

Construction of miRNA-mRNA Regulatory Network and Prognostic Signature in Endometrial Cancer

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Introduction: This bioinformatic study confirmed a new miRNA-mRNA regulatory network and a prognostic signature in endometrial cancer (EC).

Materials and Methods: We downloaded RNA-seq and miRNA-seq data of EC from the TCGA database, then used EdegR package to screen differentially expressed miRNAs and mRNAs (DE-miRNAs and DE-mRNAs). Then, we constructed a regulatory network of EC-associated miRNAs and hub genes by Cytoscape, and determined the expression of unexplored miRNAs in EC tissues and normal adjacent tissues by quantitative Real-Time PCR (qRT-PCR). A prognostic signature model and a predictive nomogram were constructed. Finally, we explored the association between the prognostic model and the immune cell infiltration.

Results: A total of 11,531 DE-mRNAs and 236 DE-miRNAs, as well as 275 and 118 candidate DEGs for upregulated and downregulated DE-miRNAs were screened out. The miRNA-mRNA network included 5 downregulated and 13 upregulated DE-miRNAs. qRT-PCR proved that the expression levels of miRNA-18a-5p, miRNA-18b-5p, miRNA-449c-5p and miRNA-1224-5p and their target genes (NR3C1, CTGF, MYC, and TNS1) were consistent with our predictions. Univariate and multivariate Cox proportional hazards regression analyses of the hub genes revealed a significant prognostic value of NR3C1, EZH2, AND GATA4, and these genes were closely related to eight types of immune infiltration cells.

Conclusion: We identified three genes as candidate biomarkers for EC, which may provide a theoretical basis for targeted therapy.

Keywords: TCGA, endometrial cancer, miRNA-mRNA network, prognostic model, immune infiltration cell

Introduction

Endometrial cancer (EC), a common malignancy, has an estimated incidence of 10–20 per 100,000 women globally and the figure is still increasing.¹ Surgical treatment is the major therapy for early-stage EC patients.² The prognosis of advanced EC is poor, and the 5-year overall survival rate is only 15–17%.³ Therefore, new biomarkers should be explored before the development of effective target treatments.

MicroRNAs (miRNAs), the 19–25 nucleotide non-coding RNAs, regulate gene expression and participate in biological processes.⁴ By base-pairing with complementary sequences within messenger RNA (mRNA) molecules, miRNA can silence RNAs and regulate post-transcriptional gene expression to promote cell

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proliferation,⁵ apoptosis,⁶ cell cycle,⁷ migration,⁸ differentiation,⁹ and energy metabolism.¹⁰ The dysregulation of miRNA is linked to multiple diseases, such as obesity, cancer, cardiovascular diseases, inflammatory diseases, and disorders of the female reproductive system.^{11–16} Many studies have verified the close relationship between miRNA and EC.¹⁷ For example, the overexpressed miR-137 suppresses tumor cell proliferation and colony formation in vitro and xenograft tumor growth in vivo.¹⁸ Despite the previous analyses on the miRNA expression in EC, this bioinformatic study aimed to confirm a new miRNA-mRNA regulatory network.

In our study, the differentially expressed miRNAs (DE-miRNAs) in EC were screened using TCGA database (Figure 1). The functions of DE-miRNA target genes were predicted by the functional and pathway enrichment analysis. Then, the regulatory network was established using EC-associated miRNAs and their target genes. The expression of unexplored EC-associated miRNAs was determined using qRT-PCR. Finally, we built a prognostic signature model using the hub genes and explored the association between the prognostic model and the immune infiltration.

Methods

Study Population

The mRNAs expression profiles of 587 (552 EC and 35 normal) tissues, and the miRNA isoform expression profiles of 568 (546 EC and 22 normal) tissues from TCGA database (<http://cancergenome.nih.gov/publications/publicationguidelines>) were downloaded and the corresponding clinical data were collected. The sequenced data were downloaded from Illumina HiSeq RNASeq and Illumina HiSeq_miRNASeq platforms.

Screening DE-mRNAs and DE-miRNAs

We used the “edgeR” package operated by R software to screen out DE-mRNAs and DE-miRNAs. DE-miRNAs were defined as $|\log_2 \text{fold change (FC)}| > 2.0$ and adjusted $P < 0.01$. DE-mRNAs were identified if $|\log_2 \text{FC}| > 1.0$ and adjusted $P < 0.05$.

Differentially Expressed Genes

To get the downstream target genes of DE-miRNAs, we used miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/php/index.php>), miRDB (<http://www.mirdb.org/>), and TargetScan

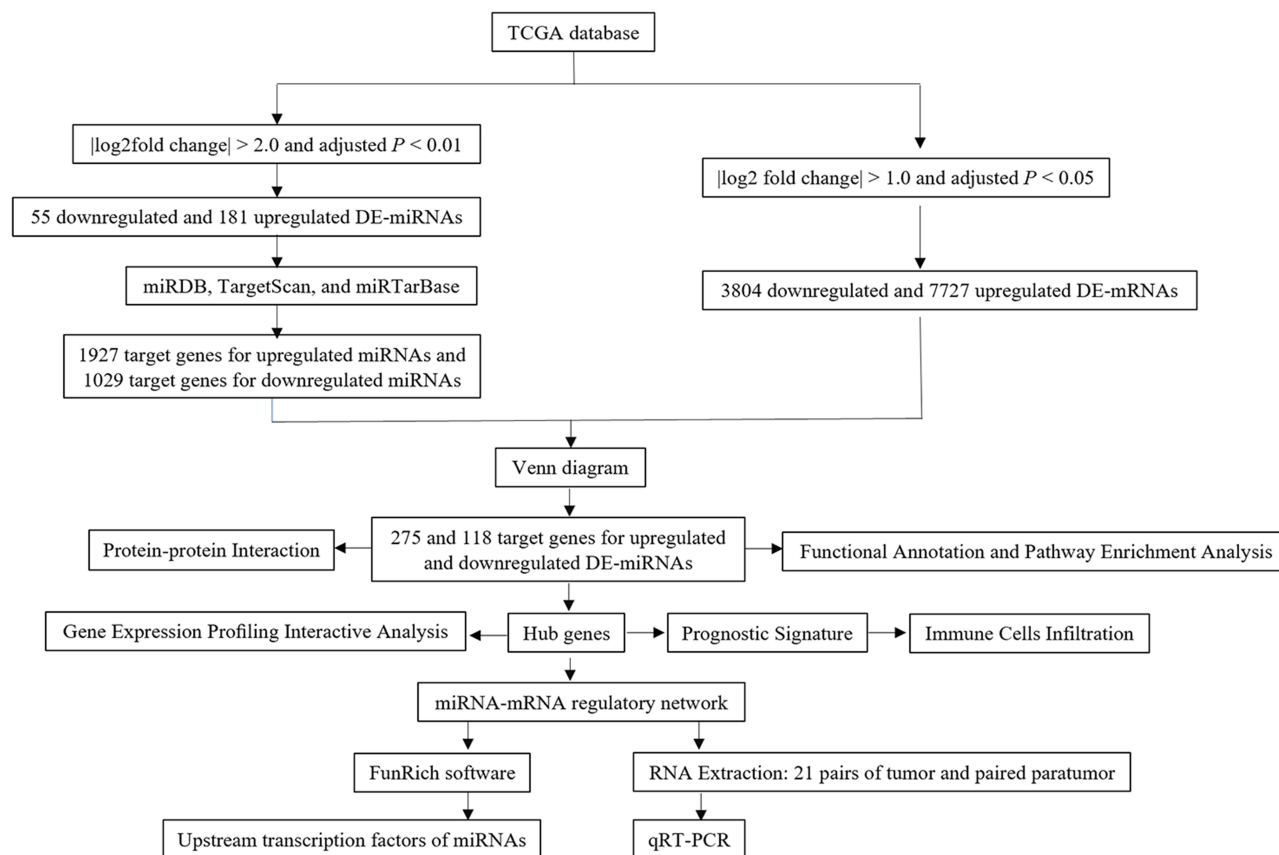


Figure 1 Flow chart of this article.

(<http://www.targetscan.org/>) to determine whether the DE-miRNAs and the target genes were paired. The differentially expressed genes (DEGs) and the overlapping genes of DE-mRNAs and miRNA target genes were identified using Venn diagram (<https://bioinfogp.cnb.csic.es/tools/venny/>).

