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Original Research

A Potential Autophagy-Related-Gene Based Signature in Patients with Preeclampsia

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Abstract

Objective: Preeclampsia (PE) is a significant cause of maternal and offspring mortality and morbidity. The purpose of this study is to identify the potential diagnostic signatures of autophagy-related genes (ATGs) in pregnancies with preeclampsia. **Methods**: The expression profile of mRNA was obtained from GSE75010 (placenta samples) and GSE48424 dataset (blood samples). The potential differentially expressed ATGs of PE were screened by R software. The gene-ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, correlation analysis, and protein-protein interactions (PPI) were applied for the differentially expressed ATGs. The diagnostic markers of PE were then screened based on least absolute shrinkage and selection operator (LASSO) logistic regression and support vector machine-recursive feature elimination (SVM-RFE). Receiver operating characteristic (ROC) analysis was used to investigate the predictive value of these diagnostic markers. Target miRNAs were predicted based on the miRDB, DIANA-micro T, Targetscan, and miRWalk databases, and were further validated in GSE84260. **Results**: A total of 20 differentially expressed ATGs were identified between PE and healthy pregnancies. Functional analysis of differentially expressed ATGs indicated several enriched terms related to autophagy, apoptosis, angiogenesis, inflammation, immune response, hypoxia-inducible factor 1 (HIF-1), forkhead box O (FoxO) and AMP-activated protein kinase (AMPK) signaling pathway. A total of 12 ATGs were recognized based on LASSO and SVM-RFE, which made an excellent distinction in both the placenta tissues (area under the curve [AUC] = 0.903) and the blood samples (AUC = 0.972). Furthermore, four feature ATGs (leptin [*LEP*], ERO1-like [*ERO1L*], phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta [*PIK3CB*], and mitogen-activated protein kinase 8 [*MAPK8*]) were screened and also shown an excellent diagnostic efficacy (AUC = 0.869 in placenta samples, and AUC = 0.914 in blood samples). Additionally, 81 target miRNAs were predicted according to the 4 feature ATGs. After evaluating the miRNA expression pattern of GSE84260, 11 miRNAs were selected. Finally, a miRNA-mRNA regulatory network was constructed, which may participate in the development of PE. **Conclusions**: We established an autophagy-related-gene based signature that may predict pregnancies with PE. And we also constructed a miRNA-mRNA regulatory network, which may deepen our understanding of the molecular mechanism underlying the development of PE.

Keywords: preeclampsia; autophagy; diagnosis; miRNA-mRNA network; bioinformatics analysis

1. Instruction

Preeclampsia (PE) is diagnosed by *de novo* onset of hypertension with blood pressure over 140/90 mmHg and substantial proteinuria of ≥300 mg in 24 hours at or after 20 weeks of gestation and affects 2% to 8% of pregnancies [1–3]. PE is associated with maternal and offspring morbidity, including fetal growth restriction, preterm birth, oligohydramnios, and maternal end-organ damage [4]. Besides, it is the second leading cause of maternal mortality just behind maternal hemorrhage. This pregnancy complication presents a major risk factor for other disorders, such as diabetes, cardiovascular disease, and renal complications [5–7]. Although the genuine etiology of PE still remains to be clarified, it is widely accepted that dysregulation of angiogenesis and trophoblast apoptosis is the major contribution to PE [8,9]. After implantation, the extravillous trophoblast (EVT) cells migrate into the decidua and then remodel uterine arteries by replacing the vascular endothelial and muscle cells. The impaired invasion of EVT cells could affect angiogenesis and disturb the remodeling of the maternal myometrial spiral artery, then inducing excessive pregnancy-incompatible factors production, such as inflammatory cytokines and anti-angiogenic factors [10,11]. The presence of inflammation can aggravate trophoblast apoptosis which can further disrupt cell migration and placental vascularization [12,13].

Autophagy, a common phenomenon in eukaryotic cells, is an important metabolic process for cytoplasmic proteins and organelles degrading into fatty acids and amino acids [14]. Previous studies have shown that autophagy is relevant to various diseases, including respiratory diseases, malignancies, and hypertension in pregnancy [15–17]. Some studies suggested that autophagy protects the placenta against pathogens and stress, and has physiologi-

cal functions for maintaining a normal pregnancy [18,19]. Hyperactivated autophagy induced by oxidative stress may affect cell invasion and placental vascularization, and then promote the development of PE [20]. Other studies demonstrated that under oxidative stress conditions, autophagy could improve cell survival and reduce cell apoptosis [21]. Thus, exploring the roles of autophagy in PE may help clinicians better understand the etiology of PE and seek for early diagnostic and prognostic markers. At present, over 30 kinds of autophagy-related genes (ATGs) are demonstrated to be closely related to autophagy. For instance, it has been shown that trophoblast-specific conditional autophagy related (Atg)7 knockout mice exhibited a significant elevation in blood pressure, a smaller placenta and reduced expression of placental growth factor [22]. However, the role of ATGs in PE has not been fully understood.

