge, there are no reports of CSF3 level changes in the AF of women with PE (43, 44). Sheikh et al. reported that *GZMB* miRNA was frequently differentially expressed in screening studies of PE (45). These studies showed that the six hub genes identified in our study were overexpressed in PE compared with normal conditions. Our study has a strong point in that it compared the AF of PE patients with FGR to that of PE patients without FGR. This condition is a manifestation of severe or uncontrolled PE. Taken together, these cytokines, encoded by 6 hub genes from 16 upregulated genes in the FGR group, were upregulated when exposed to certain conditions such as hypoxia, oxidative stress, and inflammatory stress. Likewise, in the AF of uncontrolled PE with FGR, in which the fetus is in a hypoxic state with exposure to oxidative stress and excessive inflammation, cytokines such as CCL20, CSF3, EPO, VEGFA, IL17C and

GZMB can be amplified. Moreover, this is the first study to report that CSF3 levels are increased in the AF of women with PE and FGR. Therefore, large-scale studies are warranted to validate our findings. According to our study results, changes in expression levels of *CCL20, CSF3, EPO, VEGFA, IL17C* and *GZMB* in AF may be novel potential biomarkers of PE with FGR, and may provide new insights into the pathophysiology of PE and FGR caused by PE.

5. Summary

PE is a major cause of both maternal, fetal morbidity and FGR. To investigate the molecular effect of PE on FGR, I analyzed the molecular profile of cytokines in the AF of women with PE according to the presence or absence of FGR.

In conclusion, I identified 16 differentially expressed proteins in the AF of women with PE according to the presence of FGR.

Moreover, six hub genes (*CCL20, CSF3, EPO, VEGFA, IL17C* and *GZMB*) were overexpressed in the FGR group, and these genes were mainly involved in cytokine-cytokine receptor interactions. These upregulated 6 hub genes may serve as novel biomarkers of PE with FGR. Further investigation is warranted to validate the results of our study, but they may contribute to a better understanding of the pathogenesis of FGR in women with PE.

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Profiling of Cytokines Associated with Fetal Growth Restriction in Amniotic Fluid from Women with Uncontrolled Preeclampsia

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(Abstract)

Preeclampsia (PE), a hypertensive disorder that occurs during pregnancy, is associated with chronic immune activation resulting in increased production of inflammatory cytokines. Although the frequency of fetal growth restriction (FGR) is higher in PE, the pathophysiology of FGR in PE is still not clear. To investigate the molecular effect of PE on FGR, I analyzed the molecular profile of cytokines in the amniotic fluid (AF) of women with PE according to the presence of FGR.

An observational cohort study was conducted using amniotic fluids collected from January 2017 to May 2022, provided by the Keimyung Human Bio-Resource Bank. Among a total of 46 pregnant women with PE, the FGR group consisted of 21 women and the control group

consisted of 25 women. Amniotic fluids were analyzed using proximity extension assay (PEA) technology (Olink Explore 384 Inflammation I panel) for the discovery of potential protein biomarkers.

This study identified 16 differentially expressed proteins in amniotic fluid between the FGR group and the control group. Moreover, among the 16 upregulated cytokines in the AF of the FGR group, six hub genes (*CCL20, CSF3, EPO, VEGFA, IL17C,* and *GZMB*) were identified, which are involved in cytokine-cytokine receptor interaction.

조절되지 않은 전자간증 산모의 양수에서 태아성장지연과 연관된 사이토카인의 연구

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(초록)

전자간증은 임신 중 발생하는 고혈압 질환으로, 염증성 사이토카인의 발현 증가로 인한 만성 면역 활성화와 연관되어 있다. 태아성장지연은 전자간증 산모에서 높은 빈도로 발생함에도 불구하고, 전자간증에서 태아성장지연의 병태생리학적 원인은 명확히 밝혀져 있지 않다.

저자는 전자간증의 발병에 염증성 사이토카인의 역할의 가능성을 고려하여, 전자간증 산모의 양수에서 태아성장지연과 연관된 사이토카인의 변화를 연구해 보고자 하였다.

계명 인체 자원 은행에서 2017년 1월부터 2022년 5월까지 채취된 양수를 분양받아 시행한 관찰적 코호트 연구이며, 양수는 분만 당시 양막이 노출되었을 때 주사기로 채취되었다. 총 46명의 양수 내 감염이 없는 전자간증 산모 중, 21명에서는 태아성장지연이 관찰되었고, 25명에서는 재태연령 대비 정상체중을 보였다. 양수는 단백질 생체지표를 분석하기 위해 Proximity

Extension Assay(PEA)기술을 이용한 Olink Explore 384 Inflammation I panel을 통해 분석되었다. 단백질의 발현 정도 차이는 Olink의 독자적인 단위인 Normalized Protein eXpression(NPX)로 비교하였다.

태아성장지연이 있는 전자간증 산모군과 태아성장지연이 없는 전자간증 산모군에서 평균 재태연령, 평균 산모 연령 및 평균 산모 체질량지수는 유의한 차이가 없었다.

결론적으로, 태아성장지연이 있는 전자간증 산모군과 태아성장지연이 없는 전자간증 산모군에서 16개의 차별적 발현 단백질을 규명하였으며, 16개의 단백질이 태아성장지연이 있는 전자간증 산모군에서 증가 발현되어 있었다. 또한 저자는 사이토카인-사이토카인 수용체 상호작용에서 발현 강화된 6개의 핵심 유전자(*CCL20, CSF3, EPO, VEGFA, IL17C* 및 *GZMB*)를 규명하였다.

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