Identifcation of The Ferroptosis-Related Gene Signature In Placenta of Patients With Early-Onset Preeclampsia

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Abstract

Background: The accumulation of ROS resulting from upregulated levels of oxidative stress is commonly implicated in preeclampsia (PE). Ferroptosis is a novel form of iron-dependent cell death instigated by lipid peroxidation likely plays important role in PE pathogenesis. This study aims to investigate expression profiles and functions of the ferroptosis-related genes (FRGs) in early- and late-onset preeclampsia.

Methods: The gene expression data and clinical information were downloaded from GEO database. The “limma” R package was used for screening differentially expressed genes. GO(Gene Ontology), Kyoto Encyclopedia of Genes and Genomes(KEGG) and protein protein interaction (PPI) network analyses were conducted to investigate the bioinformatics functions and molecular interactions of significantly different FRGs. Quantitative real-time reverse transcriptase PCR was used to verify the expression of hub FRGs in PE.

Results: A total number of 4,215 DEGs were identified between EOPE and preterm cases and 3,356 DEGs were found between EOPE and LOPE subtypes. 20 significantly different FRGs were identified in EOPE, while only 3 in LOPE. Functional enrichment analysis revealed that the differentially expressed FRGs was mainly involved in EOPE and enriched in hypoxia- and iron-related pathways, such as response to hypoxia, iron homeostasis and iron ion binding process. The PPI network analysis and verification by RT-qPCR resulted in the identification of the following six interesting FRGs: FTH1, HIF1A, FTL, IREB2, MAPK8 and PLIN2.

Conclusions: EOPE and LOPE owned distinct underlying molecular mechanisms and ferroptosis may be mainly implicated in pathogenesis of EOPE. Further studies are necessary for deeper inquiry into placental ferroptosis and its role in the pathogenesis of EOPE.

Background

Preeclampsia (PE) is a clinical syndrome characterized by gestational hypertension and proteinuria with maternal end-organ damage, which occurs after 20 weeks of gestation.

It threatens 5–7% of pregnancies and is a leading cause of maternal and perinatal mortality [1]. Preeclampsia can be classified into two categories [2, 3]: early-and late- onset PE. Early-onset PE, occurring before 34 weeks of gestation, have more severe manifestations or complications than late-onset PE that occurs at or near term. It is widely accepted that early-onset PE is mainly due to abnormal implantation and placentation in early gestation, whereas late-onset PE commonly results from placental dysfunction caused by maternal disease [4].

Generally, preeclampsia is considered as a two-stage disease [4, 5]. Stage 1 was composed of abnormal implantation and malplacentation while stage 2 was the clinical syndrome resulting from the release of factors by dysfunctional placenta. Local hypoxia and ischemia caused by placental maldevelopment is a

powerful inducer for oxidative stress [6]. Oxidative stress stimulates the release into maternal circulation of anti-angiogenic factors, pro-inflammatory cytokines and soluble endoglin, which may be involved in the maternal endothelial dysfunction, inflammatory response and hypertension [7–10]. Although higher levels of oxidative stress is considered to be implicated in the clinical manifestations of PE, the underlying mechanism remain largely unknown.

Ferroptosis is a novel form of iron-dependent cell death that is quite different from apoptosis, necrosis, and autophagy, in terms of the morphology, biochemistry, and genetics [11]. It is instigated by the accumulation of iron-dependent hydroxy-peroxidized phospholipids [11]. Ferroptosis has recently become a key research focus and been demonstrated to be implicated in multiple diseases, including brain injury, heart injury, acute renal failure, asthma, and cancer [12–14]. Recent studies suggested that ferroptosis might play important roles in the placental pathogenesis of preeclampsia [15–17]. However, to our knowledge, there is scarce study systematically analyzing ferroptosis in preeclampsia and its clinical subtypes. In the present study, we performed at first analysis of expression profiles in placenta of the early-and late- onset preeclampsia. Furthermore, the expression of ferroptosis regulator genes (FRGs) was comprehensively investigated and the hub FRGs were identified in early-onset preeclampsia through the bioinformatics analysis. We for the first time found that many key proteins implicated in the regulation of ferroptosis were aberrantly expressed in the placental tissues of patients with early- onset PE, but few in the placenta of late-onset preeclampsia. These results highlight the critical roles of ferroptosis in early-onset PE, which would be helpful for further elucidations of ferroptosis-related molecular mechanisms in PE pathogenesis and therapy development.

Methods

Acquisition of gene expression

The gene expression profiling dataset GSE74341, based on the GPL16699 Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray platform, was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The experiment contained 25 samples consisting of placenta tissues from patients with early-(n = 7; <34 weeks), late-onset (n = 8; >36 weeks) PE and their controls who delivered preterm (n = 5; <34 weeks) or at term (n = 5; >36 weeks). There is no need for patient consent or ethics committee approval, since all information on gene expression and samples were downloaded from public database.

Differentially Expressed Genes

The differentially expressed genes (DEGs) were identified using the “limma” R package. The cut-off values were determined according to the parameters of adjust P-value < 0.05. In order to obtain the significantly differentially expressed FRG in placenta of EOPE, DEGs in comparisons of EOPE vs. preterm was determined by the criteria of adjust P-value < 0.05 and log2 fold change > 1.

