Original Article

Immune-related gene *ANGPT1* is an adverse biomarker for endometrial carcinoma

Binbin Nong[1,2#], Tongye Su[1,3#], Mingyang Jin[2], Jintai Huang[2], Aimin Huang[2], Dalang Fang[4], Jie Wei[1]

[1]Department of Hematology, People's Hospital of Baise, Baise, China; [2]Department of Gynecology and Obstetrics, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, China; [3]Department of Gynecology and Obstetrics, People's Hospital of Baise, Baise, China; [4]Department of Breast and Thyroid Surgery, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, China

Contributions: (I) Conception and design: B Nong, T Su; (II) Administrative support: A Huang, J Wei; (III) Provision of study materials or patients: J Huang, M Jin; (IV) Collection and assembly of data: B Nong, T Su; (V) Data analysis and interpretation: D Fang, J Wei; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Jie Wei. Department of Hematology, People's Hospital of Baise, Baise, China. Email: 654680956@qq.com; Aimin Huang. Department of Gynecology and Obstetrics, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, China. Email: ham10272007@sina.com; Dalang Fang. Department of Breast and Thyroid Surgery, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, China. Email: fangdalang@stu.gxmu.edu.cn.

Background: Immunotherapy has made great strides in cancer treatment. Endometrial carcinoma (EC) has been 1 of the most common tumors among women. This study aimed to screen immune-related prognosis biomarkers for EC.

Methods: The transcriptome profiling and clinical data of EC were downloaded from The Carcinoma Genome Atlas (TCGA) public database, and differentially expressed genes (DEGs) were obtained through the limma package in R software. An immune-related genes (IRGs) list was collected from the ImmPort database. We constructed a free-scale gene co-expression network via weighted gene co-expression network analysis (WGCNA). Then, the intersection genes of the module genes which significantly related to EC, along with IRGs and DEGs were screened as the candidate genes for further analysis. We identified the hub gene via Venn analysis of the protein-protein interaction (PPI) network genes and the prognostic genes, and verified expression of the hub gene through Human Protein Atlas (HPA) and Gene Expression Omnibus (GEO) databases which provided the GSE17025 dataset. Furthermore, we used the CIBERSORT deconvolution algorithm to explore tumor immune cells infiltration in EC, and investigated correlations between the hub gene and immune cells.

Results: The differential expression analysis demonstrated that there were 900 up-regulated genes and 1,008 down-regulated genes in TCGA-UCEC (Uterine Corpus Endometrial Carcinoma) cohort. There were 74 candidate intersection genes in blue module genes, IRGs, and DEGs. Finally, angiopoietin 1 (*ANGPT1*) was identified as the hub gene in EC. Low expression of *ANGPT1* was associated with better overall survival (OS) in EC patients. The expression of *ANGPT1* was negatively correlated with regulatory T cells (Tregs), but positively correlated with resting memory cluster of differentiation 4 (CD4) T cells, activated dendritic cells (DCs), activated natural killer (NK) cells, and activated memory CD4 T cells ($P<0.05$, Spearman). A high-infiltrating regulatory T cell would improve the prognosis for EC patients.

Conclusions: The gene *ANGPT1* can increase the infiltration of T cells and improve the prognosis of EC patients.

Keywords: Endometrial carcinoma (EC); angiopoietin 1; immune infiltrates; bioinformatics analysis

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Introduction

The morbidity and mortality of endometrial carcinoma (EC) have been increasing in recent years. Global cancer statistical results have revealed that there were 380 thousand newly diagnosed cases and 89 thousand deaths of EC in 2018 (1).

The progress of cancer immunotherapy, especially in the field of lung cancer and melanoma, has grown rapidly in the past few years, and the relationship between the human immune system and cancer has been gradually exposed (2). Immune cells play a significant role within the tumor microenvironment (TME). In the early stage, immune cells can identify and kill most tumor cells (3-5), but some cancer cells remain alive due to their ability to functionally sculpt their microenvironment via the secretion of various cytokines, chemokines, and other factors (6). In this way, immune escape may occur, which means that tumor cells can survive by evading the attack of immune cells. This makes it more difficult to cure the cancer, especially in patients with relapse, metastasis, and advanced cancer. Therefore, new biomarkers for cancer immunotherapy might be discovered by investigating the tumor microenvironment.