Using published gene expression data from the Gene Expression Omnibus (GEO) database, differentially expressed ATGs were obtained in PE and healthy samples. The least absolute shrinkage and selection operator (LASSO) logistic regression and support vector machine-recursive feature elimination (SVM-FRE) algorithm were applied to select the feature genes among ATGs. Then, a predictive model was constructed based on feature genes, which could efficiently distinguish PE patients from the healthy pregnancies, and may be used for early diagnosis of hypertension in pregnancy.

2. Methods

2.1 Microarray Data Acquisition

The key word "preeclampsia" was used to search gene expression profiles of PE patients in the GEO database (https://www.ncbi.nlm.nih.gov/geo) [23]. Three GEO datasets were selected based on the following criteria: (1) The gene expression profiling contained cases and controls. (2) The number of samples should not less than 10 in each group. (3) Raw data or the processed data were provided. The gene expression dataset GSE75010, deposited by Leavey *et al.* [24], was conducted in placenta tissues from 80 PE patients and 77 healthy pregnancies and was used to perform the differential expression analysis and the other analyses. The GSE48424 dataset, provided by Textoris *et al.* [25], was conducted in blood samples from 18 PE patients and 18 controls. The miRNA expression dataset GSE84260 included 16 PE and 16 healthy placentas. The mRNA and miRNA expressions were presented by log2 conversions.

2.2 Identification of Autophagy-Related Messenger RNAs in Preeclampsia

718 ATGs were acquired from the Human Autophagy Database (HADb) (http://www.autophagy.lu/index.html) and the Molecular Signatures Database of Gene Set Enrichment Analysis (GSEA) (http://www.gsea-msigdb.org/gsea/index.jsp). 686 ATGs mRNAs were screened out by mRNAs of the training cohort GSE75010 intersected with the autophagy-related encoding gene list.

2.3 Differentially Expressed Analysis of ATGs

The "limma" package in R software (Version 3.6.3) was applied to identify the differentially expressed genes (DEGs) and differentially expressed miRNAs (DEmiRNAs). The p value < 0.05 and absolute fold-change (FC) >1.2 were considered criteria for DEGs and DEmiRNAs. The online software Venn (http://bioinformatics.psb.ugent.be/webtools/Venn) was used to identify the overlapping genes or miRNAs [26]. The heatmap and boxplot were conducted using "heatmap" and "ggplot2" packages of R software. t-distributed stochastic neighbor embedding (t-SNE) was applied to figure out the distribution of differentially ATGs between PE and controls by using "Rtsne" package in R [27].

2.4 Functional Enrichment Analysis of the Differentially Expressed ATGs

Gene ontology (GO) annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed to characterize the functions and pathways of the differentially expressed ATGs, which were conducted by using "clusterProfiler" package in R software [28,29]. The enriched GO terms and KEGG pathways were selected with the criterion of p value < 0.05.

2.5 Construction of PPI Network and Correlation Analysis of the Differentially Expressed ATGs

The protein-protein interaction (PPI) analysis of the differentially expressed ATGs was conducted using STRING (https://string-db.org/, version 11.5) and Cytoscape software (https://cytoscape.org, version 3.9.0, GitHub Inc, San Francisco, USA) [30,31]. Spearman correlation in the "corrplot" package was used to analyze the correlation of the differentially expressed ATGs.

2.6 Screening and Verification of Potential Diagnostic Markers

The least absolute shrinkage and selection operator (LASSO) logistic regression and support vector machine-recursive feature elimination (SVM-RFE) were applied to search for best parameters for gene selection among the differentially expressed ATGs [32,33]. The LASSO algorithm was conducted in "glmnet" package and SVM-RFE was established by "e1071" package of R software. Subsequently, we combined the genes from either LASSO or SVM-RFE for further analysis. The efficacy of genes in distinguishing PE from healthy pregnancies was measured via the area under the curve (AUC).

2.7 Prediction and Validation of Target miRNA

Target miRNAs were determined by using online miRNA databases miRDB, DIANA-micro T, Targetscan, and miRWalk [34–37]. Meanwhile, GSE84260 was used