GO terms and pathway enrichment analysis for FRGs of EOPE

The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed by R software. They were used for the functional enrichment analysis of FRGs, including the biological processes (BPs), cellular components (CCs), molecular functions (MFs) and pathway analysis. The Benjamini–Hochberg method was used for adjusting *P*-values. Adjusted *P*-values < 0.05 was set as the threshold values.

Gene cluster identification and protein-protein interaction (PPI) network analysis

The STRING (https://string-db.org/) was used for the PPI network analysis to obtain protein network interaction diagram [18]. The result was download from the online database of STRING and then imported into Cytoscape v3.8.0 software to select the key nodes for visualizing molecular interaction networks. The CytoHubba plugin was used to identify the hub genes from the PPI network.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To validate the key FRGs screened from above analysis, we collected 36 placenta tissues from 18 PE patients and 18 healthy volunteers (Table 1). The collection of placental sample was approved by the ethical committee of First Affiliated Hospital of Bengbu Medical College (2021KY036). This study conforms to the Ethical Review Methods for Biomedical Research involving Humans adopted by the National Health and Family Planning Commission of the People's Republic of China. PE patients were diagnosed based on the guidelines of the American College of Obstetrics and Gynecology (ACOG) [19]. Informed consent from each patient was obtained before the start of the study.

Table 1
Clinical information of patients

Category	PE	Normal	*P*-value
Age (years)	30.72 ± 1.14	32.17 ± 1.076	0.3634
Gestational age at delivery (weeks)	34.25 ± 0.6398	38.79 ± 0.2746	< 0.0001
Systolic blood pressure (mmHg)	165.6 ± 4.009	113.2 ± 1.799	< 0.0001
Diastolic blood pressure (mmHg)	107.6 ± 4.046	73.39 ± 1.403	< 0.0001
Proteinuria(%)	100	0	< 0.0001
Neonatal birth weight (g)	2115 ± 205.2	3503 ± 146	< 0.0001
1min Apgar(score)	8.278 ± 0.3598	9.889 ± 0.07622	0.0001

Total RNA from placenta tissues was extracted using TRIzol (Invitrogen, Carlsbad, Calif). The concentration and purity of extracted RNA were assessed by NanoDrop™ One/OneC (Thermo Fisher Scientific). The reverse transcription was implemented using NovoScrip® Plus All-in-one 1st Strand cDNA Synthesis Kit (Novoprotein, Shanghai, China) at 42℃ for 5 minutes ,50℃ for 15 minutes, and finally at 75℃ for 5 minutes. PCR reactions were performed on NovoStart® SYBR qPCR SuperMix Plus(Novoprotein, Shanghai, China). The primer sequences were shown in S1 Table.

Statistical analysis

All statistical analyses were presented as the means ± SEM. The R software (version 4.0.2) and GraphPad software were used to analyze the data. Continuous values and count data were analyzed using t-test and the chi-squared respectively. A *P*-value < 0.05 was considered statistically significant.

Results

Different expression genes in placenta of PE and PE subtypes

The microarray expression in placenta tissues from patients with early-onset, late-onset PE, preterm and at term was downloaded from dataset GSE74341 in GEO database (Fig. 1A). In order to explore sample features in gene expression, principal component analysis (PCA) were performed on the downloaded dataset. The results from PCA showed that EOPE samples were clustered together and separated from the LOPE subtypes and non-PE samples (Fig. 1B). The LOPE samples were also separated from non-PE placenta samples.

The DEGs between placenta tissues from EOPE, LOPE, preterm and term was analyzed using the R package of limma. DEGs were determined by the criteria: adjust P-value < 0.05. A total number of 4,215 DEGs were identified between EOPE and preterm cases, while only 556 DEGs were found between LOPE and term cases (Fig. 1C).There were 3,356 DEGs identified in the comparisons of EOPE vs. LOPE (Fig. 1C). As shown in Fig. 1D, 194 DEGs were observed both in EOPE and LOPE subtypes and 1,301 DEGs in the comparisons of EOPE vs. LOPE and EOPE vs. preterm (Fig. 1D). Besides, there were more down-regulated than up-regulated genes in the comparisons of EOPE vs. LOPE and EOPE vs. preterm (Fig. 1E).

The different expression of ferroptosis-related genes in PE and PE subtypes

In order to avoid the impact of the imbalance in the number of DEGs on the inclusion of ferroptosis-related genes (FRGs), the criteria for determining DEGs in comparisons of EOPE vs. preterm was determined as follows: adjust P-value < 0.05 and $\log_2$ fold change > 1. As shown in the volcano plot in Fig. 2A and B, there were similar number of DEGs in EOPE and LOPE. After intersection with FRGs, there were 20 differentially expressed FRGs found between EOPE and preterm samples, while only 3 between LOPE and term samples (Fig. 2C and Table 2). A total of 259 FRGs were downloaded from FerrDb

(http://www.zhounan.org/ferrdb/index.html), including drivers, suppressors and markers respectively promoting, preventing and indicating the occurrence of ferroptosis (Fig. 2D and S2 Table). As shown in Fig. 2E, almost half of FRGs (45%, 9/20) in placenta of EOPE were markers that indicate ferroptosis occurrence. The clustering analysis of significantly different FRGs showed that the EOPE samples were closely clustered together (Fig. 2F). In EOPE samples, there were 9 and 11 FRGs that were down- and up-regulated respectively (Fig. 2G and Table 2).