A few immunotherapy drugs based on the programmed cell death protein 1/programmed death-ligand 1 (PD1/PDL1) immune checkpoint have been approved by the Food and Drug Administration (FDA) for many tumors (7-9). Nevertheless, little research and limited achievements have been made in immunotherapy for EC to date. A study of immunotherapy for advanced or recurrent EC (n=24) with PD-L1 positive expression used the treatment of pembrolizumab (anti-PD1 monoclonal antibody) and found that the objective response rate was only 13% (10). Furthermore, the majority (72%) of EC patients are grouped as lacking microsatellite instability (MSI), which was a new application marker of anti-PD1/PDL1 monoclonal antibody (11). Therefore, since the increasingly popular immunotherapy has not yet achieved much in the field of EC, newly uncovered immune-related biomarkers will play a progressively important role in improving the diagnosis and treatment of EC.

In our study, we aimed at screening immune related hub genes in EC. We investigated the co-expression genes significantly associated with EC, screening out the differentially expressed genes (DEGs). We then collected immune related genes from the ImmPort database (https://www.immport.org/), where the intersection genes of 3 parts were selected as the candidate genes. Finally, we explored the candidate genes' protein-protein interaction (PPI) network and prognosis, and identified angiopoietin 1 (*ANGPT1)* as the hub immune-related gene in EC. Many studies have indicated that *ANGPT* forms new blood vessels in cancer, providing the tumors sufficient nutrition and oxygen (12-14). Saito *et al.* demonstrated that expression of *ANGPT1* was higher in normal epithelium than in endometrium adenocarcinoma (15). In immunotherapy, Grenga *et al.* found that inhibition of the angiopoietin/TIE2 pathway neutralized the binding of Ang1 and Ang2 to TIE2, making human carcinoma cell lines in the breast, prostate, and ovary significantly more sensitive to T cell-mediated attack (16). However, the specific mechanism of *ANGPT1* in EC has not been deeply investigated.

We present the following article in accordance with the REMARK reporting checklist (available at https://dx.doi.org/10.21037/tcr-21-671).

Methods

Raw data

The transcriptome profiling and clinical data of EC were downloaded from The Carcinoma Genome Atlas (TCGA) database (https://portal.gdc.carcinoma.gov/). Immune-related genes (IRGs) were collected from the ImmPort database. The gene expression profiles of GSE17025 with 91 EC samples and 12 normal samples were collected from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/).

Weighted gene co-expression network analysis

The scale-free co-expression network of EC was constructed via the R language package weighted gene co-expression network analysis (WGCNA) (https://www.r-project.org/). Based on the high throughput gene expression profiles, the WGCNA algorithm could search significantly related genes modules of carcinoma via gene co-expression network analysis (17). We selected a soft threshold power of β=3, Minimum number of module genes' size (minModuleSize) =50, and scale-free R^2=0.9 to construct the standard scale-free network. The first principal component of genes expression matrix of each corresponding module were defined as the module eigengenes (MEs). The clinically significant module was identified from the correlation between MEs and clinical disease types. We used the most

significant module for subsequent analyses.

Screen of DEGs

We identified the DEGs via the limma package (3.44.0 version) in R software with the thresholds of log2 Fold Change |logFC| >2.0 and false discovery rate (FDR <0.05). The heatmap and volcano plot were constructed using the ggplot2 package.

Venn analysis of DEGs, IRGs, and MEs

The VennDiagram package was used to explore the intersections genes of DEGs, IRGs, and MEs. The intersection genes were deemed the candidate genes.

Enrichment function analysis

The R packages of clusterProfiler, enrichplot, and ggplot2 were used to explore the potential enrichment functions of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) of the intersection genes. A p-value and q-value less than 0.05 were selected as significant enrichment functions.

PPI network construction

The PPI network of intersection genes of blue module genes in the co-expression network, DEGs, and IRGs were constructed through the STRING website with a confident score >0.4 (https://string-db.org/). The nodes of the top 10 degree genes were calculated using the cytoHubba plug-in in Cytoscape software (https://cytoscape.org/).

Survival analysis

We used the R packages rms, survival, and survminer to analyze the prognosis of EC patients. Univariate cox regression analysis was visualized by using the forest map of the ggplot2 package. The survival curve was established by Kaplan-Meier method, and the difference was statistically significant (log-rank P<0.05).