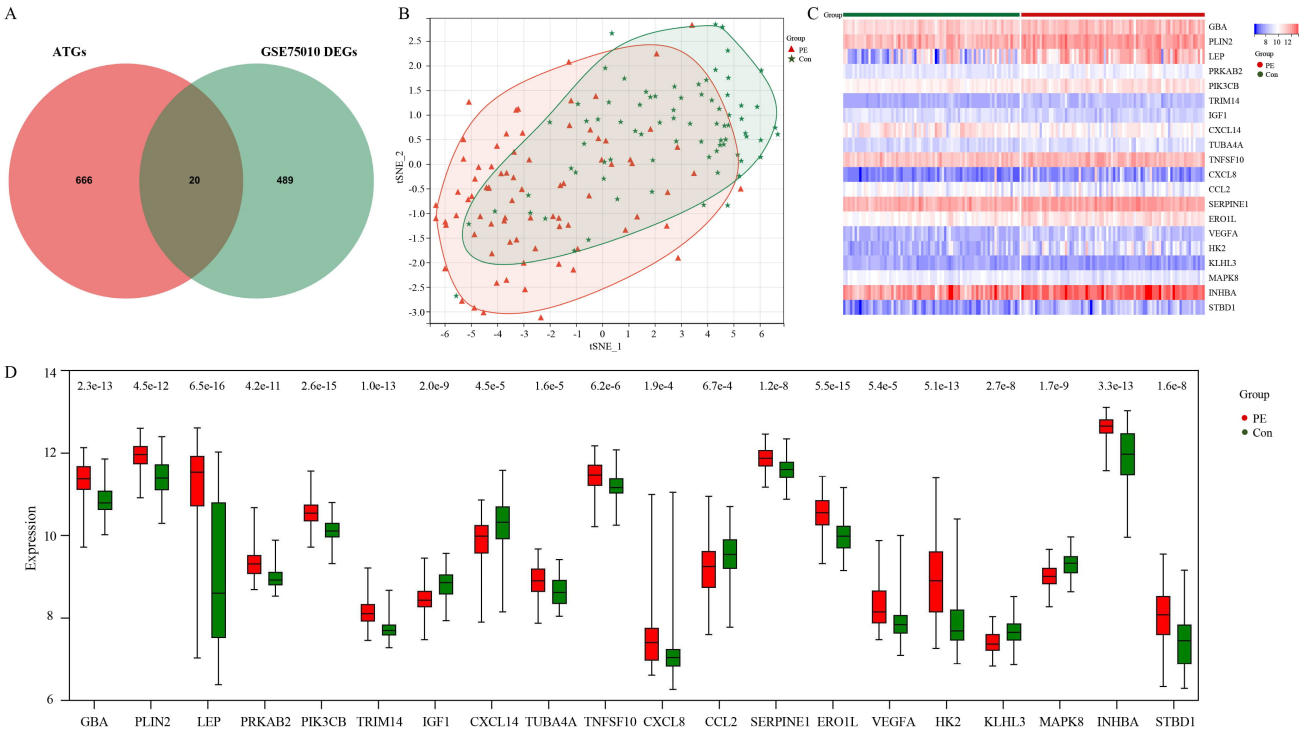

Fig. 1. Construction of differentially autophagy-related genes (ATGs) signature in preeclampsia (PE). (A) Venn of overlapping ATGs between well-known ATGs and differentially expressed genes (DEGs) in GSE75010 dataset. (B) t-SNE of differentially expressed ATGs between PE and healthy samples. (C) Heatmap of the 20 differentially expressed ATGs in PE and healthy pregnancies. (D) Twenty differentially expressed ATGs were shown with median expression levels.

to identify the DEmiRNAs between PE patients and the healthy pregnancies. The overlapping miRNAs, which were acquired among above four miRNA databases and verified in GSE84260 dataset, were considered as target miRNAs.

3. Results

3.1 Construction and Definition of the ATGs Signature in Preeclampsia

R software was used to extract a total of 509 DEGs from GSE75010 dataset with the criteria of p value < 0.05 and FC >1.2 after data preprocessing (**Supplementary Table 1**). Via using online tool Venn, 20 differentially expressed ATGs were identified (Fig. 1A), including 15 up-regulated genes and 5 down-regulated genes. tSNE was used to figure out the distribution of these differentially expressed ATGs between PE and controls graphically (Fig. 1B). Following the analysis of GSE75010 dataset, the 20 differentially expressed ATGs between PE and controls were presented in heatmap and boxplot (Fig. 1C,D).

3.2 Functional Analysis and Correlation Analysis of the Differentially Expressed ATGs

To explore the potential functions of these 20 differentially expressed ATGs, GO and KEGG enrichment analysis was carried out by using R software (**Supplementary Table 2**). As shown in Fig. 2A, the top 10 significantly enriched terms of GO biological process included process utilizing autophagic mechanism, leukocyte migration, regulation of leukocyte chemotaxis, apoptotic process, regulation of autophagy, anatomical structure formation involved in morphogenesis, regulation of vasculature development, regulation of immune system process, tube morphogenesis, and regulation of cell death. In the KEGG pathway analysis, these ATGs were mostly involved in AGE-RAGE signaling pathway in diabetic complications, HIF-1 signaling pathway, Chagas disease, FoxO signaling pathway, Non-alcoholic fatty liver disease, viral protein interaction with cytokine and cytokine receptor, cytokine-cytokine receptor interaction, AMPK signaling pathway, Yersinia infection, and typer II diabetes mellitus (Fig. 2B). To determine the interactions among differentially expressed ATGs, PPI analysis was performed. Except for *TRIM14* (tripartite motif containing 14), *KLHL3* (kelch-like family member 3), and *TUBA4A* (tubulin alpha 4a), the other 17 ATGs interacted with each other (Fig. 2C). Furthermore, correlation analysis was performed to explore the expression correlation patterns of these differentially expressed ATGs. Fig. 2D revealed the relationship of the 20 ATGs in GSE75010 dataset.