Table 2
The different expressed ferroptosis related genes in EOPE

Up-regulated			**Down-regulated**		
Gene symbol	**Adjusted P-value**	**logFC**	**Gene symbol**	**Adjusted P-value**	**logFC**
DRIVER					
EGFR	6.64E-03	1.447	IREB2	8.71E-03	0.289
CDO1	8.76E-03	1.365	CYBB	6.25E-03	1.215
HILPDA	2.07E-03	1.660	SCP2	5.63E-03	1.382
			DPP4	1.18E-02	1.010
			MAPK8	1.35E-04	1.000
MARKER					
GPT2	2.49E-03	1.871	FTH1	2.65E-03	1.272
SLC7A5	2.20E-02	1.473	VLDLR	2.96E-03	1.189
HERPUD1	9.48E-03	1.256	FTL	8.90E-03	1.004
GDF15	2.74E-02	2.787			
ARRDC3	1.35E-03	1.388			
SLC2A1	1.62E-02	1.104			
SUPPRESSOR					
LINC00336	1.39E-03	1.084	FTH1	2.65E-03	1.272
PLIN2	2.68E-03	2.864	ENPP2	5.07E-03	1.333
			ACSL3	2.38E-02	0.372
			HIF1A	2.95E-02	1.088

Functional enrichment analysis of DEGs

To investigate the biological functions and pathways of FRGs in EOPE, GO and KEGG enrichment analysis was performed on the 20 genes. The GO analysis showed that differentially expressed FRGs were mainly enriched in hypoxia- and iron-related pathways, such as response to hypoxia, iron homeostasis and iron ion binding process (Fig. 3A,C and S3 Table). KEGG results showed that the differentially expressed FRGs were closely enriched in central carbon metabolism in cancer, HIF-1 signaling pathway, necroptosis and ferroptosis (Fig. 3B, D).

PPI Network Analysis of DEGs

The significantly different FRGs were analyzed using the STRING online database and a PPI network with 22 nodes and 66 edges was obtained (Fig. 4A). We used cytoHubba plugin in Cytoscape to identify the hub FRGs involved in EOPE. As shown in Figs. 5B and Table 3, the top 10 hub FRGs including mitogen-activated protein kinases 8 (MAPK8), epidermal growth factor receptor (EGFR), Solute carrier family 2 member 1 (SLC2A1), hypoxia-inducible factor 1A (HIF1A), ferritin heavy chain 1 (FTH1), growth differentiation factor 15(GDF15), solute carrier family 7 member 5 (SLC7A5), iron responsive element binding protein 2 (IREB2), ferritin light chain (FTL), Perilipin 2 (PLIN2) were identified (Fig. 4B and Table 3) .

Table 3 Top 10 genes in network ranked by degree method.

Rank	Name	Score
1	EGFR	10
1	MAPK8	10
3	SLC2A1	8
4	HIF1A	6
5	FTH1	3
6	FTL	2
6	IREB2	2
6	SLC7A5	2
6	GDF15	2
6	PLIN2	2

Validation of DEGs in PE

The top 10 hub FRGs were validated in placenta samples of PE using RT-qPCR analysis. Consistent with the prediction, the results showed that the mRNA expression of FTH1, HIF1A, FTL, IREB2 and MAPK8 in placenta samples of PE were significantly up-regulated compared with that of healthy controls, while PLIN2 was significantly increased in PE placentas (Fig. 5).

Discussion

Ferroptosis, distinct from apoptosis and autophagy, is an iron-dependent programmed cell death initiated by iron-dependent hydroxy-peroxidized phospholipids [11]. Oxidative stress and cell damage and death resulting from hypoxia and mitochondrial dysfunction are the major causes of placental pathogenesis in preeclampsia (PE) [20]. Although ferroptosis has been well characterized in various cancers [21, 22], its role that plays in PE is much less clear. At the present study, we systematically analyzed the expression of ferroptosis genes in placenta of patient with early- and late-onset preeclampsia. Our results showed that: 1) the gene expression profile in EOPE was very different from that in LOPE; 2) the significantly different FRGs was mainly involved in EOPE compared with LOPE; 3) these FRGs mainly enriched in hypoxia- and iron-related pathways, such as response to hypoxia, iron homeostasis and iron ion binding process.

As we are known, EOPE is often associated with impaired placentation in as early as the first trimester, while abnormalities in the maternal vasculature is associated with LOPE. Previous studies showed that EOPE and LOPE shared different gene expression profile underlying the differential pathogenesis of the two PE subtypes. In this study, we observed the similar results. The principal component analysis (PCA) showed that EOPE were clustered together and separated from the LOPE subtypes and non-PE samples. The number of DEGs in comparisons of EOPE vs. preterm (4,215 DEGs) was much more than that of LOPE vs. term (556 DEGs). Besides, only 7 DEGs was found between preterm and term, which suggests that the gestational age may exert little influence on their gene expression. Importantly, a total number of 3,356 genes were found to be differentially expressed in EOPE compared with LOPE. All these results strongly implied the different molecular mechanisms involved in the two clinical subtypes.

