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Original Article

Identification of key genes in preeclampsia-associated trophoblast cells using bioinformatics analysis

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Abstract: To understand preeclampsia (PE) at the molecular level, this study was conducted to identify the genes and key pathways associated with invasive trophoblast cells in PE via integrated bioinformatics analysis. The gene expression profiles GSE93839 and GSE74341 were downloaded from the Gene Expression Omnibus (GEO). Differentially expressed genes (DEGs) between PE trophoblast cell samples and normal controls were identified by GEO2R. A series of functional and pathway enrichment analyses were performed with DEGs. A protein-protein interaction (PPI) network based on DEGs was constructed and visualized, and hub gene nodes of the network were screened. Finally, the hub genes for the PPI network were confirmed and further investigated to study the potential relationships between DEGs and PE. A total of 472 DEGs were discovered in PE, including 292 upregulated genes and 180 downregulated genes. GO analysis indicated that upregulated DEGs were mainly involved in female pregnancy and embryonic neurocranium morphogenesis. KEGG pathway analysis showed that the DEGs were mainly enriched in ovarian steroidogenesis, metabolic pathways, and biosynthesis of antibiotics. The top 10 hub gene nodes in the PPI network were IL8, CAT, ADCY3, GNGT1, BDKRB1, HCAR2, NPW, P2RY12, TAS2R19, and TAS2R31. In conclusion, we identified the critical genes and pathways that participated in PE initiation and progression from trophoblast cells and placenta samples. We identified ADCY3 and CAT as key genes and the pathways that were most likely to be involved in PE initiation and progression. Our results provide new insights into the development of PE at the molecular level.

Keywords: Preeclampsia, trophoblast cell, molecular biology, functional module

Introduction

Preeclampsia (PE) is a pregnancy-unique, systemic, hypertensive disorder that affects 3%-5% of all pregnancies worldwide. It is the leading cause of maternal and perinatal morbidity and mortality, and an estimated 50,000-60,000 PE-related deaths are reported per year worldwide [1, 2]. PE is defined as new-onset hypertension and proteinuria after 20 weeks of gestation. However, the clinical manifestations of PE are highly variable among patients, and signs and symptoms in ≥1 organ systems [3, 4] reflect the disease in diagnostic guidelines. There is no cure or effective treatment for PE apart from expectant management and delivery of infants and the placenta.

The placenta is a transient apparatus with multiple vital roles throughout gestation in the maintenance and protection of the developing fetus [5]. The polyhedral cells of the placenta have been proven by animal models and human pregnancy studies to be crucial for the survival and health of embryos and mothers [6]. The placenta serves as the interface between the mother and the growing fetus and is involved in the exchange of gases, nutrients, and waste products [7]. Moreover, as an endocrine organ, the placenta produces several pregnancy-related hormones and growth factors to protect the fetus from maternal immune attack [8].

The exact etiology of PE remains elusive [9-11], but the placenta plays an important role in the development of the disease. In the process of embryo implantation, the undifferentiated state of trophoblast cells is assigned to the outer layer of the blastocyst. Cytotrophoblast cells invade the mother's decidua to reach the uter-

ine artery and replace the vascular endothelial cells and smooth muscle cells in the differentiated endothelial-like phenotype [12]. Accumulating evidence showed that defects in remodeling of the uterine arteries are caused by the invasion of inefficient cytotrophoblasts, which support the fetal demand of nutrients and growth [9, 10]. Abnormal remodeling of the spiral arterioles during early placental development results in poor placental perfusion in the pathogenesis of PE. However, the mechanism behind this phenomenon remains unclear even in the change of PE and the invasion of differentiated trophoblast cells [13].