Verify the protein expression of hub gene

The Human Protein Atlas (HPA) is an open-access database, which uses transcriptomics and proteomics techniques to study protein expression in different human tissues and organs from RNA and protein levels (https://www.proteinatlas.org/). All the data of HPA are free for scientists to use. We investigated the hub gene's protein expression levels between uterine tumor and normal tissue from immunohistochemistry (IHC) analysis provided by the HPA database.

Immune infiltration analysis

The CIBERSORT deconvolution algorithm is a method for characterizing the cell composition of complex tissues from their gene expression profiles to estimate the relative abundance of 22 immune cells types (18). We used the CIBERSORT algorithm to explore the immune infiltration in 552 EC samples. Then, to achieve quality filtering, 203 tumor samples with CIBERSORT algorithm P-values of less than 0.05 were selected for further analysis using their complete clinical information.

Statistical analysis

The soft threshold power of weighted gene co-expression network analysis were set as β=3, minModuleSize =50, and scale-free R^2=0.9 (WGCNA package). The DEGs were identified via limma package with the thresholds of log2 Fold Change |logFC| >2.0 and false discovery rate (FDR <0.05). Wilcoxon was used for difference analysis, Spearman was used for correlation analysis, P<0.05 was considered statistically significant. Kaplan-Meier method was used to plot the survival curve, and log-rank P<0.05 was considered statistically significant.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Results

Construction of the WGCN and identification of significant MEs

The TCGA-UCEC cohort with 575 samples was included to construct the WGCNA. We identified 12 modules with the average linkage hierarchical clustering, and used a dynamic pruning method to determine the MEs. Finally, we ascertained that the blue MEs with a 0.73 score were the