There are circumstances that may induce ferroptosis during the development of the placenta, including free iron [23, 24], hypoxia-reoxygenation [25, 26], trophoblastic lipid peroxidation [6, 27] and a failure of the ferroptosis-mitigating guards [28]. Indeed, the potential role of ferroptosis in placental dysfunction and trophoblast injury has been established in recent studies [15–17]. In this study, we systematically analyzed the expression profile of FRGs in EOPE and LOPE. Interestingly, we found that the differential expression FRGs mainly enriched in EOPE but not in LOPE. 30% FRGs (6/20) as the markers indicating the occurrence of ferroptosis were up-regulated in placenta of EOPE, while only 10% (2/20) down-regulated. These results implied the great potential roles of ferroptosis in early-onset PE.

The essence of ferroptosis is metabolic cell death instigated by excessive peroxidation of polyunsaturated fatty acids catalyzed by iron [11]. Non-enzymatic lipid peroxidation is essential to initiate the oxidation of polyunsaturated fatty acids [29]. Besides, enzymatic lipid peroxidation, mediated by lipoxygenase (LOX) family, is another catalyzed chain reaction of polyunsaturated fatty acids [30]. The

consequence induced by serial oxidation is the destruction of the membrane, which ultimately results in the occurrence of ferroptosis. The hypoxia-reoxygenation and production of reactive oxygen species (ROS) commonly occur during implantation and placentation [31, 32]. The accumulation of ROS and lipid peroxidation resulting from upregulated levels of oxidative stress is commonly involved in impaired placenta function [6]. Besides, iron is rich in placental trophoblasts even in the case of iron deficiency because it is actively transferred to fetus through the placenta [23, 24]. Previous studies have shown that iron imbalance is related to the impaired placental function that characterizes preeclampsia [23, 33, 34]. Consistent with these evidences, functional enrichment analysis at the present study revealed that the differentially expressed FRG in EOPE were mainly enriched in hypoxia- and iron-related reactions. These data support the link between ferroptosis and EOPE that emanate from abnormal implantation and placentation, which highlights the need for deeper study the role of ferroptosis in preeclampsia and other obstetrical diseases.

In the present study, 10 differentially expressed FRGs were identified as the most significant hub genes. Consistent with the prediction, downregulated genes including FTH1, HIF1A, FTL, IREB2 and MAPK8 and the upregulated PLIN2 were validated by RT-qPCR in PE. FTH1, FTL and IREB2 were mainly responsible for iron metabolism. FTL and FTH1 are light and heavy chain of ferritin respectively. The aberrant expression of the two iron-related genes induce the disorder of iron uptake and intracellular storage, which facilitates cell ferroptosis [35]. In particular, FTH1 as a key subunit of ferritin was reported to be impacted in a variety of biological process, including regulating immunity [36] and inhibiting apoptosis [37]. IREB2 is an important iron-binding protein and mainly involved in regulation of iron transporters [38]. HIF1A, as the main transcriptional regulator of hypoxia response, regulates cell survival in response to stresses. In addition, studies showed that HIF1A plays an important role in reducing fatty acid β-oxidation and promoting lipids storage [39, 40], which may induce peroxidation-mediated endometrial damage and inhibit ferroptosis [41]. MAPK8 belongs to the family of mitogen-activated protein kinases (MAPK), which can be activated by environmental stressors to regulate a variety of signaling pathways and play an important role in cell function, from cell survival to cell death [42, 43]. Perilipin 2 (PLIN2), also known as adipogenic differentiation-related protein (ADRP), is wrapped in the lipid droplets together with phospholipids and participates in neutral lipid storage in lipid droplets [44]. Recent studies showed that PLIN2 in gastric cancer played pivotal roles in the regulation of ferroptosis induced by abnormal lipid metabolism [45].

Taken together, this study provided molecular-level evidences that the two clinical subtypes EOPE and LOPE owned distinct underlying molecular mechanisms. Importantly, differentially expressed ferroptosis-related genes in the EOPE were identified, which provides a link between placental ferroptosis and PE. However, further studies are necessary for deeper inquiry into placental ferroptosis and its role in the pathogenesis of EOPE.

Abbreviations

EOPE: Early-onset preeclampsia; LOPE: Late-onset preeclampsia; PE: Preeclampsia; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: Differentially expressed genes; FRGs: Ferroptosis-related genes; PPI: Protein protein interaction (PPI); qRT-PCR: Quantitative reverse transcription polymerase chain reaction; PCA: Principal component analysis

Declarations

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Availability of data and materials

All datasets generated for this study are included in the manuscript and the supplementary files.

Ethics approval and consent to participate

The collection of placental sample was approved by the ethical committee of First Affiliated Hospital of Bengbu Medical College (2021KY036). This study conforms to the Ethical Review Methods for Biomedical Research involving Humans adopted by the National Health and Family Planning Commission of the People's Republic of China.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors’ contribution

NY, QH and XL conceived and designed the idea, analyzed the data and drafted the manuscript. BD, YG and SP did sample and data collection. YW, GX and LL performed the experiments. FZ did literature review. DD and XL contributed to the reviewing of the final manuscript. All authors approved the final format of the submitted manuscript.