Numerous attempts have been made to investigate the roles of trophoblast cells in PE. However, studies concerning the gene profiles in PE-associated trophoblast cells are insufficient [10-14]. Integrin α1β1, a receptor for collagen 1, collagen IV, and laminin, is expressed in invasive extravillous trophoblasts (EVTs) in normal pregnancy. However, integrin α1β1 is downregulated in PE, resulting in the impaired invasion of EVTs as the failure to obtain the vascular repertoire of adhesion molecules [14]. Early in the first trimester (<10 weeks), placental oxygen tension is at a low level (~2%; 25.6 mmHG O_2) [15], which mediates the proliferation of immature trophoblasts in TGF-β3 through the HIF-1α pathway [16]. At the gestational age of over 10 weeks, placental oxygen tension increases, the number of proliferating trophoblasts decreases, and the number of invasive trophoblasts increases. Caniggia et al. reported that the decreased expression of HIF-1α and TGF-β3 and the failure of TGF-β3 production at a gestational age of 9 weeks are caused by increased placental oxygen tension, thereby leading to shallow trophoblast invasion. However, Lyall et al. reported no change in TGF-β1, TGF-β2, and TGF-β3 expression in the placenta and placental bed in PE and fetal growth restriction compared with those in normal pregnancy [16]. However, reliable and accurate results with respect to PE-associated trophoblast cells are insufficient.

The purpose of this research was to identify the key pathways and genes in PE. We downloaded the gene microarray datasets GSE93839 and GSE74341, and bioinformatics analysis was conducted. Differentially expressed genes (DEGs) were screened between severe PE invasive cytotrophoblasts and normal tissues, which were selected for functional and pathway enrichment analyses. We constructed a protein-protein interaction (PPI) network and further module analysis based on DEGs. Our results provide further insights into the development of PE at the molecular level.

Materials and methods

Gene expression profiles

Gene expression profile GSE93839 was downloaded from the National Center of Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/). The array data of GSE93839 included three samples of invasive cytotrophoblasts with severe PE and four samples of noninfected preterm birth invasive cytotrophoblasts.

Identification of DEGs

We used GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) for background correction, quantile normalization, and log2 transformation of the raw data. Affymetrix probe IDs were converted to the official gene symbol. The threshold was $P<0.05$ and $|\log 2\ FC| > 0.58$, which means that gene expression quantity in PE samples changed >1.5 fold compared with normal samples. The heat maps of the DEGs were constructed using the Morpheus online tool (https://software.broadinstitute.org/morpheus/).

Functional and pathway enrichment analyses

Three independent GO ontologies (biological process, molecular function, and cellular component) and systematic pathway analysis of gene functions were processed using the Database for Annotation, Visualization, and Integrated Discovery (http://david.abcc.ncifcrf.gov/home.jsp) [18]. Biological function and related gene pathways were further analyzed with a threshold of $P<0.05$.

Construction of PPI network and module analysis

In this study, the PPI network based on DEGs was constructed by the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org/cgi/) with the cutoff criterion of the confidence score >0.7. The PPI network was visualized by Cytoscape. We then calculated the degree of the nodes in the net-