Functional Annotation and Pathway Enrichment Analysis

Gene Ontology (GO) analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were used to detect the functions of the target genes. The categories of GO functional annotation included biological process (BP), cellular component (CC), and molecular function (MF). The Enrichr database (<http://amp.pharm.mssm.edu/Enrichr/>) was applied in GO functional annotation and KEGG pathway enrichment analysis for the overlapping target genes and DE-mRNAs. $P < 0.05$ was used as the threshold.

Construction of Protein-Protein Interaction

To reveal functional interactions and relationships between protein products of DEGs in EC, we uploaded all the overlapping genes screened from the Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://string-db.org/>). Subsequently, the hub genes in the network were identified according to the connectivity using Cytoscape software (version 3.6.1). We used “MCODE” plug-in to visualize and select modular hubs in the PPI network (degree = 4, node score = 0.2, k-core = 2, and max. depth = 100). The node in the network represented a gene or a protein and the line between the nodes represented their interaction. Central nodes represented key or significant proteins or genes with important functions. We recognized the top 10 hub nodes as hub genes.

Validation of Hub Gene Expression Levels

The expression levels of top 20 hub genes were validated using the Gene Expression Profiling Interactive Analysis (GEPIA).¹⁹ The hub genes with $|\log_2FC| > 2$ and $p < 0.01$ were considered statistically different.

Establishment of miRNA-mRNA Regulatory Pathways and Corresponding Transcription Factors

We built a new miRNA-mRNA network to identify the most functional miRNAs, then used the Cytoscape

software (version 3.6.1) to establish the miRNA-hub gene network and to investigate the association between the 20 hub genes and the DE-miRNAs. The selected upregulated and downregulated DE-miRNAs were input into FunRich software to predict the upstream transcription factors of the screened DE-miRNAs.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

EC tissues and pericarcinomatous tissues were obtained from patients of the First Affiliated Hospital of Nanjing Medical University. The clinical program was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University. All patients individually signed the informed consent. Total RNA of 42 (21 pairs of tumor and paired paratumor) tissues was separated using Trizol reagent (Thermo Fisher Scientific, Waltham, MA). The RNA quantity control and concentration were evaluated using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The reverse transcription of total RNA was performed using the Reverse Transcription Kit (Takara, Tokyo, Japan). qRT-PCR was conducted (SYBR Premix Ex Taq, TaKaRa, Dalian, China) on Light Cycler 480 (Roche, Switzerland) to detect the relative miRNA and mRNA expressions using the $2^{-\Delta\Delta Ct}$ method, in which GAPDH and U6 were used as endogenous control for mRNA and miRNA, respectively. The PCR primers are listed in [Supplemental Table 1](#).

Establishment of Prognostic Signature Model Using Hub Genes

The association between the hub gene expression and the overall survival was assessed using the univariate Cox proportional hazards regression analysis. Then, the prognosis-related genes ($p < 0.05$) were taken as candidate variables. Stepwise multivariate Cox proportional hazards regression analysis was employed to predict the risk score of the genes. The model was developed using the `coxph()` function in survival package.²⁰ The risk score for predicting the overall survival was calculated as follows: Risk score = $\exp(\text{RNA1}) * \beta_{\text{RNA1}} + \exp(\text{RNA2}) * \beta_{\text{RNA2}} + \exp(\text{RNA3}) * \beta_{\text{RNA3}} + \dots + \exp(\text{RNA}_n) * \beta_{\text{RNA}_n}$ (\exp RNA: relative expression value after transforming z score; β_{RNA} : regression coefficient obtained via the multivariate Cox proportional hazards regression model). The patients fell into either a low-risk group or a high-risk group based on the mean risk score. We used the “survival” package in

R to generate the overall survival curves of both groups. The 1-, 3-, and 5-year receiver operating characteristic (ROC) curves were used to calculate the predictive value of the model. The sensitivity and specificity of the risk model of EC were calculated by the ROC curve using the “survival ROC” R package. The areas under the ROC curve (AUC) presenting sensitive and specific cities were used to indicate the predictive value. The predictive model, established with the AUC >0.6, was considered to have explanatory and informative efficacy. In addition, comprehensive survival analysis was implemented to analyze the relationship between the different clinical parameters (age, race, tumor stage, histological type, grade, and tumor status) and the risk score model. Clinical-based subgroup survival analysis stratified by clinical factors was also conducted. All analyses were conducted using the R. $P < 0.05$ was considered statistically significant in the prognostic signature analysis. Finally, we built and validated a predictive nomogram.²¹ Clinical parameters like age, race, tumor stage, histological type, grade, and tumor status, and the risk score model, were used to build a nomogram to investigate the probability of 1-, 3-, and 5-OS of EC. Validity of the nomogram was assessed by discrimination and calibration.

Association of Prognostic Signature with Immune Cell Infiltration

To investigate the prognostic signature, CIBERSORT was used to search the most significant tumor-infiltrating immune cells and prognostic signature of the risk score.²² We explored the relationship between the risk score and the significant immune cells, including B cells memory fraction, B cells naive fraction, dendritic cells activated fraction, dendritic cells resting fraction, eosinophils fraction, macrophages M0 fraction, macrophages M1 fraction, macrophages M2 fraction, mast cells resting fraction, monocytes fraction, neutrophils fraction, NK cells activated fraction, NK cells resting fraction, plasma cells fraction, T cells CD4 memory activated fraction, T cells CD4 memory resting fraction, T cells CD8 fraction, T cells follicular helper fraction, T cells gamma delta fraction, and T cells regulatory (Tregs) fraction. The Tumor Immune Estimation Resource 2.0 (TIMER 2.0) (<http://timer.cistrome.org/>) provides more robust estimation of immune infiltration levels for The Cancer Genome Atlas (TCGA). It can be used to perform Spearman correlation analysis between multiple TIICs

and specific gene, which is used to validate the immunologic correlation in this study.

Results

Analysis of DE-miRNAs

Totally, 55 downregulated and 181 upregulated miRNAs were screened out in EC samples ([Supplemental Figure 1A](#)). [Supplemental Figure 1B](#) showed the heat map of DE-miRNAs.

Target Genes of DE-miRNAs

DE-miRNA-gene pairs in the three databases were chosen to obtain reliable miRNA-gene pairs. As a result, 1927 target genes were paired with upregulated miRNAs, and 1029 target genes with downregulated miRNAs ([Supplemental Table 2](#)).

Candidate DE-mRNAs and Their Target Genes

We screened out 3804 downregulated and 7727 upregulated mRNAs, and confirmed the corresponding target genes ([Supplemental Figure 2A](#)). The heat map of DE-mRNAs was presented in [Supplemental Figure 2B](#).

Differentially Expressed Genes

Abundant studies have proved the inverse relationship between miRNAs and target genes.²³ Our study screened out 1927 target genes for upregulated miRNAs and 1029 target genes for downregulated miRNAs. We also identified 7727 upregulated and 3804 downregulated target genes of DE-mRNAs. After analyzing DE-mRNAs and target genes of DE-miRNAs, 275 and 118 candidate DEGs for upregulated and downregulated DE-miRNAs were identified, respectively ([Figure 2](#)).

Functional and Pathway Enrichment Analysis

The GO functional annotation included BP, CC, and MF. [Figure 3](#) presented the top 10 enriched GO items. In BP analysis, the candidate target genes of upregulated DE-miRNAs were significantly enriched in the cell migration involved in sprouting angiogenesis and the negative regulation of DNA-templated transcription ([Figure 3A](#)). In CC analysis, these genes were significantly enriched in chromatin and protein kinase complex ([Figure 3C](#)). The MF analysis revealed these genes were associated with vascular endothelial growth factor-activated receptor activity and transcription of RNA polymerase II ([Figure 3E](#)). BP