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Figures

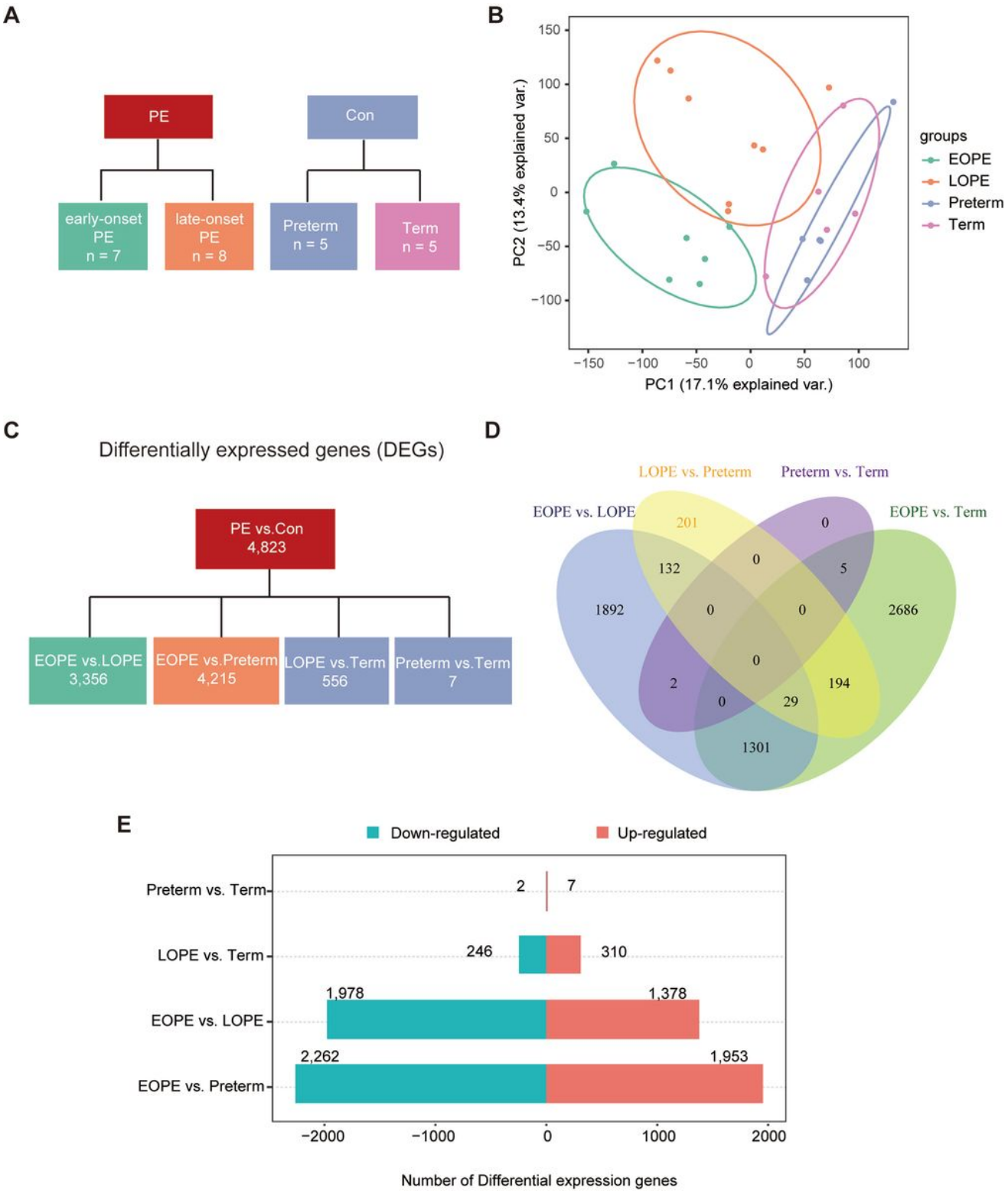

Figure 1

Differentially expressed genes in placenta of preeclampsia. (A) Clinical subtypes of PE patients and their controls who delivered preterm or at term. (B) Principal component analysis (PCA) of the gene expression datasets. (C) The differentially expressed genes (DEGs) for different comparisons. (D) The overlapping genes between the comparisons of EOPE vs. LOPE, LOPE vs. Preterm, Preterm vs. Term and EOPE vs. Term. (E) Numbers of up- and down-regulated genes in comparisons.