3.3 Screening and Verification of Predictive ATGs in PE

We used LASSO to identify 13 genes from differentially expressed ATGs as diagnostic markers for PE

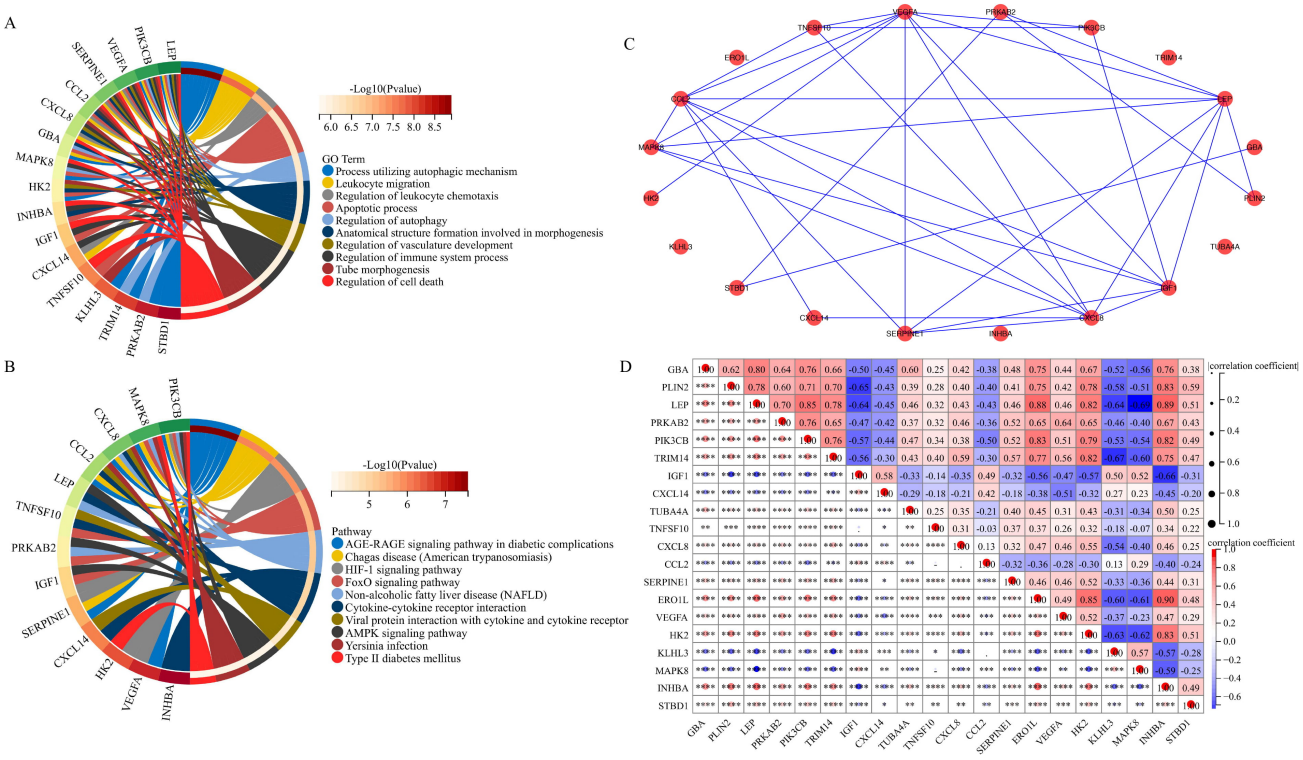

Fig. 2. Functional analysis of 20 differentially expressed ATGs in PE. (A) Circos plot of top 10 enriched GO biological process terms. (B) Circos plot of top 10 enriched KEGG terms. (C) The protein-protein interactions among 20 differentially expressed ATGs. (D) Spearman correlation analysis of the 20 differentially expressed ATGs.