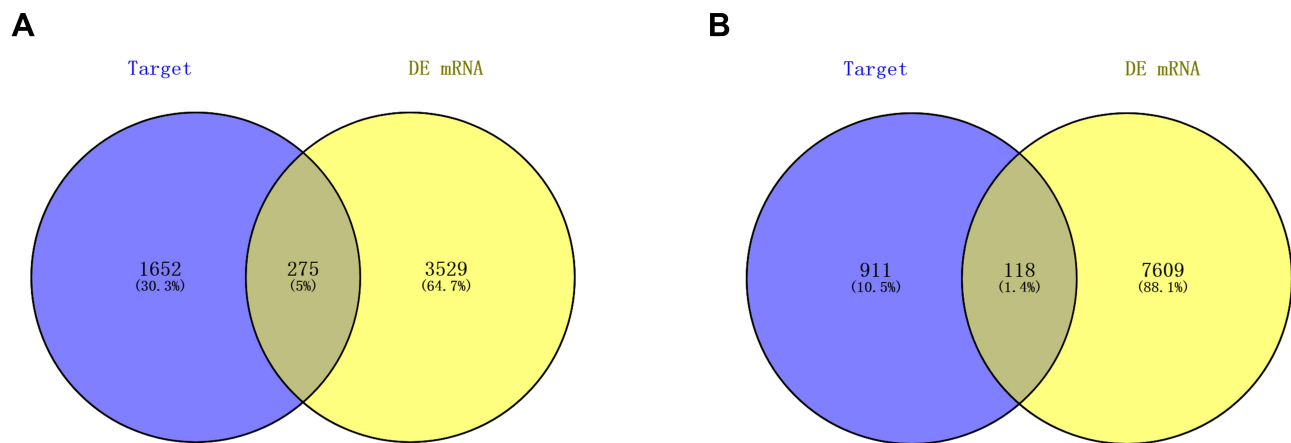


Figure 2 Screening of candidate DEGs. **(A)** The intersection of target genes of upregulated DE-miRNAs and downregulated target genes of DE-mRNAs; **(B)** the intersection of target genes of downregulated DE-miRNAs and upregulated target genes of DE-mRNAs. Blue represents target genes of DE-miRNAs. Yellow represents DE-mRNAs.

Abbreviations: DE-miRNAs, differentially expressed microRNAs; DE-mRNAs, differentially expressed messenger RNAs; DEGs, differentially expressed genes.

analysis showed that the candidate target genes of downregulated DE-miRNAs were involved in the positive and negative feedback regulation of DNA-templated transcription (Figure 3B). In CC analysis, these genes were significantly enriched in nuclear transcription factor complex and chromatin in telomeric region (Figure 3D). MF analysis indicated that these genes were significantly enriched in AT DNA binding and retinoid X receptor binding (Figure 3F). KEGG pathway enrichment analysis showed that the upregulated DE-miRNAs were enriched in FOXO signaling pathway, and Focal adhesion (Figure 3G). The downregulated DE-miRNAs were enriched in pathways in cancer and cell cycle (Figure 3H).

PPI Network and Hub Genes

PPI network of these genes was constructed after mapping these candidate DEGs into the STRING database (Figure 4A and B). Totally, 275 and 118 node pairs of upregulated and downregulated DE-miRNAs for candidate target genes were obtained. The top 10 hub genes of upregulated DE-miRNAs are listed in Supplemental Figure 3A. The top 10 hub genes of downregulated DE-miRNAs are shown in Supplemental Figure 4B.

Hub Gene Expression Levels

Using GEPIA database, we found the expression of the 10 hub genes of downregulated DE-miRNAs was significantly higher in EC tissues than in normal ones (Figure 5A–Q). Marked upregulation was found in 7 out of 10 hub genes of upregulated DE-miRNAs in EC tissues compared with normal tissues. No significant difference was

observed in MYC, PRKACB, and GATA4 expression between the EC and normal tissues ($p > 0.05$).

miRNA-mRNA Regulatory Pathways and Corresponding Transcription Factors

We established an miRNA-mRNA regulatory network about EC occurrence and progression (Figure 6A). Different genes might be regulated by the same miRNA. For example, miR-424-5p targeted the largest number of genes (KIF23, CHEK1, CEP55, CDC25A, and CCNE1) i, suggesting that miR-424-5p might play a significant part in the development of EC. On the other hand, different miRNAs could regulate the same gene, indicating their vital role in the progression of EC. For example, NR3C1, FOXO1, and JUN were, respectively, targeted by three miRNAs. Via FunRich software, the top five transcription factors for upregulated and downregulated DE-miRNAs were obtained (Figure 6B and C).

Since 12 of the 18 miRNAs in the network were verified in EC, and their expression levels were consistent with our results, we assessed the remaining six miRNAs in EC. The results (Figure 7A–L) demonstrated that expression levels of miRNA-18a-5p, miRNA-18b-5p, miRNA-449c-5p and miRNA-1224-5p were markedly upregulated in EC tumors compared with those in paratumor tissues. Of note, miRNA-636 expression was significantly downregulated, which was inconsistent with our prediction. The expression of miRNA-6715a-3p showed no difference between EC and adjacent normal tissues (Figure 7F). qRT-PCR revealed that NR3C1, CTGF, MYC, TNS1, PRKACB, and FOXO1 had a higher expression in tumors

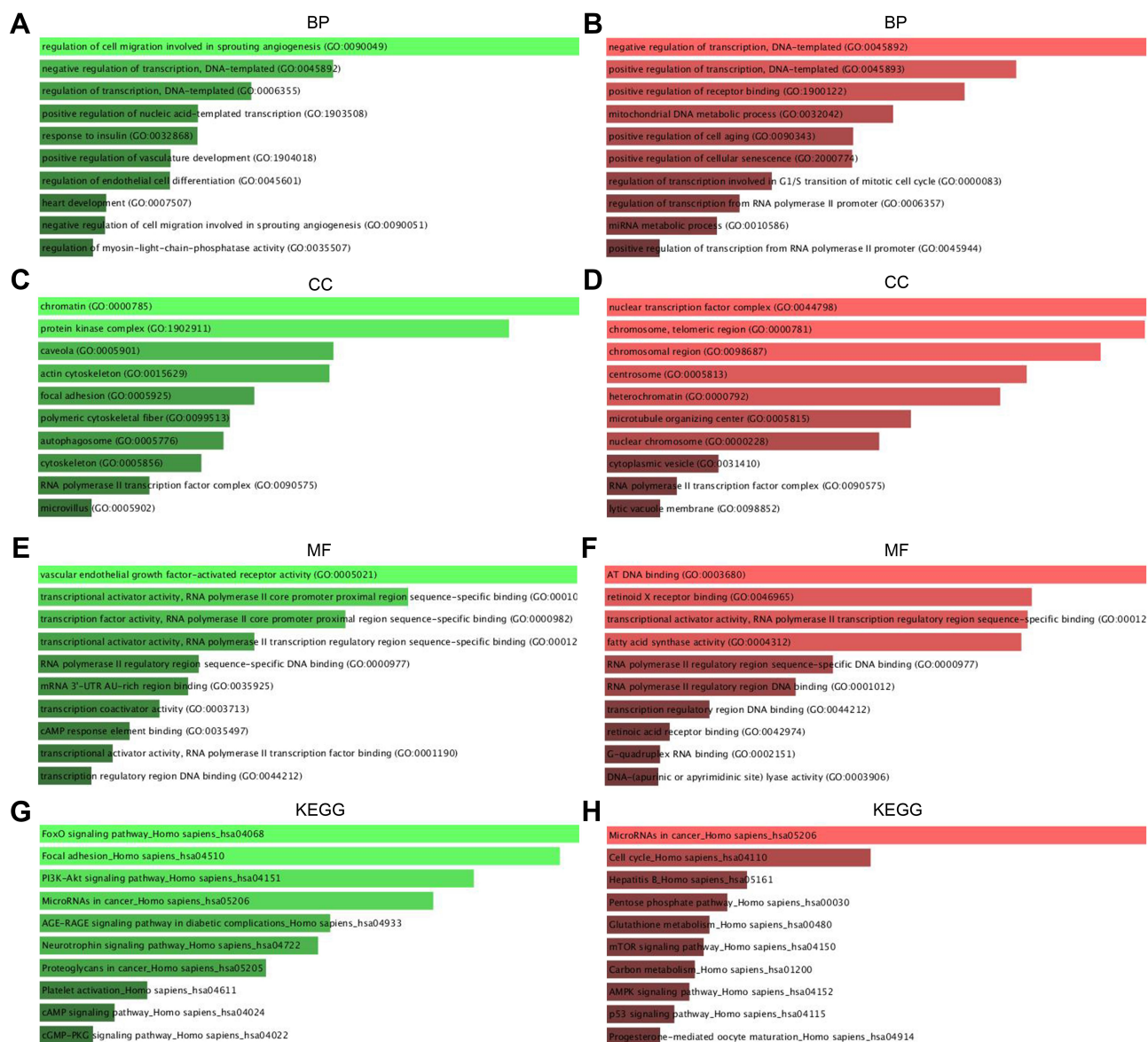


Figure 3 The enriched functions for DEGs of DE-mRNAs and DE-miRNAs. **(A)** The top 10 enriched BP items of downregulated candidate genes; **(B)** the top 10 enriched BP items of upregulated candidate genes; **(C)** the top 10 enriched CC items of downregulated candidate genes; **(D)** the top 10 enriched CC items of upregulated candidate genes; **(E)** the top 10 enriched MF items of downregulated candidate genes; **(F)** the top 10 enriched MF items of upregulated candidate genes (GO: gene ontology); **(G)** the top 10 enriched KEGG items of downregulated candidate genes; **(H)** the top 10 enriched KEGG items of upregulated candidate genes.