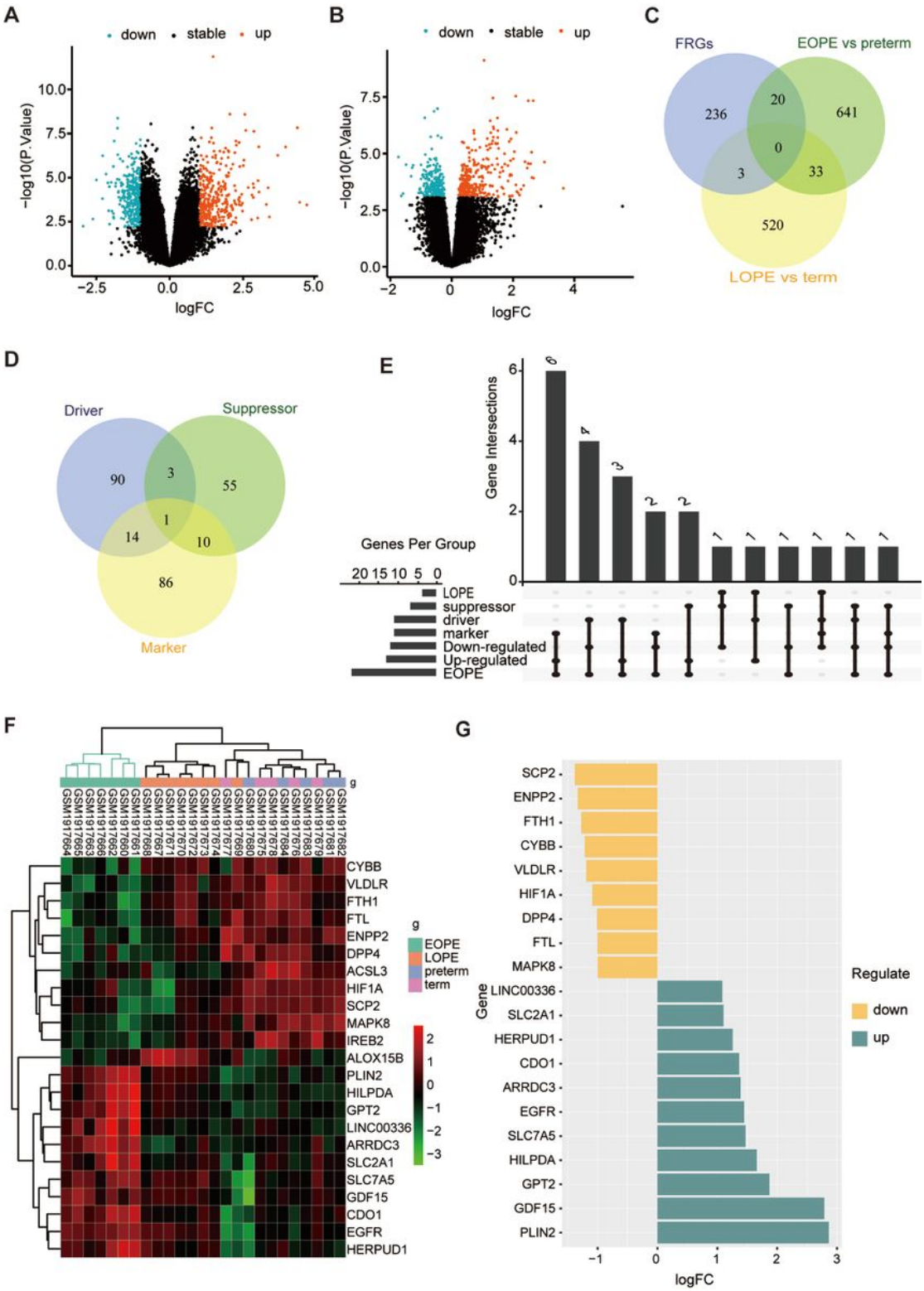

Figure 2

Differentially expressed ferroptosis-related genes in placenta of preeclampsia. (A) Volcano plot of differentially expressed genes in placenta samples of EOPE. (B) Volcano plot of differentially expressed genes in placenta samples of LOPE. (C) The overlapping genes between FRGs and comparisons of EOPE vs. Preterm and LOPE vs. Term. (D) The overlapping FRGs between driver, suppressor and marker. (E) The distribution of up- and down-regulated ferroptosis regulators and markers in EOPE and LOPE. (F) The

heatmap of differentially expressed FRGs in placenta of preeclampsia and the dendrogram based on clustering analysis. Green represents down-regulation while red represents up-regulation of genes (G) Deviation plot of up- and down-regulated FRGs in placenta samples of EOPE.