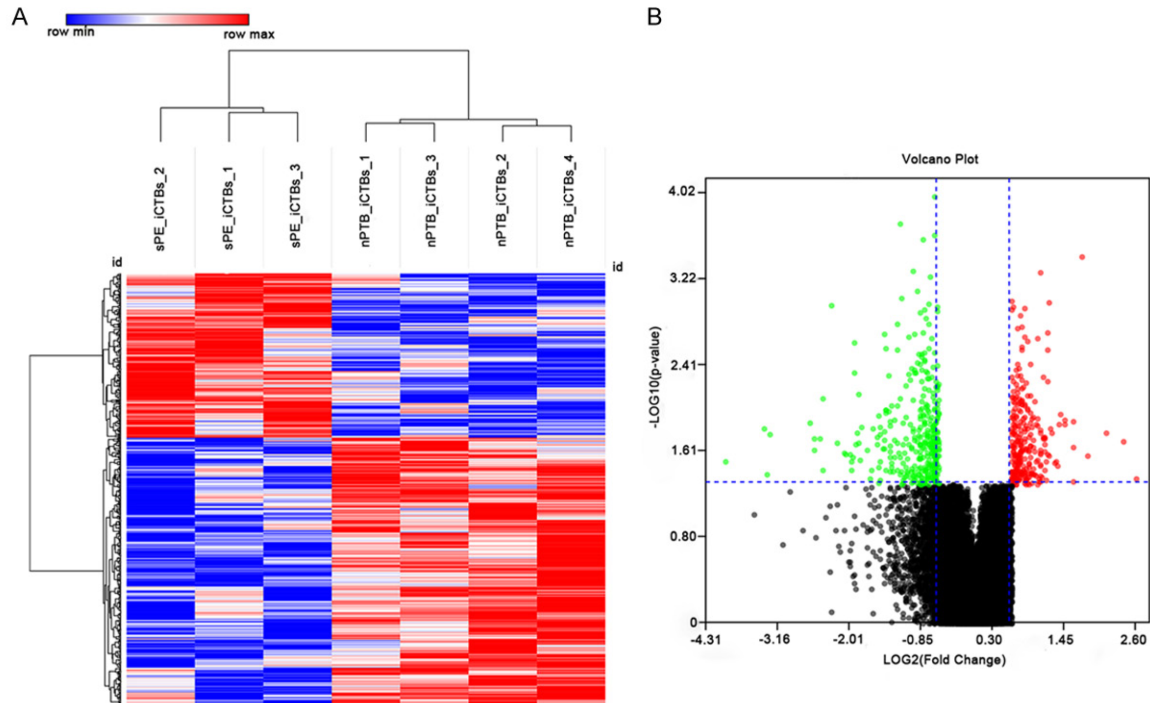

Figure 1. Heat map and volcano plot of gene expression in PE-associated trophoblasts and control trophoblast cells. Rows of heat map (A) indicate the expression levels of genes, and columns indicate different samples. Dysregulated expressed genes in volcano plot (B). Red, upregulated genes; green, downregulated genes.

work by the MCODE algorithm to screen the hub genes of the PPI network.

External valid dataset

The GSE74341 dataset from the GEO database was used as an external validation of hub genes identified in GSE93839 at the placenta level. GSE74341 consists of five normal placenta samples at term, five preterm normal placenta tissues, seven EOPE placenta tissues, and eight LOPE placenta samples.

Protein expression data

Immunohistochemistry images were obtained from Human Protein Atlas (http://www.proteinatlas.org) to confirm the protein expression of trophoblast cells in the placenta.

Statistics analysis

Statistical analyses were performed using GraphPad Prism 6. Statistical significance between groups with or without PE was presented using Student's t-test or one-way ANOVA. P<0.05 was considered statistically significant.

Results

Screening of DEGs in PE

By using GEO2R, a total of 472 DEGs were identified from GSE93839, including 292 downregulated ones and 180 upregulated ones. Hierarchical cluster analysis clearly showed the expression levels of the DEGs between the PE and normal samples (**Figure 1A**). The volcano plot showed the significant distinction of DEG expression between the PE and normal samples (**Figure 1B**).

GO term enrichment analysis

We uploaded both upregulated and downregulated DEGs to publicly available DAVID for the enrichment of GO categories and KEGG pathways (**Table 1**). In the biological process (BP), DEGs were associated with female pregnancy, embryonic neurocranium morphogenesis, and estrogen biosynthetic process. In cell component (CC) ontology, DEGs were enriched in P granule, lysosomal membrane, and extracellular region. In molecular function (MF) analysis, DEGs were significantly enriched in gamma-tubulin bind-