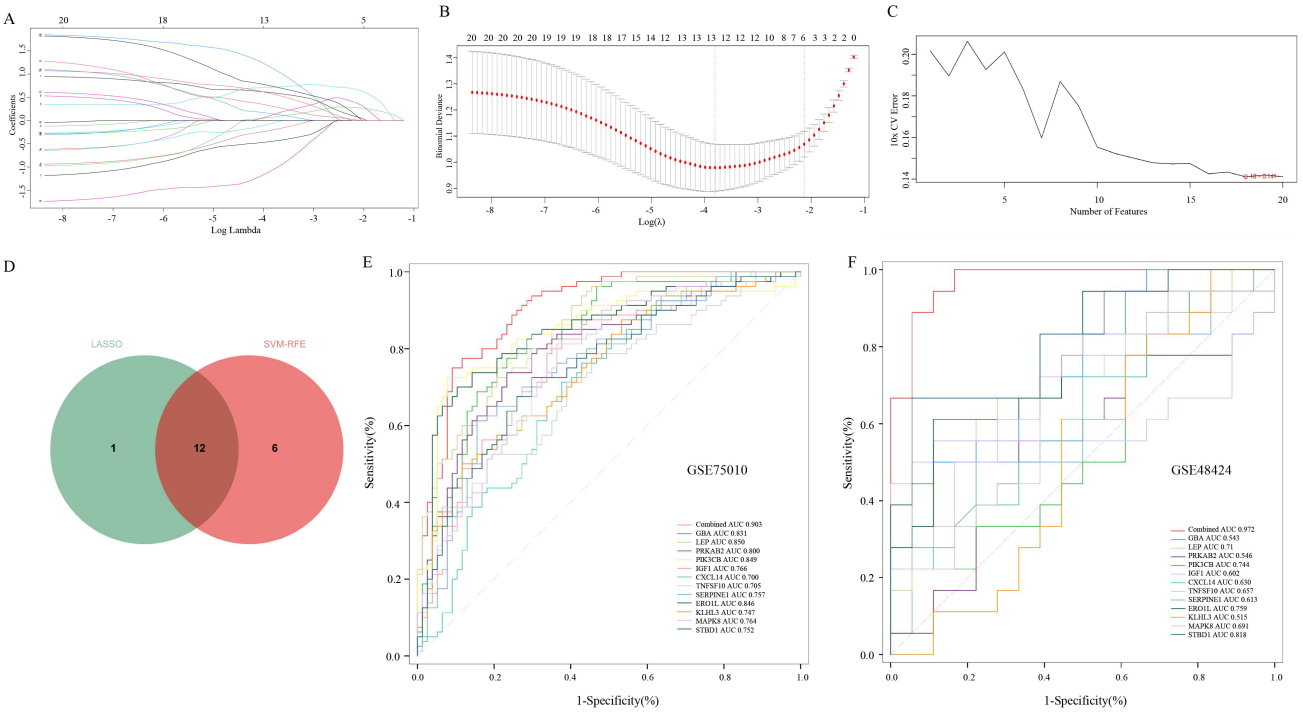

Fig. 3. Screening and verification of predictive markers. (A,B) Optimized lambda determined in LASSO regression model. 13 indexes were selected. (C) SVM-RFE was used to screeen diagnostic markers. (D) Venn shows the intersection of predictive markers obtained by the two algorithms. (E,F) The ROC curve of the dignostic efficacy verification after combining 12 predictive markers to one variable in GSE75010 dataset and GSE48424 dataset.