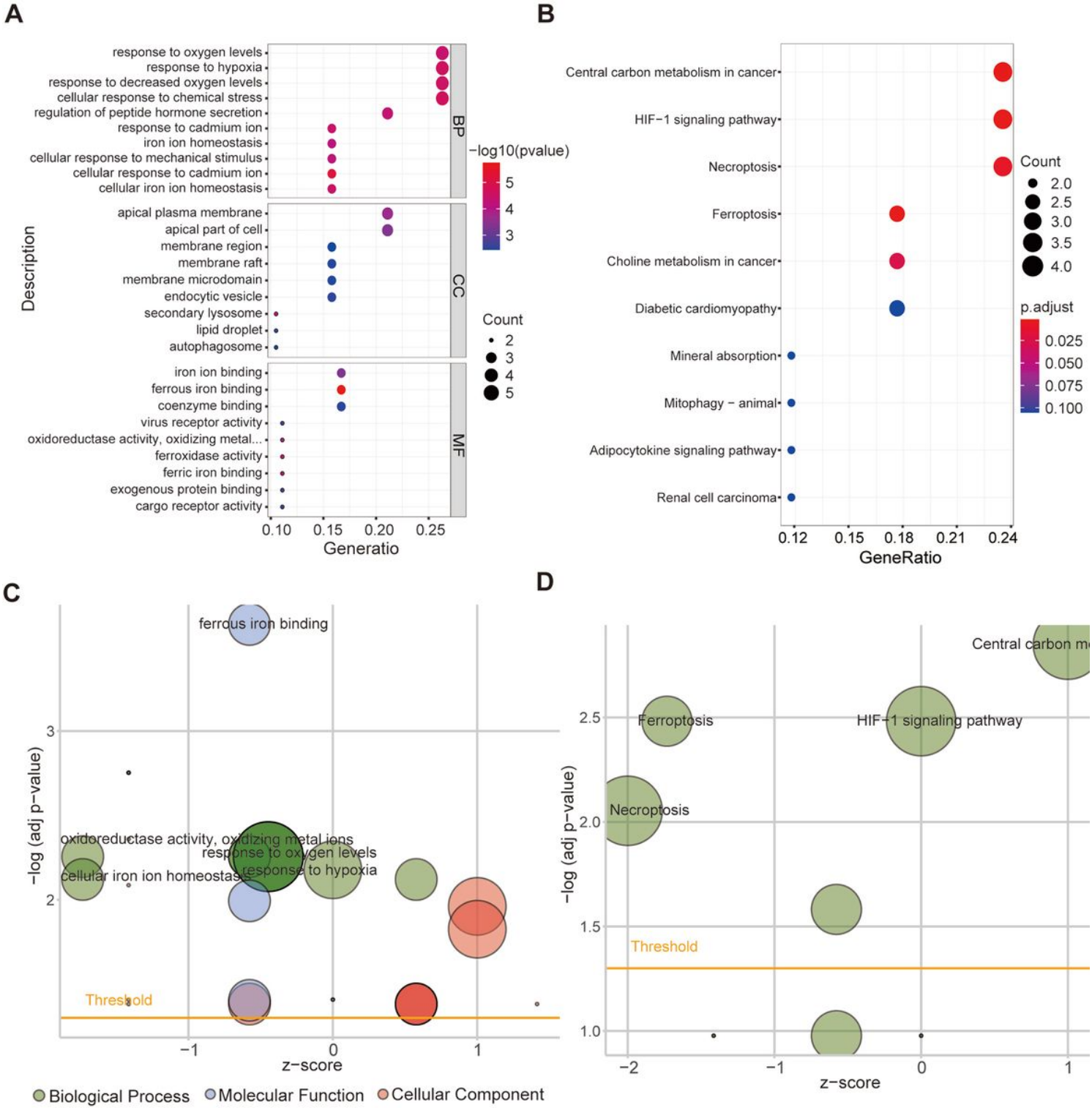

Figure 3

Representative results of GO and KEGG. (A) Bubble plots of GO analyses. (B) Bubble plots of KEGG analyses. (C) Results of GO analyses. (D) Results of KEGG. The larger bubble represents the more enrichment.