Table 1. Functional and pathway analyses

	Term	*P*-value	Genes
BP	GO:0007565~female pregnancy	0.001098606	LNPEP, INSL4, PSG6, STAT5B, CRH, PSG3, PSG1, FOSB, PSG11
	GO:0048702~embryonic neurocranium morphogenesis	0.013799241	TULP3, NDST1, SPEF2
	GO:0006390~transcription from mitochondrial promoter	0.021506034	TFAM, SLC25A33, MTERF3
	GO:0006703~estrogen biosynthetic process	0.025885458	HSD17B14, HSD17B1, CYP19A1
	GO:0019228~neuronal action potential	0.026677515	CATSPER4, ANK3, CHRNA1, KCNMB2
	GO:0006164~purine nucleotide biosynthetic process	0.030591153	ADSSL1, ADSL, HPRT1
	GO:0007005~mitochondrion organization	0.033687816	MAN2A1, RAB3A, TFAM, P2RX7, SLC25A33, TFB1M
	GO:0007420~brain development	0.034056053	ATP2B1, IMMP2L, SHROOM2, TULP3, ARNT2, TGFBR2, BRCA2, ABCB6, CLN5, NEURL1
	GO:0048147~negative regulation of fibroblast proliferation	0.034790522	CD300A, DACH1, PMAIP1, FTH1
	GO:0006878~cellular copper ion homeostasis	0.035605632	MT2A, SLC31A1, SCO1
	GO:0006694~steroid biosynthetic process	0.03774639	HSD17B2, HSD17B1, NR0B1, CYP19A1
	GO:0002755~MyD88-dependent toll-like receptor signaling pathway	0.040826324	IRAK4, IRAK2, LY96, TRAF6
	GO:0007585~respiratory gaseous exchange	0.044029057	MAN2A1, FUT8, NDST1, DACH1
	GO:0060434~bronchus morphogenesis	0.046028963	TULP3, TGFBR2
	GO:0090288~negative regulation of cellular response to growth factor stimulus	0.046028963	CASK, SLIT2
	GO:0031167~rRNA methylation	0.046494144	HENMT1, NSUN3, TFB1M
	GO:2000679~positive regulation of transcription regulatory region DNA binding	0.046494144	KAT2A, TRAF6, TWIST1
	GO:0034587~piRNA metabolic process	0.046494144	HENMT1, PIWIL2, PIWIL4
	GO:0034162~toll-like receptor 9 signaling pathway	0.046494144	IRAK4, IRAK2, TRAF6
CC	GO:0043186~P granule	0.035250669	HENMT1, PIWIL2, PIWIL4
MF	GO:0043015~gamma-tubulin binding	0.017873023	CEP57L1, BRCA2, NDRG1, PIFO
	GO:0004622~lysophospholipase activity	0.019965261	PLA2G4A, LGALS13, ABHD12, GDPD1
	GO:0004303~estradiol 17-beta-dehydrogenase activity	0.021704728	HSD17B2, HSD17B14, HSD17B1
	GO:0042803~protein homodimerization activity	0.02250453	GGCT, HELT, TYW5, HPRT1, SDIM1, ANXA6, APOE, HSF2, PKD2, CEACAM5, BHLHE40, CAT, TWIST1, IRAK2, ODC1, ADSSL1, PDXK, STC2, MSH3, LDB1, NR0B1, SLIT2, ABCG2, P2RX7, NLGN4X, KIF20B, CD200
	GO:0005179~hormone activity	0.022968331	GH2, CSHL1, CGA, INSL4, STC2, CRH, C1QTNF9
	GO:0070888~E-box binding	0.044566509	BHLHE40, ARNTL2, AHR, TWIST1
	GO:0004386~helicase activity	0.049088773	MCM9, FANCM, HELZ2, DDX10, ZRANB3, DDX52
KEGG	hsa04913:Ovarian steroidogenesis	0.007802996	ADCY3, CGA, PLA2G4A, HSD17B2, HSD17B1, CYP19A1
	hsa01100:Metabolic pathways	0.014915859	LDHB, GALNT1, FUT8, HSD17B2, NDST1, HSD17B1, PIP5K1B, KMO, HPRT1, ASAH1, CMPK2, CDS2, PIGK, AGPS, PIGB, FUT1, ACAD8, SPAM1, GCNT1, BPNT1, AGL, ACSL5, FH, CYP19A1, ODC1, ADSSL1, PDXK, MSMO1, NDUFA4L2, BCKDHB, AK6, PIGN, MAN2A1, ATP6V1C2, PLA2G4A, PYCR2, PLA2G7, PON2, ADSL, GPT, MAT2B, UROC1, APIP, SMPD4
	hsa01130:Biosynthesis of antibiotics	0.019210672	ODC1, LDHB, PYCR2, MSMO1, BCKDHB, AK3, PLA2G7, ADSL, CAT, AK6, BPNT1, FH
	hsa04978:Mineral absorption	0.028916631	VDR, MT2A, SLC31A1, FTH1, MT1G
	hsa05133:Pertussis	0.0417932	IRAK4, LY96, CXCL8, CALML5, TRAF6, C1QC

Figure 2. Protein-protein interaction network and hub gene selection of differentially expressed genes in preeclampsia-related trophoblast cells. Size of node indicates the number of interaction. Size of nodes is determined by the degree of interaction.