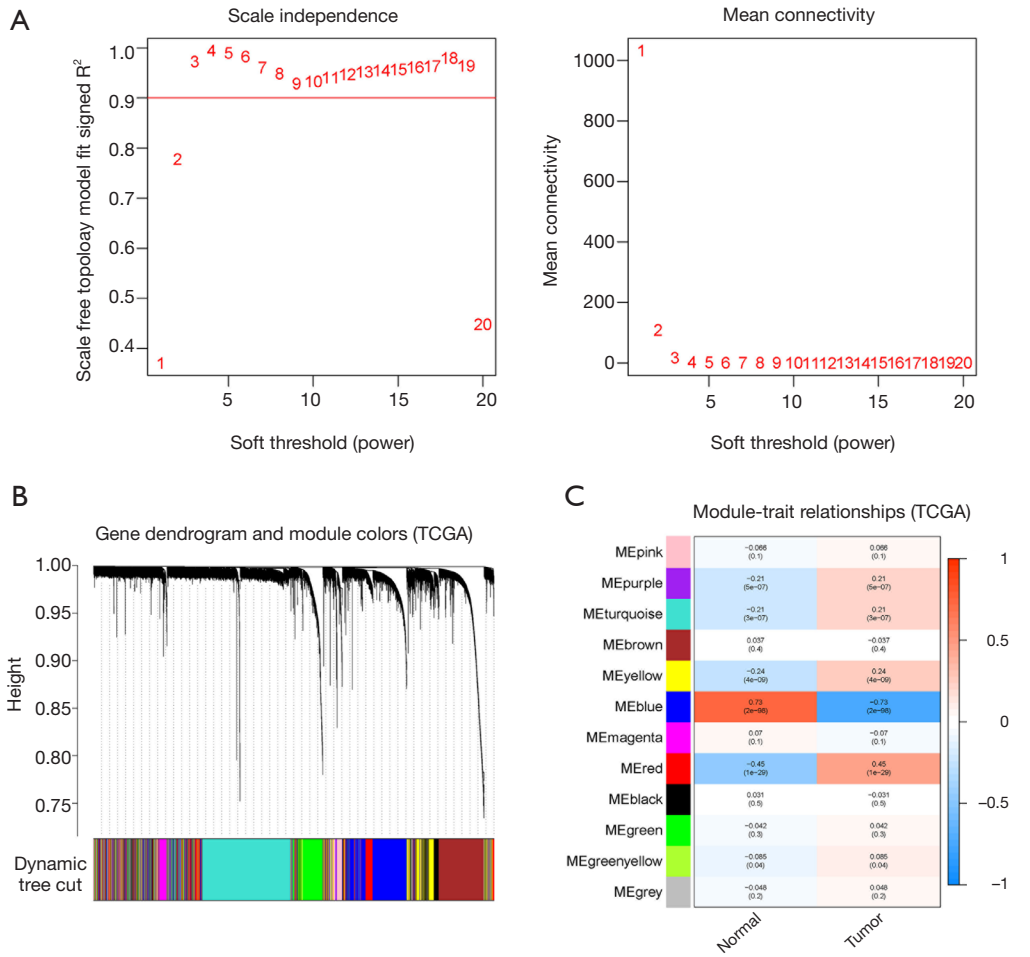

Figure 1 Identification of the soft threshold power in WGCNA. (A) Scale-free fitting index β and average connectivity of various soft threshold capabilities; (B) dendrogram of all genes clustered based on a dissimilarity measure; (C) the correlation heatmap between module eigengenes, each column contains the correlation coefficients and P value. WGCNA, weighted gene co-expression network analysis.

greatest correlation coefficients, and the genes of blue MEs were selected for the further analysis (*Figure 1*).

DEGs between endometrial tumor and normal tissues

The TCGA-UCEC cohort contains 552 tumor samples and 23 normal samples. A total of 1,008 down-regulated genes and 900 up-regulated genes (tumor *vs.* normal) were identified (*Figure 2*).

The Venn genes of blue MEs' genes in the WGCN and DEGs and IRGs

The results showed that there were 1,908 DEGs of EC, 3,162 genes in blue MEs of the co-expression network, and 1,744 genes of IRGs (ImmPort database). Finally, 74 intersection genes were selected as the candidate genes for further analysis (*Figure 3*).

Functional enrichment analysis (GO and KEGG) of candidate genes

The findings indicated that the top 5 biological processes (BP) were calcium ion homeostasis, leukocyte migration, cellular calcium ion homeostasis, cellular divalent inorganic cation homeostasis, and epithelial cell proliferation. The top 5 cell components (CC) were the external side of plasma membrane, membrane raft, membrane microdomain,

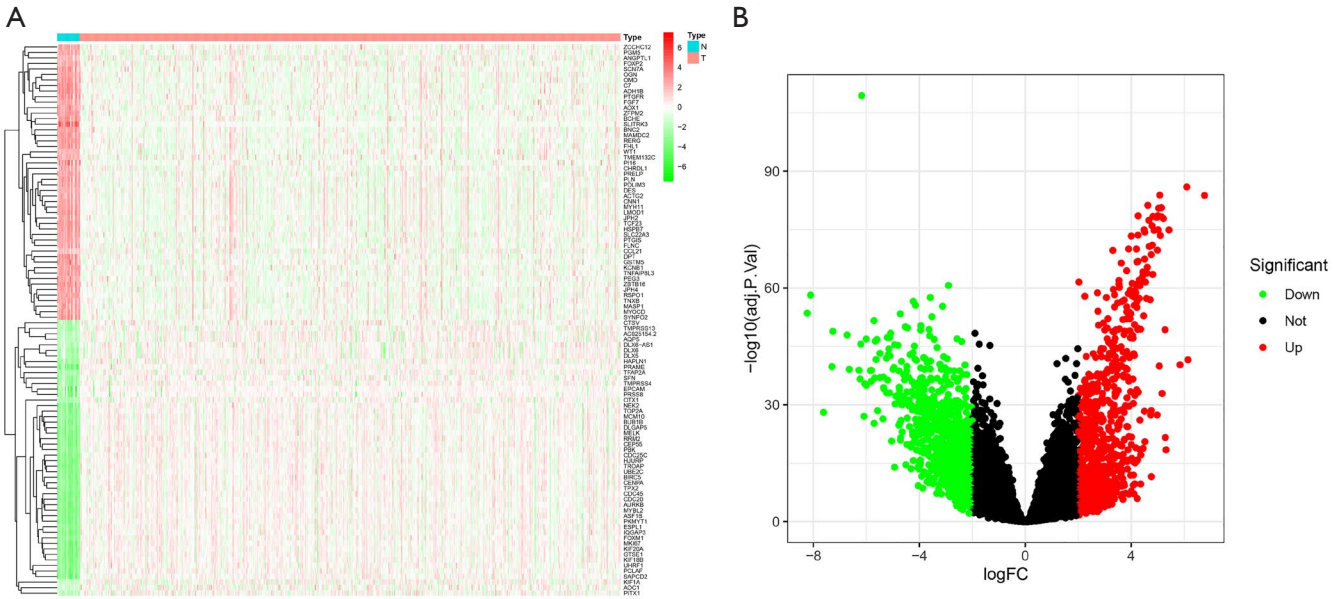

Figure 2 Analysis of DEGs of EC. Heatmap plot of the top 50 (up-regulated and down-regulated) DEGs of EC (A). Volcano plot of DEGs (B). The red represents the up-regulated genes and the green represents the down-regulated genes. DEGs, differentially expressed genes; EC, endometrial carcinoma.