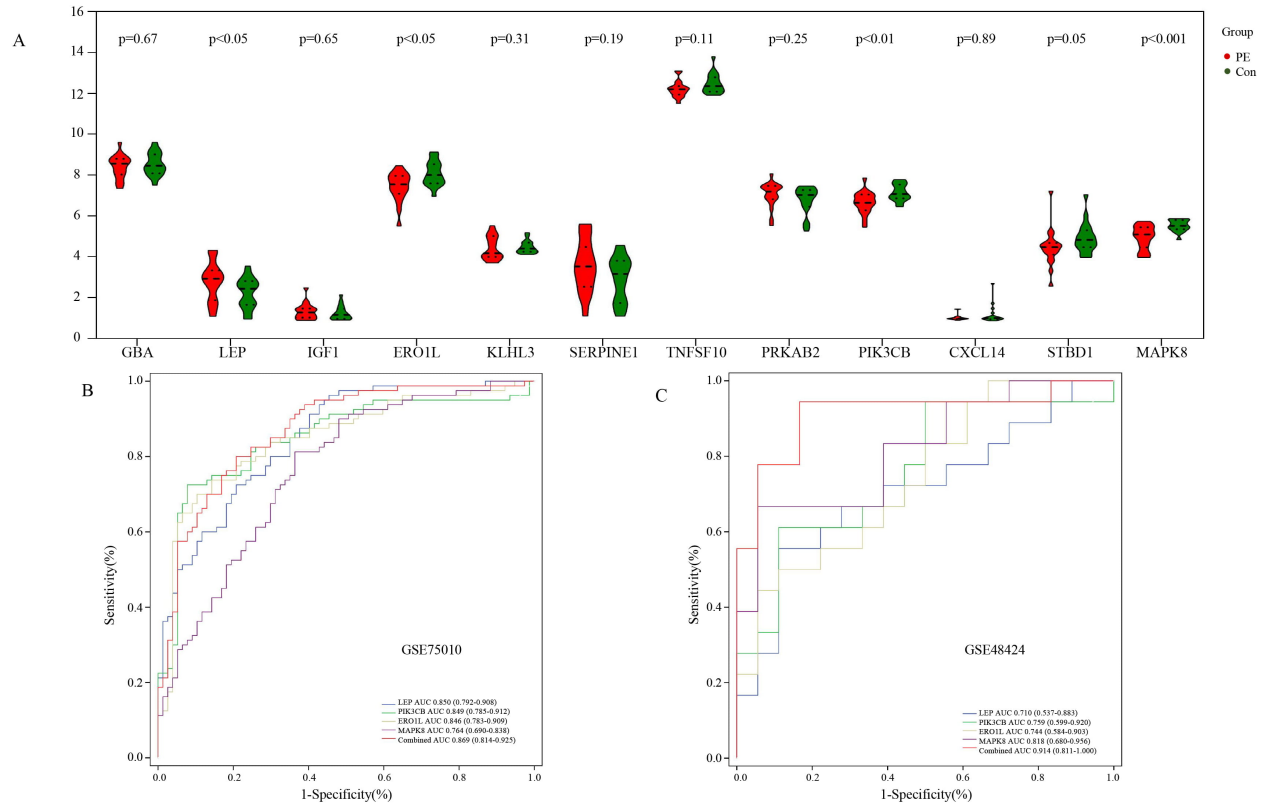

Fig. 4. Screening and verification of feature ATGs. (A) Twenty ATGs were shown with median expression levels in GSE48424 dataset, of which 4 ATGs were differentially expressed. (B,C) The ROC curve of the diagnostic efficacy of these 4 diagnostic markers in GSE75010 dataset and GSE48424 dataset.

(Fig. 3A,B). While 18 genes were determined as diagnostic markers using the SVM-RFE algorithm (Fig. 3C). The overlapping gene markers between the two algorithm were obtained using Venn, and 12 diagnostic related genes were finally selected for further analysis (Fig. 3D). When combining these 12 genes into one variable, a higher level of diagnostic efficiency was reached in dataset GSE75010 (AUC = 0.903) (Fig. 3E). Since placenta tissues are obtained invasively, we wonder whether the expression of ATGs in maternal peripheral blood can be used to predict preeclampsia. Thus, we evaluated the diagnostic efficiency of these 12 genes in dataset GSE48424, which contained blood samples from PE and healthy pregnancies. Results showed a high diagnostic value of ATGs in blood samples (AUC = 0.972) (Fig. 3F). We next tested the expression patterns of these 12 genes in maternal blood. Results showed that the expression of four genes differed significantly between PE and controls, including *LEP* (leptin), *ERO1L* (ERO1-like), *PIK3CB* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta), and *MAPK8* (mitogen-activated protein kinase 8) (Fig. 4A). In accordance with GSE75010, *LEP* and *MAPK8* showed same expression patterns both in GSE75010 and GSE48424. While the expression patterns of *ERO1L* and *PIK3CB* were quite contrary. This phenomena may be caused by different clinical samples. Despite the different expression patterns of these four genes, the logistic regression model based on *LEP*, *ERO1L*, *PIK3CB*, and *MAPK8* could also efficiently distinguish patients with PE from the healthy subjects either in placenta samples or in blood samples, with the AUC of 0.869 and 0.914, respectively (Fig. 4B,C). Moreover, this model demonstrated reliable diagnostic values not only in the unattainable placenta samples but also in the accessible blood samples.

3.4 Prediction and Validation of Targeted miRNA

Four online miRNA databases were used to predict the target miRNAs of four hub genes, including miRDB, DIANA-micro T, Targetscan, and miRWalk. A total of 81 overlapping miRNAs were identified after intersection of these four databases (Fig. 5A, **Supplementary Table 3**). The miRNA-mRNA regulatory network was then constructed using CytoHubba (Fig. 5B). DEmiRNAs in patients with preeclampsia were obtained by analyzing GSE84260 dataset. After intersection with the above 81 miRNAs, 11 miRNAs were identified (Fig. 5C). Finally, a co-expressed network on PE was established on the predicted miRNA-RNA pairs (Fig. 5D). Results showed that *ERO1L* was not a target gene of these 11 common miRNAs and three autophagy-related genes were all regulated by *hsa-miR-557* and *hsa-miR-936*.