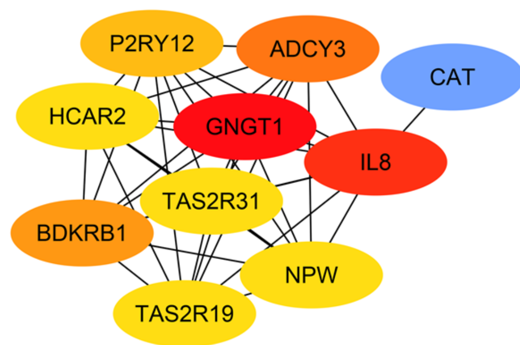

Figure 3. Hub gene selection of differentially expressed genes in preeclampsia-related trophoblasts cells.

ing, lysophospholipase activity, and estradiol 17-beta-dehydrogenase activity. KEGG pathway analysis showed that DEGs were mainly enriched in ovarian steroidogenesis, metabolic pathways, and biosynthesis of antibiotics.

PPI network construction and hub gene selection

The PPI network with 157 nodes and 286 edges based on DEGs was identified by the STRING database (**Figure 2**). Ten hub genes with high degrees were selected from DEGs (**Figure 3**), which indicated the strong

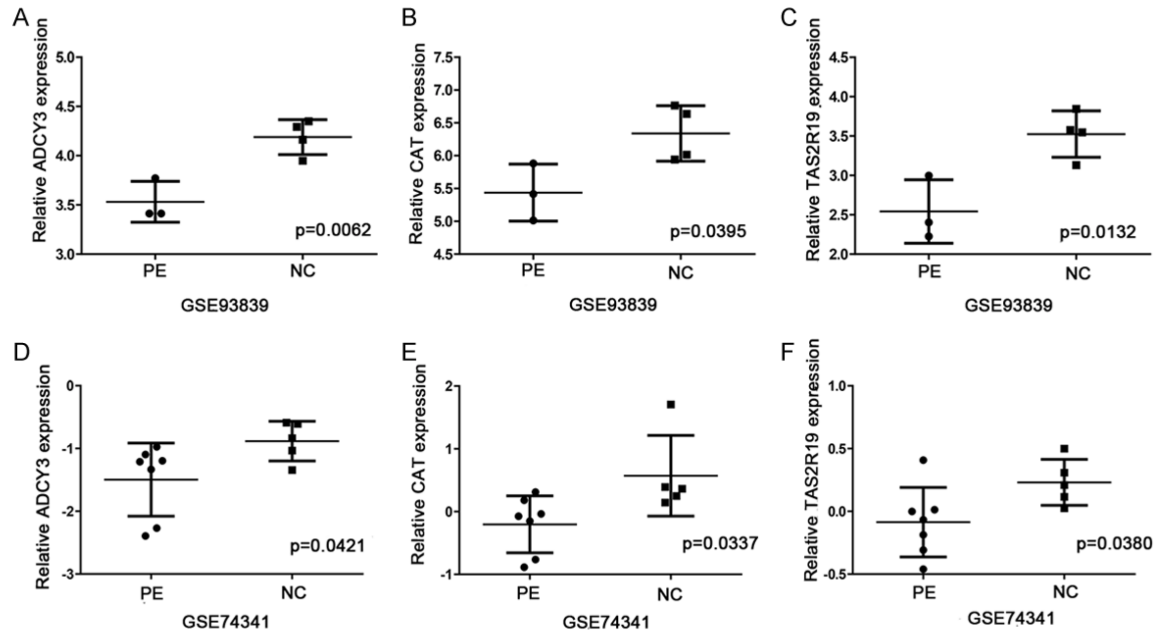

Figure 4. Relative mRNA expression level of three hub genes in GSE93839 and GSE74341. A: mRNA expression of ADCY3 in GSE93839; B: mRNA expression of CAT in GSE93839; C: mRNA expression of TAS2R19 in GSE93839; D: mRNA expression of ADCY3 in GSE74341; E: mRNA expression of CAT in GSE74341; F: mRNA expression of TAS2R19 in GSE74341.

interaction between the hub genes and other proteins. In the network, GNGT1 showed 23 node degrees, which was the highest.