Abbreviations: KEGG, The Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

than in paratumor tissues (Figure 7J–L). The above results indicated that miRNA-18a-5p, miRNA-18b-5p, miRNA-449c-5p and miRNA-1224-5p and their target genes might play a more powerful role than miRNA-636 and miRNA-6715a-3p and their target genes in EC development.

Prognostic Signature Model

A prognostic analysis was conducted based on the 20 hub genes (Supplemental Figure 3). The top eight significant prognostic factors were ESPL1, NR3C1, CCNE1, GATA4,

KIF23, EZH2, CDC25A, and BIRC5, as shown in the univariate Cox proportional hazards regression analysis (Supplemental Table 3). The genes NR3C1, EZH2, and GATA4 exhibited a significant prognostic value in the multivariate Cox proportional hazards regression analysis. Immunohistochemistry also confirmed the differential expression of NR3C1, EZH2, and GATA4 in normal endometrial tissues (Supplemental Figure 4A, 4C, and 4E) and endometrial carcinoma tissues (Supplemental Figure 4B, 4D, and 4F) based on the Human Protein Atlas (HPA,

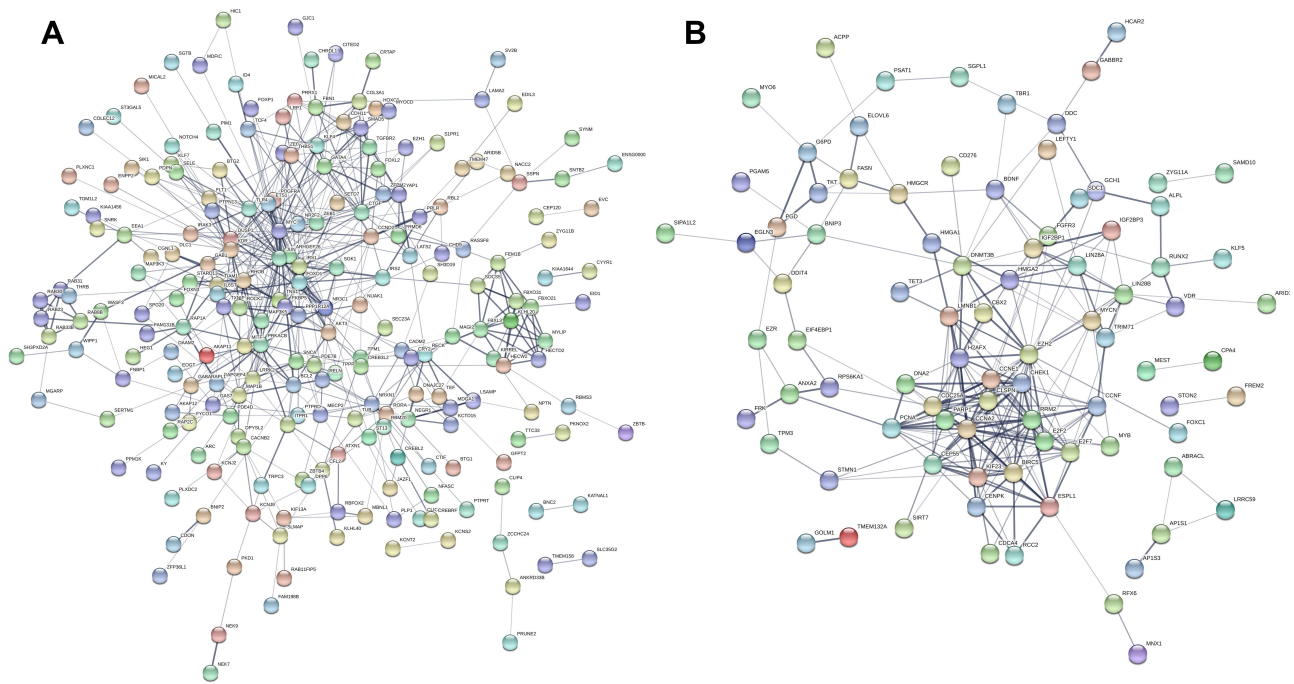


Figure 4 PPI network of the candidate target genes. **(A)** Upregulated DE-miRNAs; **(B)** downregulated DE-miRNAs. **Abbreviation:** PPI, protein–protein interaction.

<https://www.proteinatlas.org/>). The hazard ratios of NR3C1, EZH2, and GATA4 were positive, suggesting their negative correlation with prognosis. The following formula was used to calculate the patient's risk score:

$$\text{Risk score} = 0.284183 * \text{NR3C1} + 0.401466 * \text{EZH2} + 0.089382 * \text{GATA4}$$

Kaplan–Meier curve was plotted to compare the survival time of low-risk patients and high-risk patients. The results showed that the low-risk patients had significantly longer overall survival time than the high-risk ones ($p < 0.0001$; **Figure 8A**). The areas under the time-dependent survival ROC curves were more than 0.656 in both groups, indicating the good performance of our risk model in predicting 1-, 3-, and 5-year survival (**Figure 8B**). We also analyzed the risk score distribution, survival status, and the expression of three genes for each patient (**Figure 8C–E**). The heatmap (**Figure 9A**) showed comparison of clinicopathological features between the two groups. Significant differences were found between the two group in terms of grade, age, stage, histological type, and tumor status ($p < 0.01$). Univariate Cox regression analysis and multivariate Cox regression analysis proved that this prognostic model could be an independent prognostic indicator of EC (**Figure 9B and C**). It was also found that high-risk patients in age ≥ 60 years old and stage III/IV subgroups were prone to a worse OS (**Figure 9D and**

E). A nomogram was then constructed to predict 1-year, 3-year, and 5-year OS in 482 EC patients using seven prognostic factors including age, race, stage, histological type, grade, tumor status, and the risk score (**Figure 10A–D**). The nomogram, combined with the model, might be used to predict survival time of EC patients in clinical practice.

Prognostic Signature Associated with Immune Cells Infiltration

We used CIBERSORT method to search the most significant tumor-infiltrating immune cells and prognostic signature of risk score. The genes NR3C1, EZH2, and GATA4 in the prognostic model were associated with the enumeration and activation of immune cells. A close relationship was also confirmed between the three genes and the eight types of immune infiltration cells, including B cells naive fraction, macrophages M1 fraction, neutrophils fraction, T cells CD4 memory resting fraction, T cells follicular helper fraction, T cells gamma delta fraction, and T cells regulatory (Tregs) fraction (**Figure 11A–G**). In addition, TIMER 2.0 was also used to validate the correlation between three hub genes and tumor-infiltrating immune cells (TIICs). The results showed that NR3C1, EZH2, and GATA4 were all correlated with some certain TIICs (**Supplemental Figure 5A–C**).