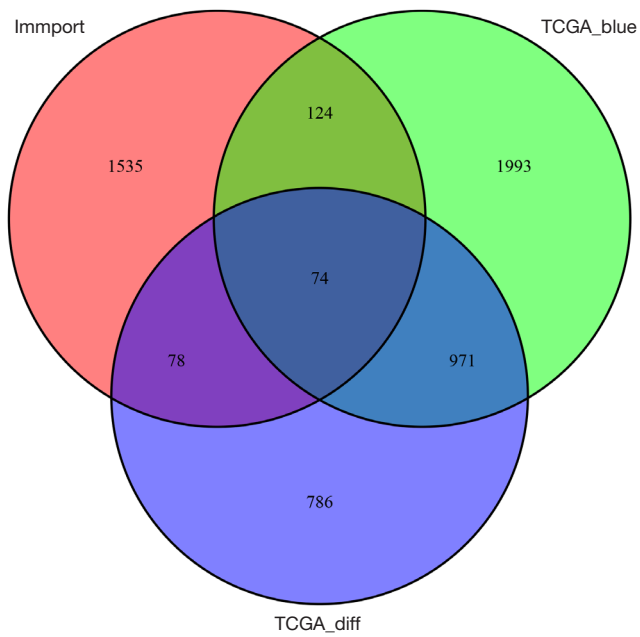

Figure 3 Venn diagram of candidate genes of EC. The pink represents of IRGs, the green represents blue MEs of co-expression network, the blue represents DEGs of EC. EC, endometrial carcinoma; IRGs, immune-related genes; MEs, module eigengenes; DEGs, differentially expressed genes

membrane region, and collagen-containing extracellular matrix. The top 5 molecular functions (MF) were the receptor ligand activity, signaling receptor activator activity, growth factor binding, G protein-coupled peptide receptor activity, and peptide receptor activity.

The top 10 pathways were the neuroactive ligand-receptor interaction, Ras signaling pathway, PI3K-Akt signaling pathway, Rap1 signaling pathway, calcium signaling pathway, MAPK signaling pathway, cytokine-cytokine receptor interaction, focal adhesion, gastric carcinoma, and cAMP signaling pathway (*Figure 4*).

PPI network

The top 10 ranking genes of degree (connecting numbers with other nodes) were respectively C-X-C motif chemokine ligand 12 (*CXCL12*), secreted phosphoprotein 1 (*SPP1*), fibroblast growth factor 2 (*FGF2*), hepatocyte growth factor (*HGF*), *ANGPT1*, fibroblast growth factor 7 (*FGF7*), cadherin 1 (*CDH1*), thrombospondin 1 (*THBS1*), platelet derived growth factor receptor alpha (*PDGFRA*), and transforming growth factor beta receptor 2 (*TGFBR2*) (*Figure 5*).