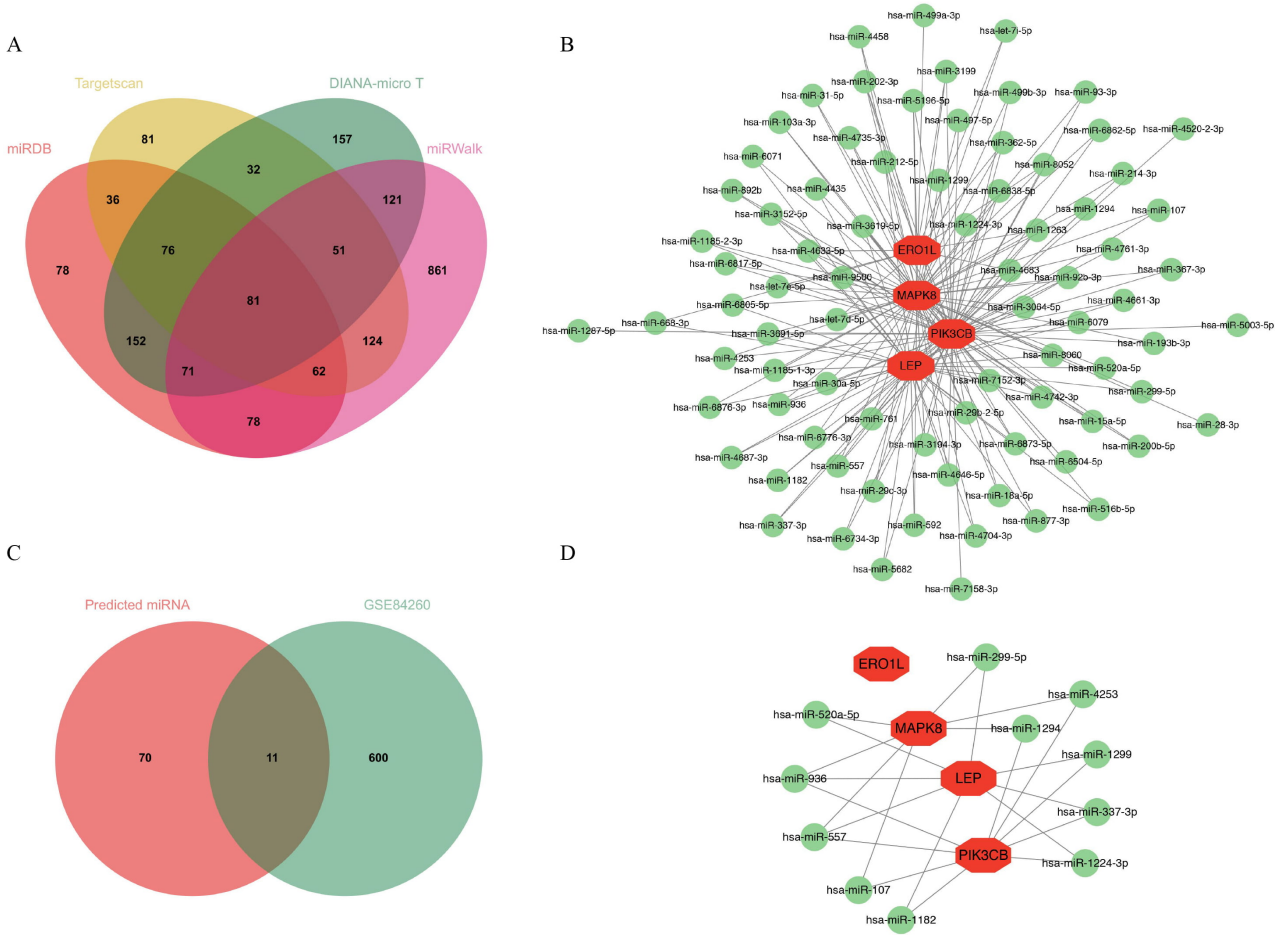

Fig. 5. Prediction and validation of target miRNAs. (A) Venn diagram presenting the intersection of predicted miRNAs based on the miRDB, DIANA-micro T, Targetscan, and miRWalk databases. (B) The miRNA-mRNA regulatory network showing relationship between 4 feature ATGs and 81 miRNAs. (C) Venn diagram showing the intersection between miRNAs and DEmiRNAs in PE pregnancies from the GSE84260 dataset. (D) The relationship between 4 feature ATGs and 11 validated miRNAs (miRNA-mRNA regulatory network).

4. Discussion

Preeclampsia is characteristic of placental shallow implantation and insufficient spiral artery recasting, which is considered to be of placental origin [38]. It remains a significant contributor of short- and long-term maternal and fetal morbidity. It is generally accepted that impaired angiogenesis and trophoblast apoptosis play important roles in the occurrence of PE. In recent decades, an association between autophagy and pregnancy has been demonstrated. Autophagy is the most fundamental phenomenon in eukaryotes, through which senescent or damaged structures are degraded to maintain microenvironment stability. Studies showed that autophagy can influence the trophoblast cell homeostasis via maintaining the reactive oxygen species (ROS) balance so to preserve the angiogenic capacity [39]. It is also reported that autophagy could preserve the function of trophoblast cells by regulating vascular endothelial growth factor A (VEGFA) and fms-related tyrosine kinase 1 (FLT1) expression and protecting against cell apoptosis at the maternal-fetal interface [17]. However excessive or impaired autophagy can result in pregnancy related disorders. Li *et al.* [17] found that excessive autophagic activity in trophoblasts or endothelial cells could affects trophoblast invasion and the placenta vasculature, thus participating in the development of preeclampsia. This suggests that to some extent the development of preeclampsia is regulated by the autophagic mechanism. To better understand the role of autophagy in PE, we used published gene expression data from GEO database to construct bioinformatics analysis of ATGs in PE.

In this study, 20 differentially expressed ATGs were obtained in placentas from women diagnosed with PE. Most of these autophagy genes were highly-expressed, indicating that hyperactivated autophagy may be dominant in the development of PE. Functional analysis indicated these genes were related to behaviors like autophagy, apoptosis, angiogenesis, inflammation, and immune response. The inflammatory response is a well-recognized contributor of PE and