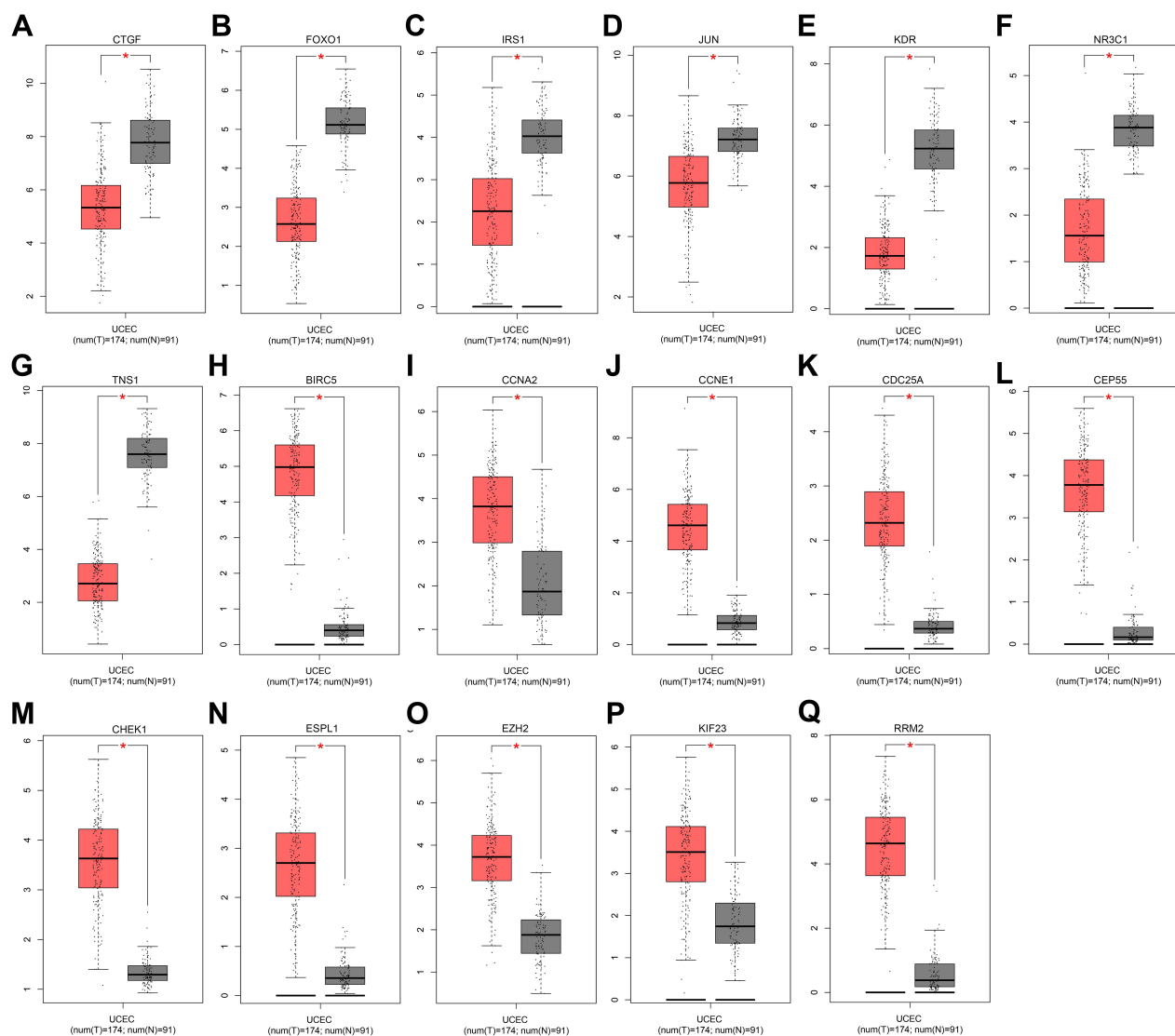


Figure 5 The expression levels of 17 hub genes from the GEPIA database. (A) CTGF expression; (B) FOXO1 expression; (C) IRS1 expression; (D) JUN expression; (E) KDR expression; (F) NR3C1 expression; (G) TNS1 expression; (H) BIRC5 expression; (I) CCNA2 expression; (J) CCNE1 expression; (K) CDC25A expression; (L) CEP55 expression; (M) CHEK1 expression; (N) ESPL1 expression; (O) EZH2 expression; (P) KIF23 expression; (Q) RRM2 expression. * $p < 0.05$.

Discussion

Recent studies have mentioned that the expression of miRNAs and their downstream targets is closely related to EC development. For the first time in this study, we constructed a regulatory network using EC-associated genes and DE-miRNAs. Besides, we built a prognostic model based on hub genes, and confirmed the relationship between immune cell infiltration and the prognostic signature of risk score.

In our study, Five downregulated DE-miRNAs and 13 upregulated DE-miRNAs were finally determined, among which miR-424-5p targeted the largest number of genes (KIF23, CHEK1, CEP55, CDC25A, and CCNE1) in the

regulatory network, suggesting its significant role in the development of EC. Our analytic results on the expression of DE-miRNAs were mostly consistent with the previous studies. For example, the miR-424-5p and let-7c-5p expression levels were lower in EC tissues than in normal tissue.^{24,25} The significant down-regulation of miR-542-3p in EC^{26,27} promoted the morphological change of endometrial stromal cells.²⁸ The lowered expression of miR-101-3p was significantly correlated with a poor overall survival; miR-101-3p regulated the cell proliferation in the carcinogenesis of endometrial serous carcinoma.²⁹ miR-96-5p, miR-182, miR-7, and miR-183-5p were significantly up-regulated in EC

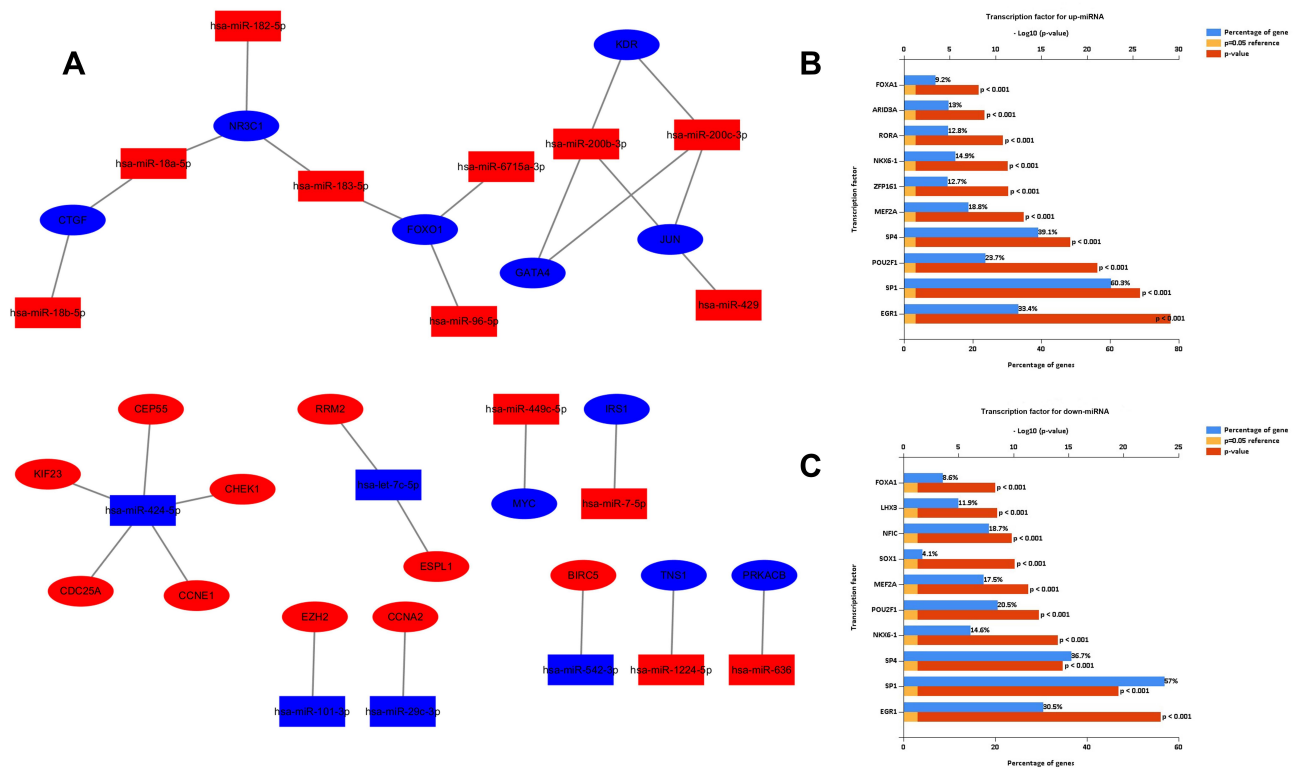


Figure 6 Regulatory network of miRNA-mRNAs and corresponding transcription factors. (A) Regulatory network of RC-associated genes and their target miRNAs (red: upregulation, blue: downregulation, oval: mRNA, rectangle: miRNA); (B) predicted transcription factors of upregulated DE-miRNAs; (C) transcription factors of downregulated DE-miRNAs.

compared with normal endometrium.^{30,31} The miR-200 family (miR-200b, miR-200c, and miR-429) in EC was upregulated compared with that in normal endometrial tissues.³² Li et al identified that miRNA-29c-3p (a tumor suppressor) was significantly lower in EC cells and was linked to the low paclitaxel sensitivity of EC.³³ However, some miRNAs were found to be related to other tumors rather than EC. For instance, a research revealed the significantly higher expression of miR-636 and the significantly lower expression of miR-18a-5p in breast cancer than in normal samples.³⁴ Wu et al detected a lower miR-449c-5p level in gastric cancer (GC), which was correlated with a lower survival rate.³⁵ miR-18b-5p was upregulated in breast cancer.^{36,37} Compared with normal tissues, miR-1224-5p was significantly downregulated in lung cancer tissues.³⁸ Although most miRNAs in the network have been reported to be associated with cancer, miR-6715a-3p has not yet been reported. According to the present network, miR-6715a-3p might be related to EC. However, further qRT-PCR found a higher expression of miR-6715a-3p in EC tissues than in the adjacent tissues but with no significant difference, which might be caused by the small sample size.