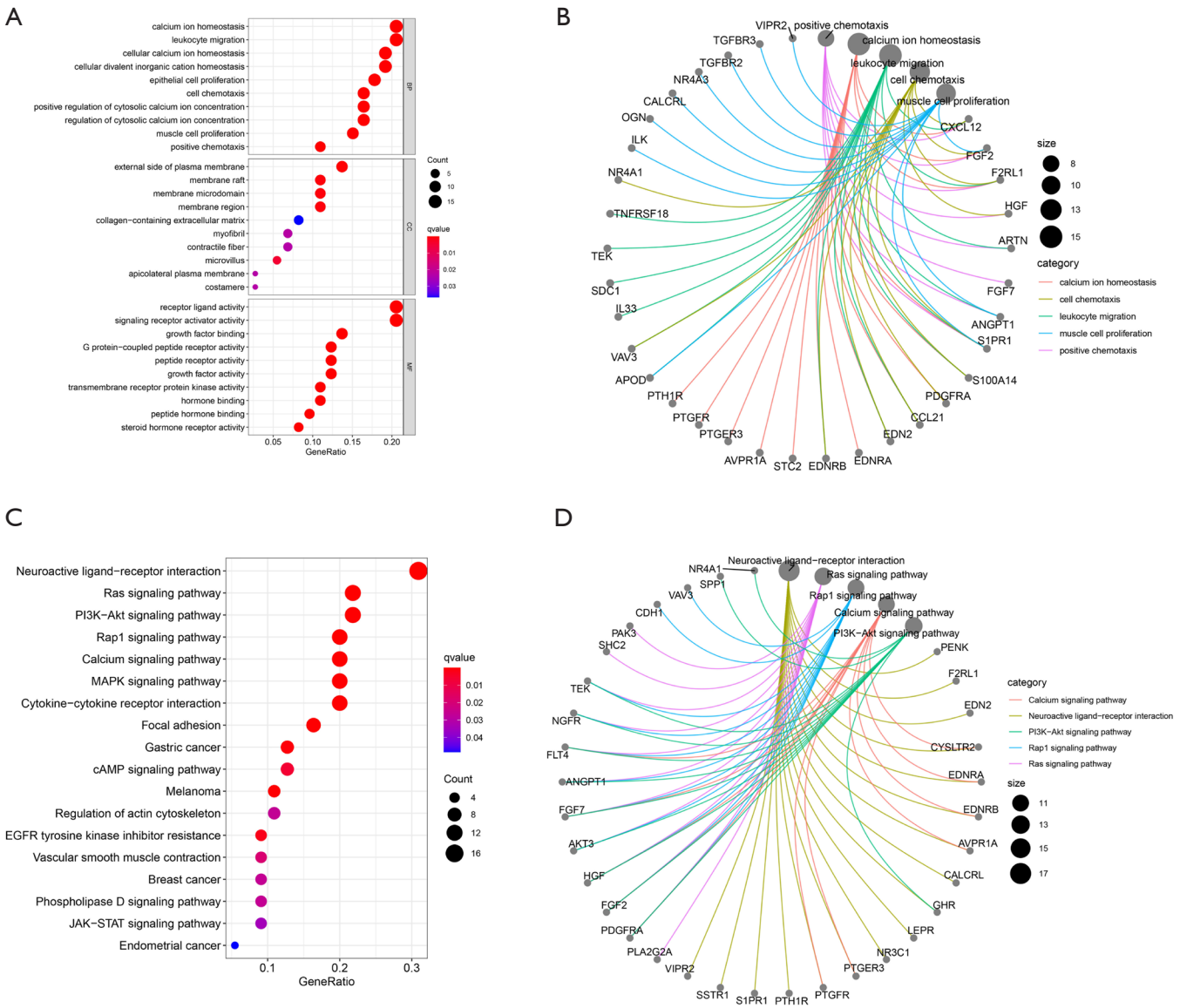

Figure 4 GO and KEGG enrichment analysis of candidate genes. The bubble diagram of GO (top 10 functions of BP, CC, and MF) enrichment analysis, red and blue dots represent q-value, the radius size of dots represent the genes count (A). The circus diagram of top 5 GO's BPs, the radius size of dots represent the genes count, and each line of color represents a different BPs (B). The bubble diagram of top 18 pathways of candidate genes (C). The circus diagram of top 5 pathways of candidate genes (D). GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological processes; CC, cell components; MF, molecular functions

Prognosis analysis of candidate genes and identification of the hub gene of EC

Prognosis analysis results showed that there were 2 protective genes and 4 detrimental genes on EC patients' overall survival (OS). We identified *ANGPT1* as the hub immune-related gene of EC via Venn analysis of the prognosis genes and the top 10 degree PPI genes (*Figure 6*).

The expression and survival analysis of ANGPT1

The expression level of *ANGPT1* was lower in EC than in normal tissue, and a lower expression of *ANGPT1* was associated with a better OS (*Figure 7*).

The verified results of ANGPT1 expression in EC

The IHC results of the HPA database showed that *ANGPT1* had high protein expression in normal endometrial tissues (*Figure 8A*) and low protein expression in EC tissues (*Figure 8B*); the GSE17025 dataset demonstrated that *ANGPT1* also had a low expression in EC (*Figure 8C*).