Validation of the trophoblastic cell hub gene expression

To validate the gene expression level of trophoblastic cell signature resulting from microarray analyses, we selected five preterm and seven EOPE placenta tissues from GSE74341. As shown in **Figure 4**, the expression levels of ADCY3, CAT, and TAS2R19 detected in the placentas were consistent with those detected in the trophoblastic cells, whereas BDKRB1 and NPW demonstrated opposite expression levels between the placentas and trophoblastic cells (**Figure 5**). The other five genes showed similar expression patterns in both datasets, but no statistical difference in EOPE placentas was found compared with normal controls.

Protein expression of trophoblastic cell genes

To evaluate the protein expression level of the identified and validated genes in trophoblastic cells, immunohistochemistry data were downloaded from the Human Protein Atlas (http://www.proteinatlas.org/). All the proteins, including ADCY3, CAT, and TAS2R19, detected in the trophoblast cell genes and further validated by GSE74341, were highly expressed in normal placenta tissues, especially in trophoblastic cells (**Figure 6**).

Discussion

PE, a common complication during pregnancy, significantly influences the maternal-fetal outcome. The main pathological manifestations of PE are shallow trophoblastic invasion into the endometrium and remolding of uterine spiral arteries. Understanding PE at the etiological and molecular levels is crucial to improve its prevention and treatment. With the rapid advance of microarray technologies, we employed integrated bioinformatics methods to explore the potential targets for the diagnosis, therapy, and prognosis of PE.

In this study, the GEO dataset GSE93839 with three severe PE invasive cytotrophoblasts and four noninfected preterm birth invasive cytotrophoblasts was analyzed. A total of 472 DEGs were identified between PE and normal invasive cytotrophoblast cells, including 292 upregulated genes and 180 downregulated genes. To determine the biological meaning behind these