We built the PPI network and identified the top 20 hub genes (10 upregulated and 10 downregulated ones). The expressions of these hub genes in EC were assessed using GEPIA database, which included more normal samples than TCGA.¹⁹ Inspiringly, the expression levels of these genes were generally consistent with our results of TCGA mRNA data. Most of these genes were key modulators of EC. For example, c-myc was found activated in multiple human tumors (including EC) with a poor prognosis,^{39,40} c-myc was proved to promote EC growth through many pathways.^{41–43} The histone methyltransferase EZH2 promoted EC cell growth through H3K27 trimethylation.⁴⁴ CCNA2 knockdown decreased the cell proliferation in human EC cell lines.⁴⁵ Based on the above results, we built an miRNA-mRNA regulatory network. In this network, most miRNA-mRNA pairs might contribute to the pathogenesis of EC, making them potential therapeutic targets.

Since miRNA expression is modulated by transcription factors,^{46,47} we speculated that these DE-miRNAs could be regulated by the transcription factors. Specific protein-1 (Sp1), a zinc-finger transcription factor of the Sp/KLF family, can bind to GC-rich promoter elements like

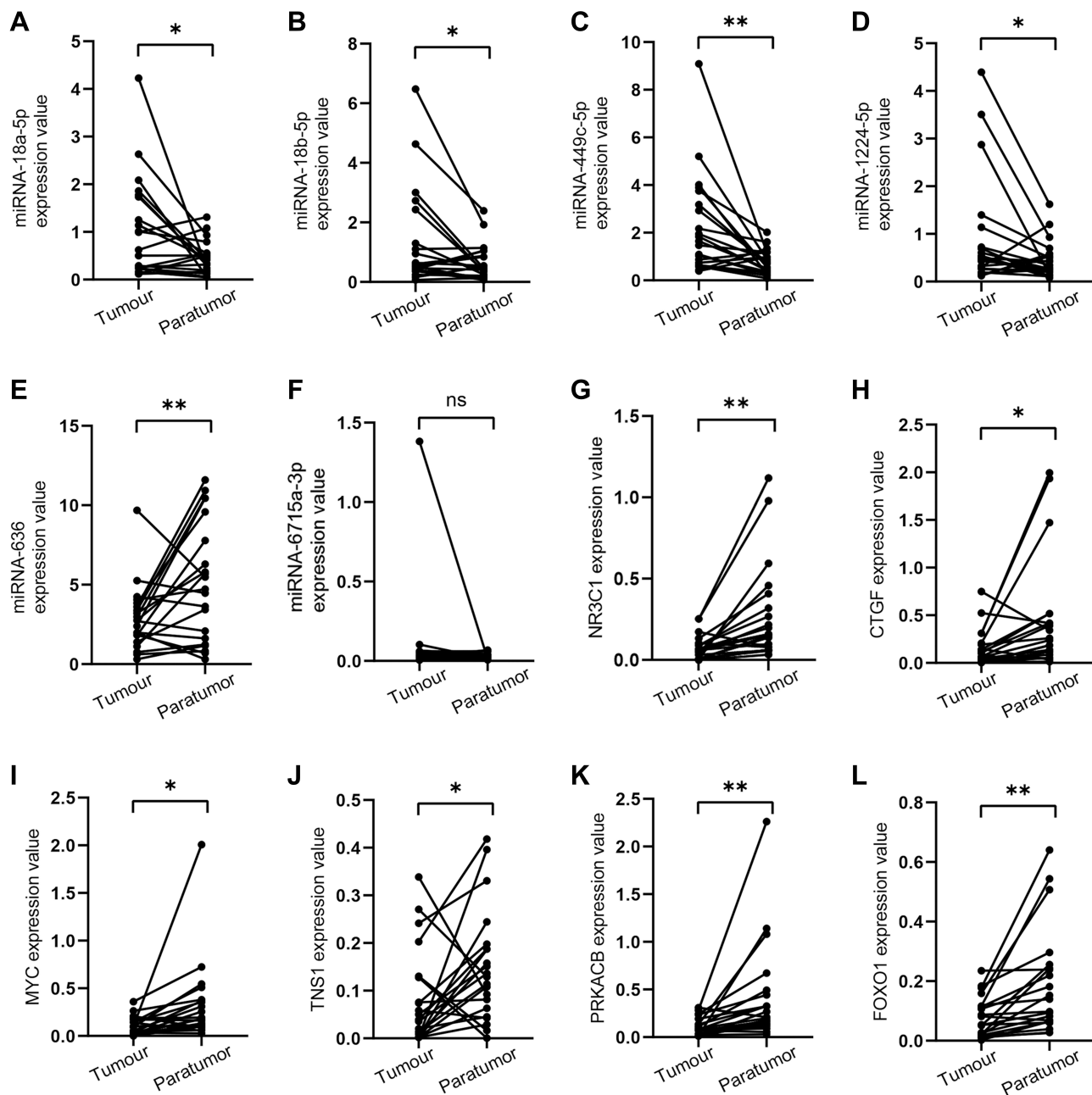


Figure 7 Expression of potential miRNAs and target genes in EC tissues and normal adjacent tissues. (A–F) miRNA expression levels; (G–L) mRNA expression levels. * $p < 0.05$; ** $p < 0.01$.

Abbreviation: ns, no statistic.

GC-boxes, CACCC-boxes and related motif.^{48–50} Sp1, which regulates the expression of genes involved in cell cycle, proliferation and death,^{51,52} has been reported as an miRNA target.^{53,54} But few studies have reported its role in miRNA expression modulation and its function in EC. For instance, Sp1 was verified as a target of miR-490, and Sp1 knockdown could reverse the effects of miR-490 inhibition on the malignant behaviors of EC, providing a new strategy for EC therapy.⁵⁵ The functions of these

transcription factors in EC need further experimental exploration in the future.

In this study, we developed a three-gene signature (GATA4, NR3C1, and EZH2) from the 20 hub genes. These three key genes are correlated to tumorigenesis, development, and metastasis. The abnormal expression of GATA4 has been reported to be associated with the onset and progression of some solid tumors, but whether GATA4 is an oncogenic driver or a tumor suppressor in