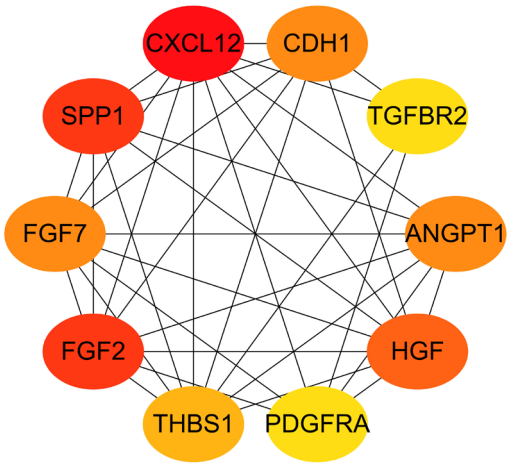

Figure 5 The top 10 ranking degree of PPI network. The color represents the connected numbers: red represents a higher number of connections number; yellow represents a lower number of connections. PPI, protein-protein interaction.

The immune infiltration analysis of EC cohort (CIBERSORT algorithm)

The CIBERSORT algorithm could calculate the relative abundance of 22 immune cells in each EC sample, and we selected 203 samples with P-values of less than 0.05 according to the CIBERSORT algorithm for further analysis. The relative abundance of 22 immune cells in 203 EC samples are shown in the histogram (*Figure 9*).

Furthermore, we explored the correlation between *ANGPT1* and immune cells, and the results showed that the expression of *ANGPT1* was negatively correlated with regulatory T cells (Tregs) (*Figure 10A*) and activated natural killer (NK) cells (*Figure 10B*), while it was positively correlated with the resting memory cluster of differentiation 4 (CD4) T cells (*Figure 10C*), activated memory CD4 T cells (*Figure 10D*), and activated dendritic cells (DCs) (*Figure 10E*).

The Kaplan-Meier (grouping by the median of abundance) survival analysis results of 22 immune cells indicated that high infiltration of Tregs (P=0.005) and CD8 T cells (0.066) improved OS in EC patients (*Figure 11*).

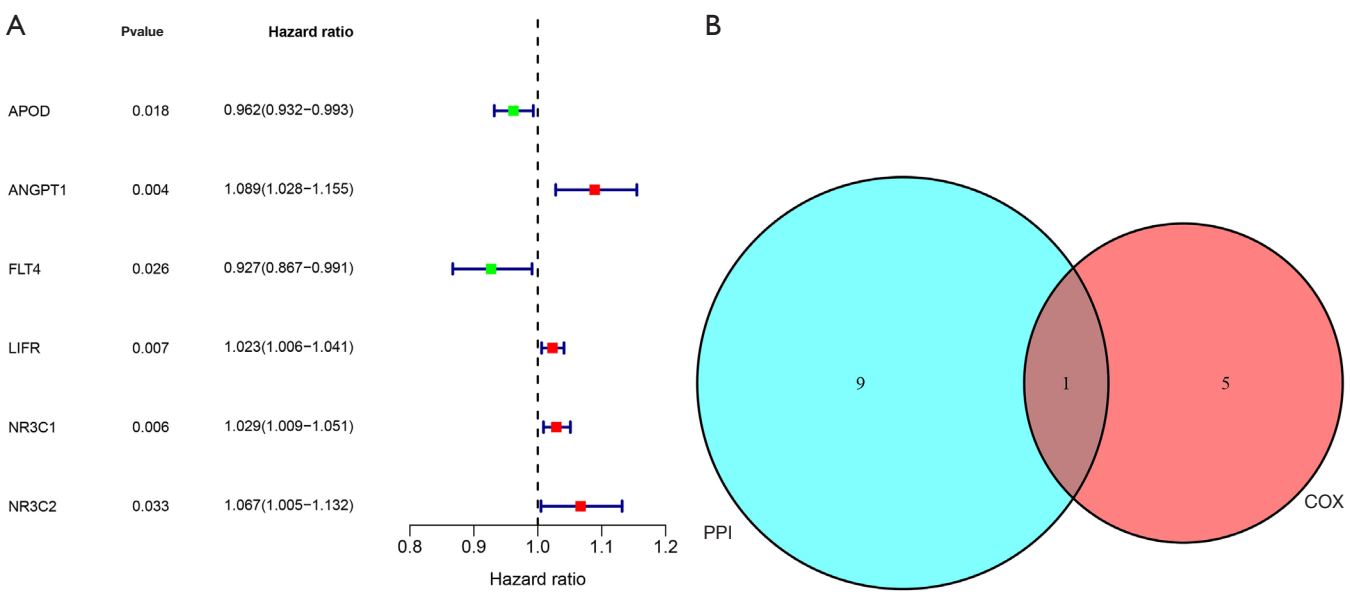

Figure 6 Identification of the hub immune-related gene of EC. Forest diagram showed the prognostic genes of EC. The HR represents the OS ratio (A). Venn diagram the top 10 degree PPI genes and prognostic genes of EC (B). EC, endometrial carcinoma; HR, hazard ratio; OS, overall survival.