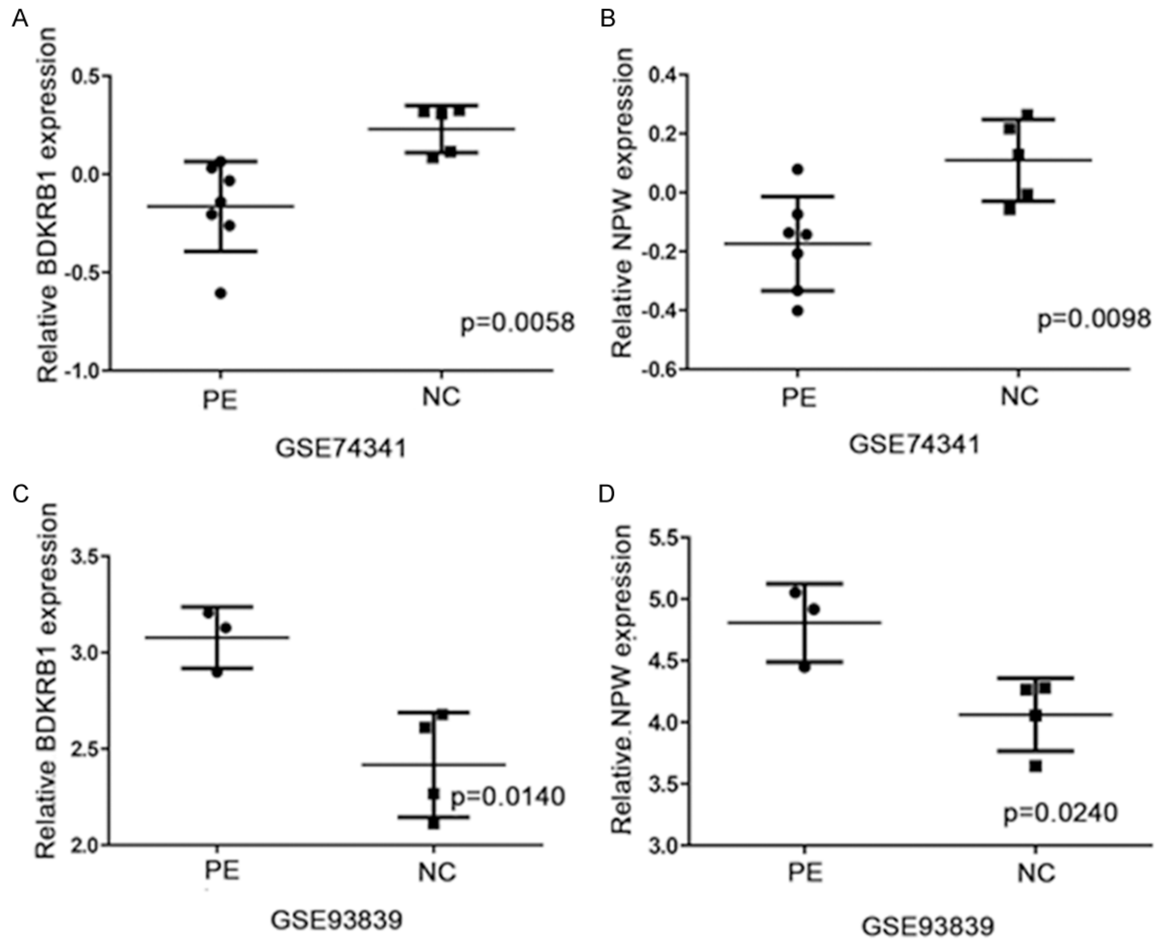

Figure 5. Differently mRNA expression level of two hub genes in GSE93839 and GSE74341. A: mRNA expression of BDKRB1 in GSE93839; B: mRNA expression of NPW in GSE93839; C: mRNA expression of BDKNP1 in GSE93839; D: mRNA expression of NPW in GSE74341.

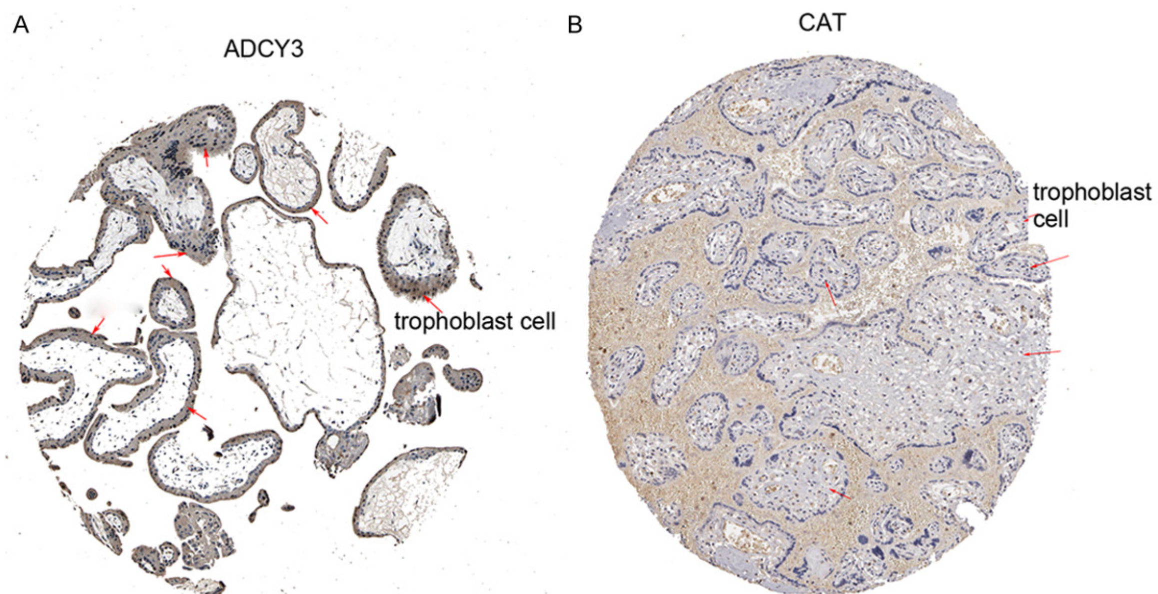

Figure 6. Immunohistochemistry validation of proteins for hub genes using the Human Protein Atlas database. A: Protein of ADCY3 in human trophoblast cells. B: Protein of CAT in human trophoblast cells.