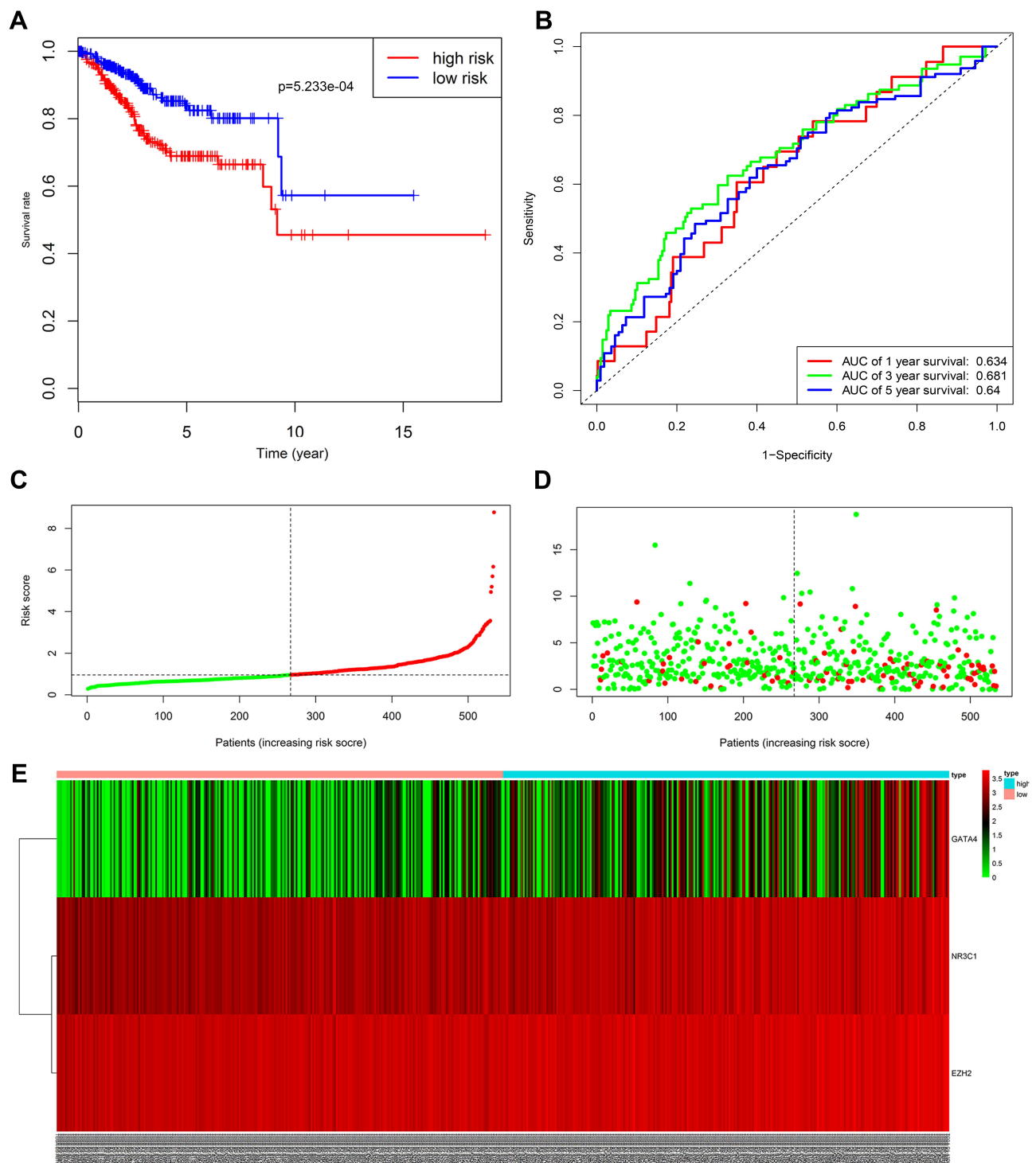


Figure 8 Analysis of the prognostic model between low- and high-risk groups. **(A)** Kaplan-Meier survival curve of overall survival between the high-risk group and low-risk group; **(B)** the time-dependent survival ROC curves; **(C)** the distributions of risk score; **(D)** survival status; **(E)** the heatmap showing the expression levels of the three genes in low- and high-risk groups.

Abbreviation: ROC, receiver operating characteristic.

oncogenesis is unclear. Chia et al⁵⁶ found that KLF5/GATA4/GATA6 might promote GC development by engaging in mutual crosstalk, and maintaining a pro-oncogenic transcriptional regulatory network in GC. A recent study

also demonstrated that GATA4 promoted oncogenesis by inhibiting miR125b-dependent suppression of DKK3 expression.⁵⁷ However, Han et al discovered that GATA4 could suppress tumor by repressing the NF- κ B signaling in

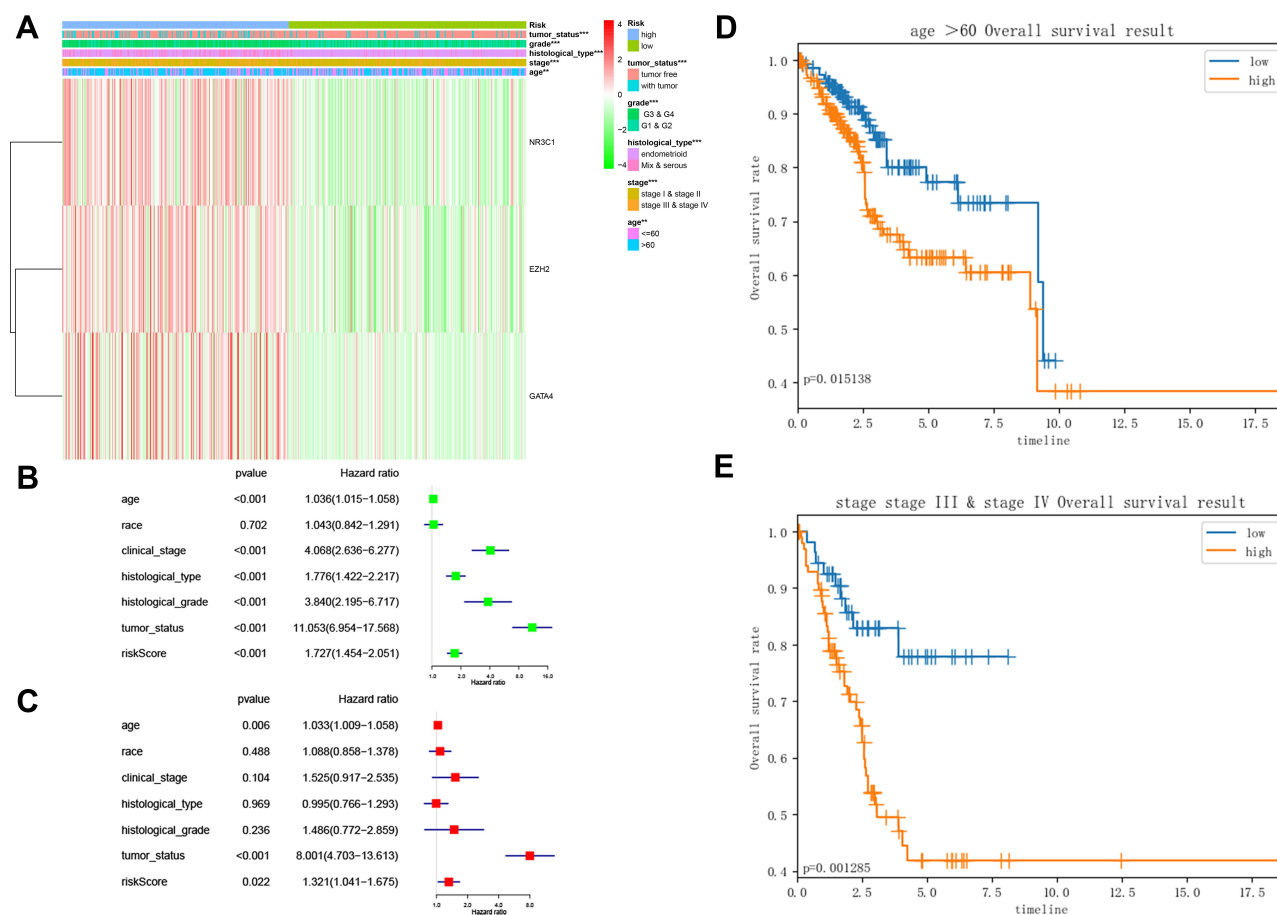


Figure 9 Study of the factors impacting EC prognosis. **(A)** The heatmap comparing the distribution of clinicopathological features in the low- and high-risk groups. ****** $p < 0.01$; ******* $p < 0.001$; **(B)** Forest Plot for univariate Cox regression analysis of clinical factors and risk score; **(C)** Forest Plot for multivariate Cox regression analysis of clinical factors and risk score; **(D)** Kaplan-Meier survival curve of age ≥ 60 years old subgroup between the low- and high-risk groups for the overall survival rate in EC; **(E)** Kaplan-Meier survival curve of stage III/IV subgroup between the low- and high-risk groups for the overall survival rate in EC.

Abbreviation: EC, endometrial cancer.

cells of breast cancer.⁵⁸ Compared with samples of non-neoplastic endometrium, the EC group showed significant higher methylation in GATA4 gene.⁵⁹ In addition, nuclear receptor subfamily 3 group C member 1 (NR3C1), a member of the nuclear hormone receptor super family of ligand-activated transcription factors, regulated the glucocorticoid hormone activities and changed gene expression in target cells and tissues.⁶⁰ NR3C1 was identified as epigenetically deregulated gene in gastrointestinal tumorigenesis.^{61,62} NR3C1 was correlated with liver metastasis of GC⁶³ and highly methylated in GC, indicating that it might play a pivotal role in the initiation and progression of GC. The variants of NR3C1, namely rs4912913, rs33388 and rs12521436, might contribute to GC susceptibility.^{64,65} EZH2, the enzymatic subunit of the polycomb-repressive complex 2 (PRC2) that catalyzes H3K27 methylation, is a most frequently mutated epigenetic regulator in hematologic malignancies. A recent

study indicated that EZH2, as a potential anti-EC therapeutic target, could drive EC progression by regulating miR-361/Twist signaling, which is consistent with our research.⁶⁶ These findings suggest that the three-genes signature can help clinicians to select high-risk patients from those with identical clinical or molecular characteristics and make rational treatment decisions.