imbalance in immune response may cause abnormalities in angiogenesis and placental structure [40]. Studies demonstrated that inflammation can further activate and promote trophoblast apoptosis and the activation of apoptosis can backfire on trophoblast through disrupting cell migration and placenta vasculature, indicating that autophagy damage may play a pivotal role in the progress of PE via inducing inflammation status and affecting angiogenesis and apoptosis. KEGG analysis showed that these genes may be involved in hypoxia-inducible factor 1 (HIF-1), forkhead box O (FoxO) and AMP-activated protein kinase (AMPK) signaling pathway. Severe or persistent hypoxia in PE placenta induces the overexpression of hypoxia-inducible factor 1-alpha (HIF-1α), which further causes an increase in soluble fms-like tyrosine kinase 1 (sFlt-1) and ultimately lead to placental dysfunction [41]. Studies also illustrated that hypoxia induces autophagy in a HIF-dependent induction of BCL2/adenovirus e1B 19 kDa protein interacting protein 3 (BNIP3) and BCL2/adenovirus e1B 19 kDa protein interacting protein 3-like (BNIP3L) [42]. AMPK, a crucial kinase regulating energy homeostasis, plays a pivotal role in promoting autophagy via regulating phosphorylated autophagy-related proteins in mTORC1, ULK1 or indirect proteins to stimulate the expression of autophagy transcription factors such as FoxOs [43,44]. Overall, these findings indicate that autophagy and ATGs may play a significant role in the develpoment of PE.

Since PE remains a serious threat to the health and life of the mother and the fetus, it is of vital importance for early diagnosis in order to expand the window of treatment and reduce complications. The predictive effect of well-known sreening indicators is not satisfactory, such as placental growth factor (PIGF) and soluble fms-like tyrosine kinase 1 (sFlt-1) [45]. Therefore, there is an urgent need for novel biomarkers with high specificity and sensitivity. To elucidate the sensitivity and specificity of the autophagy genes in predicting PE, 12 feature genes were screened out and presented a remarkable predictive efficiency of PE and normal pregnancies. Considering the difficulties in accessing placenta samples during pregnancy, we assessed the discrimination power of these selected feature genes in maternal blood samples. Results shown that feature ATGs also succeeded in separating patients with PE from the healthy subjects. Ultimately, four ATGs, *LEP*, *ERO1L*, *MAPK8*, and *PIK3CB*, were selected as hub diagnostic genes of ATG-based signature, which were both differentially expressed in placenta and blood. Upregulation of leptin (*LEP*) was detected in placenta samples from PE patients when compared with those from normal pregnancy and depletion of *LEP* could repress apoptosis and promote proliferation, migration, and invasive capacity [46]. The protein product leptin of *LEP* was also increased in the serum of PE patients in comparison to those with normotensive pregnancies [47]. *MAPK8* is reported to dissociate the complex of BCL2L1 (BCL-like 1) and BECN1 (beclin 1) to trigger autophagy and *TNFSF10* (tumor necrosis factor superfamily member 10)-induced *MAPK8* activation and autophagy can be effectively suppressed by knockdown of TRAF2 or RIPK1 [48]. Several studies shown an association of decreased *MAPK8* activity with pulmonary hypertension [49,50]. Yang *et al*. [51] comfirmed a decreased expression level of *MAPK8* in PE placentas. The role of *MAPK8* in pre-capillary pulmonary hypertension gives us a hint that it might also exert function on placenta vasculature. The hypermethylation of *ERO1L* has been reported to be triggered by increased binding of DNA (cytosine-5)-methyltransferase 1 (DNMT1) to the *ERO1L* promoter which contributes to trophablast cell apoptosis in the placenta of PE rats [52]. However, the role of *PIK3CB* in PE has not been well studied. In this study, the expression patterns of *ERO1L* and *PIK3CB* in PE placental samples and blood samples were quite contrary. We assumed that this phenomena may be caused by different microenvironments. Gene expression is quite a complex process, which is related not only to the gene itself, but also to the upstream and downstrem regulators.

There are also some shortcomings in our study. Firstly, this study was based on bioinformatics analysis and the expression patterns of ATGs differed between blood and placental samples. Therefore, further validation from both *in vivo* and *in vitro* experiments is needed. Secondly, though the ATGs-based signature model presented a powerful predictive value in PE, the efficiency remains to be further validated in multicenter, large-scale prospective studies.

5. Conclusions

In this study, we identified 20 potential autophagy-related genes of preeclampsia via bioinformatics analysis. Moreover, four feature genes *LEP*, *ERO1L*, *PIK3CB*, and *MAPK8* may be used as potential biomarkers for preeclampsia prediction. Identification of these ATGs might help to understand the molecular mechanism underlying the development of preeclampsia and might provide a new perspective on the management and treatment of preeclampsia.

Availability of Data and Materials

The raw data of the present study are downloaded from the GEO data portal (https://www.ncbi.nlm.nih.gov/geo/; Accession number: GSE75010, GSE48424, GSE84260), which is a publicly available database.

Author Contributions

LQW made substantial contributions to conception and design. JYS and XYT collected and analyzed the data, as well as draft the paper. JYZ and YLF made contributions to interpretation of data for the work and reviewing the manuscript for important intellectual content. LQW gave final approval for the version to be published. All authors

contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The raw data of the present study are downloaded from the GEO data portal, which is a publicly available database. Neither ethics approval nor consent is needed.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2807132.

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