DEGs, we conducted functional enrichment of GO analysis and KEGG pathway analysis. GO term enrichment analysis showed that nine DEGs were enriched in female pregnancy and three DEGs were enriched in embryonic neurocranium morphogenesis. KEGG pathway analysis showed that six DEGs were significantly enriched in ovarian steroidogenesis, and 44 DEGs were enriched in metabolic pathways. Moreover, DEGs with high node degrees were obtained from the PPI network: ADCY3, IL8, BDKRB1, CAT, GNGT1, NPW, HACR2, P2RY12, TAS2R19, and TAS2R31 were identified as hub nodes. The expression levels of three hub genes, namely, ADCY3, CAT, and TAS2R19, were further verified in EOPE placentas, which showed a high liability of microarray data. However, the BDKRB1 and NPW genes exhibited reverse mRNA expression patterns in GSE93839 and GSE74341. The protein expression levels of ADCY3, CAT, and TAS2R19 were confirmed to be specifically expressed in trophoblastic cells of the placenta. As the most significant hub gene, CAT is a key antioxidant enzyme defense against oxidative stress (OS) in the body. It was proven to play a critical role in the pathogenesis of PE. The pathogenesis of PE can be divided into two stages characterized by generalized endothelial dysfunction. The first stage is poor trophoblast invasion during which spiral artery remodeling occurs in the placentation process, causing transformation failure of the placental bed arteries from high resistance vessels to low resistance vessels. One important characteristic of this stage is the abnormal proliferation and apoptosis of placental trophoblast cells. In C28/I2 chondrocytes, CAT exerts powerful anti-TNF-α-induced apoptosis effects by interfering with the TNF/TNFR-mediated extrinsic apoptosis pathway [19]. Recent studies indicated that catalase (CAT) reverses the concentration-dependent decrease in cancer cell invasion and migration [20]. This phenomenon is also observed in prostate cancer (PCa); overexpression of catalase in tumor microenvironment reverses the inhibitory effect of extracellular superoxide dismutase on cancer cell invasion [21]. The downregulation of CAT in trophoblast cells may lead to abnormal proliferation, apoptosis, and invasion during the first stage. Poor trophoblast invasion leads to local ischemia, reperfusion damage, and OS. The second stage is inflammation response, endothelial activation, systemic OS, and disturbed production of angiogenic and anti-angiogenic factors. Previous studies have reported reduced glutathione, superoxide dismutase, and CAT [21, 22] in PE compared with normal pregnant women, and the reduced level of placental CAT may result in increasing amounts of reactive oxygen species in the placenta that can contribute to the pathogenesis of PE [23].

In addition, ADCY3 is a membrane-associated protein involved in catalyzing the formation of cyclic adenosine-3',5'-monophosphate (cAMP). Recent studies have revealed that overexpression of ADCY3 stimulates HEK293 cell migration, invasion, proliferation, and clonogenicity; conversely, downregulation of ADCY3 reduces these phenotypes in SNU-216 cells [24, 25]. Our study found that ADCY3 was downregulated in trophoblast cells and the placenta of patients with PE, and cAMP expression in PE placentas was lower than that in controls [26]. Moreover, cAMP has been described as a second messenger highly regulating the modulation of human first-trimester trophoblast functions in multiple ways, such as inducing differentiation of cytotrophoblast to both syncytiotrophoblast and extravillous trophoblast (EVT) [27, 28] and regulating the secretory activity of syncytiotrophoblast, as well as the proliferation, migration, and invasiveness of EVT cells in multiple pathways [29]. The production of cAMP in human placental multipotent mesenchymal stromal cell increases MMP9 expression and activates protein kinase A (PKA)-Rap1-integrin β1 pathways in trophoblasts, leading to the elevation of trophoblast cell migration [30]. And cyclic AMP (cAMP)-dependent pathway enhanced trophoblast differentiation by regulating the expression and post-translational modifications activity of Glial cells missing Drosophila homolog, a the GCM transcription factor [31].

Conclusions

In conclusion, we screened the critical genes and pathways participating in PE initiation and progression from trophoblast cell and placenta samples. We identified ADCY3 and CAT as key genes and the ovarian steroidogenesis, metabolic pathways and biosynthesis of antibiotics pathways as the key pathways involved in PE. Our results provide new insights into the development of PE at molecular level.

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Disclosure of conflict of interest

None.

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