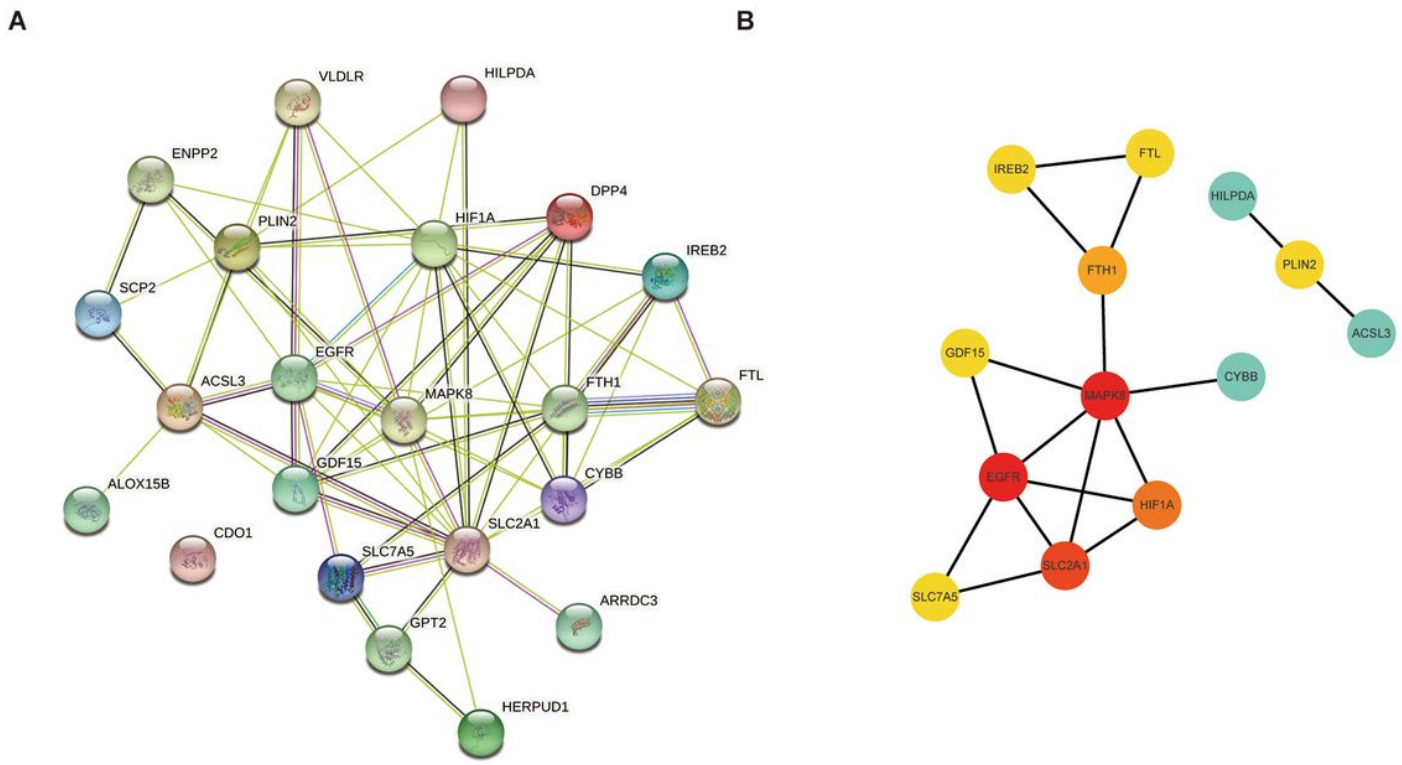

Figure 4

Gene interactions of differentially expressed ferroptosis-related genes (FRGs) in preeclampsia. (A) The network of the differentially expressed FRGs downloaded from the STRING database. (B) The top 10 hub FRGs distinguished using the color shading from yellow to red according to the score.

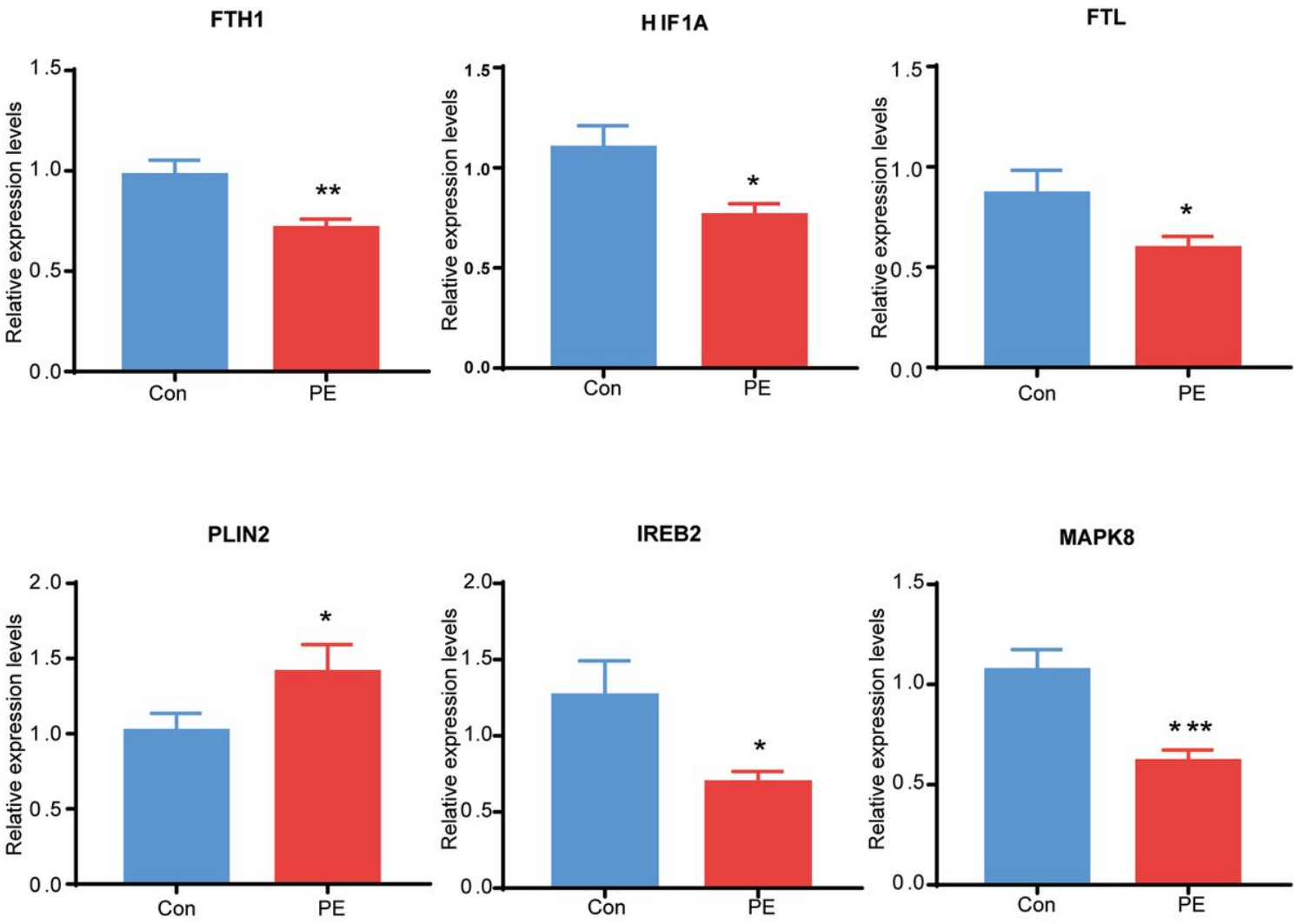

Figure 5

The relative expression of differentially expressed ferroptosis-related genes in placenta. The control group respects normal placenta samples and the PE group respects the placenta samples of preeclampsia.

Supplementary Files

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- Additionalfile1.doc