EC is a group of heterogeneous tumors with distinct characteristics. High-throughput technologies bring EC with a possibility of targeted therapies. Currently, immunotherapies, such as anti-cancer vaccines, anti-PD-1 monoclonal antibody, and adoptive cell transfer, have emerged as alternative treatments for EC.⁶⁷ Therefore, it is critical to identify molecular subgroups that are amenable to targeted therapies, including immunotherapy. We established a prognostic model to compare the enumeration and activation of immune cell subtypes between high-risk and low-risk groups. Many studies have supported the

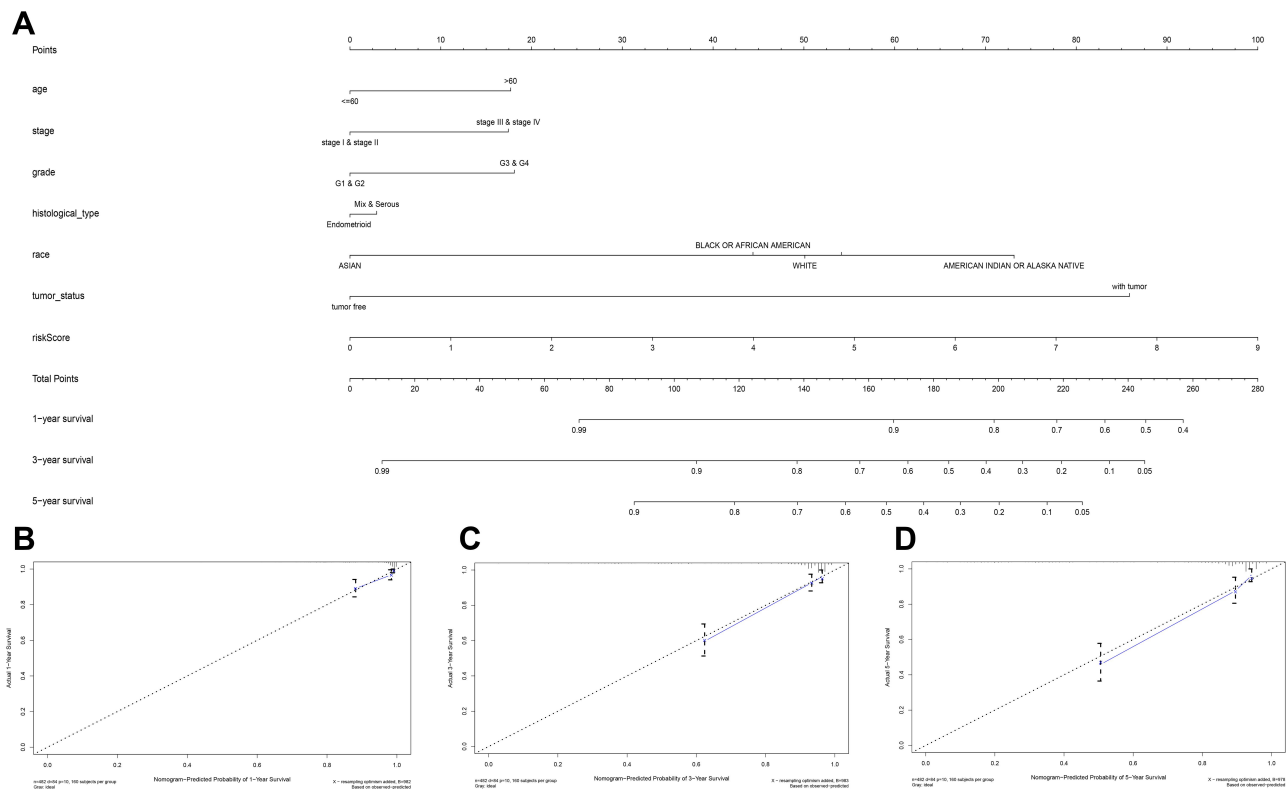


Figure 10 Nomogram predicting overall survival for EC patients. **(A)** For each patient, seven lines are drawn upward to determine the points received from the seven predictors in the nomogram. The sum of these points is located on the ‘Points’ axis. Then a line is drawn downward to determine the possibility of 1-, 3-, and 5-year overall survival of EC. **(B–D)** The calibration plot for internal validation of the 1-, 3-, and 5-year overall survival nomogram. Y-axis: actual survival; X-axis: nomogram-predicted survival.

Abbreviation: EC, endometrial cancer.

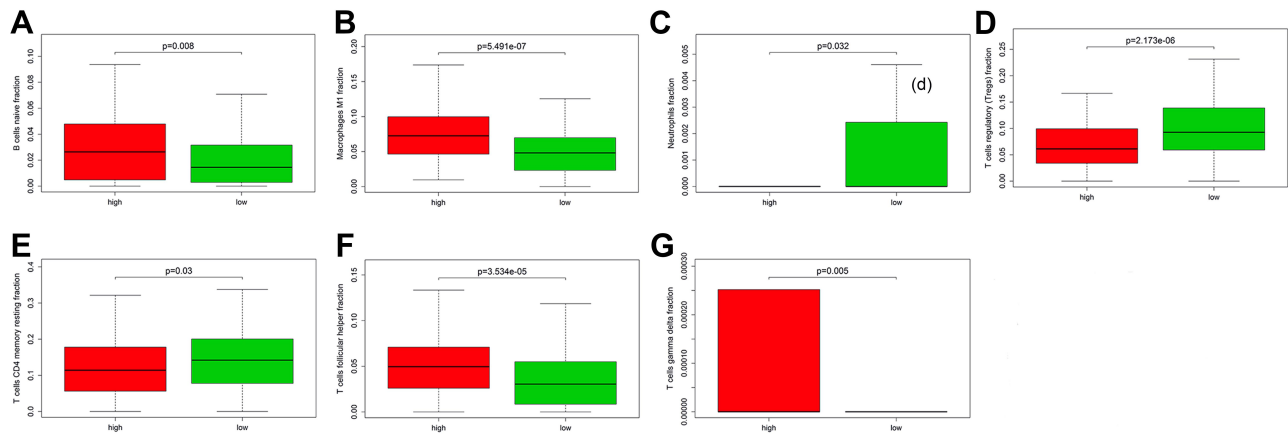


Figure 11 Relationship of risk score with the significant immune cell. **(A)** B cells naive fraction; **(B)** macrophages M1; **(C)** neutrophils; **(D)** T cells regulatory (Tregs); **(E)** T cells CD4 memory resting; **(F)** T cells follicular helper; **(G)** T cells gamma delta.

key role of immune infiltration in the development of cancer. For example, T cells could induce dormancy or promote multistage carcinogenesis. The degree of T cell infiltration in tumor could predict the patient’s response to cancer immunotherapy.⁶⁸ High T cell density in tumor and immune cells was once considered as an active anti-tumor

response.⁶⁹ Tumor-associated macrophages, especially M1 macrophages, became tumoricidal in a less hypoxic environment and less exposed to tumor-derived cytokines.^{70,71} Besides, in the advanced stage of tumor, M1 macrophages tended to polarize into M2 macrophages, thus having protumor function.⁷²

The present study has some limitations. First, the sample size in TCGA database is not large enough. Second, the association of these miRNA-mRNA pairs needs to be validated by experiments. In the future, more in vitro and in vivo functional experiments should be performed on the miRNA-mRNA regulatory pathways in EC.

Conclusion

The miRNA-mRNA regulatory network in EC was established and three hub genes were screened out from hundreds of candidate genes by bioinformatics analysis. Moreover, we identified a three-gene prognostic signature as a prognostic predictor for EC. The findings of this study may provide theoretical reference for the exploration of the biomarkers for the diagnosis and prognosis of EC.

Abbreviations

EC, endometrial cancer; DE-miRNAs, differentially expressed; DE-mRNAs, differentially expressed mRNAs; DEGs, the differentially expressed genes; FC, fold change; GO, gene ontology; KEGG, The Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function; microRNAs, microRNAs; mRNAs, messenger RNAs; PPI, protein-protein interaction; FDR, false discovery rate.

Data Sharing Statement

EC mRNA and miRNA data can be downloaded from TCGA database (<http://cancergenome.nih.gov/publications/publicationguidelines>).

Ethics Approval and Consent to Participate

Endometrial cancer tissues and pericarcinomatous tissues were obtained from patients of the First Affiliated Hospital of Nanjing Medical University. The clinical program was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University. All patients individually signed informed consent.

Consent for Publication

All parties consented for publication.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest for this work.

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