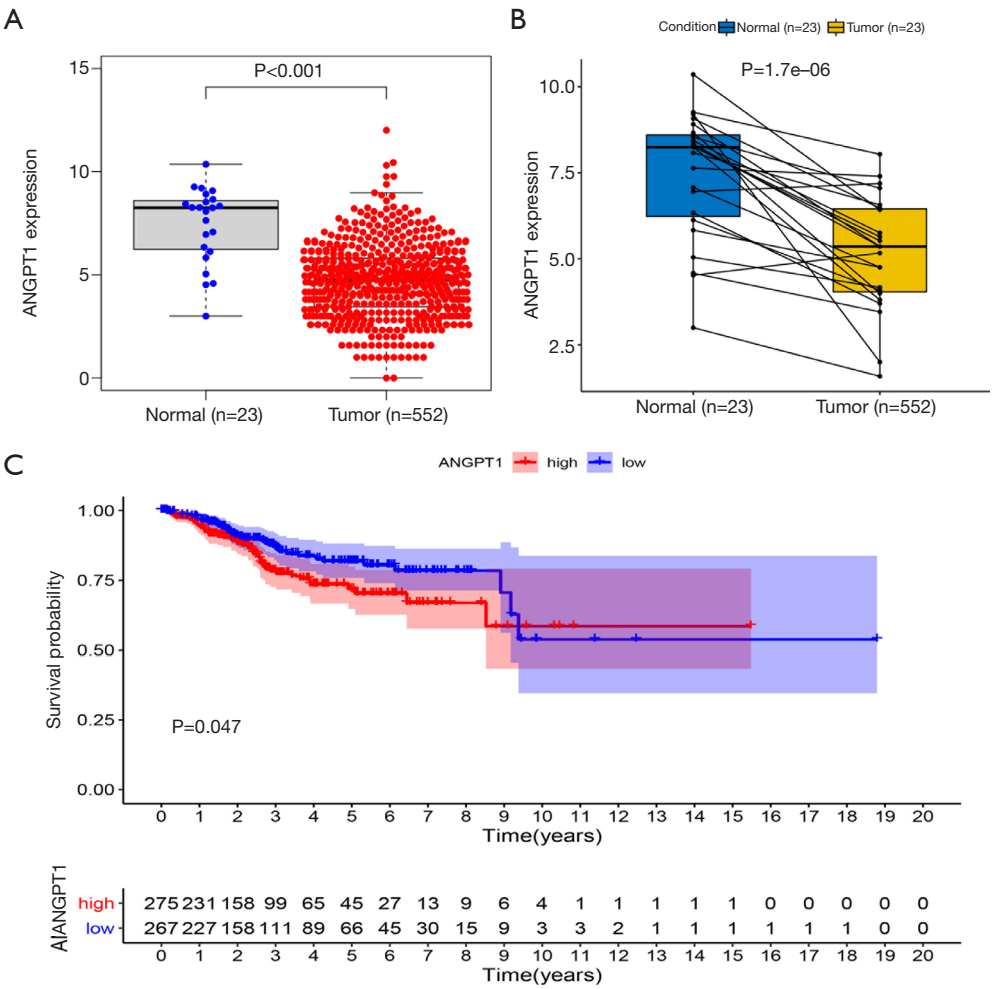

Figure 7 The expression and survival analysis of *ANGPT1*. There was low expression of *ANGPT1* in EC tissue, TCGA-UCEC cohort with 552 tumor samples compare to 23 normal samples (A). Comparison between 23 paired samples (B). Low expression level of *ANGPT1* had a better OS in EC patients (C). EC, endometrial carcinoma; TCGA-UCEC, The Carcinoma Genome Atlas-Uterine Corpus Endometrial Carcinoma; OS, overall survival.

Discussion

In the present study, we identified *ANGPT1* as hub immune-related gene in EC. Firstly, we identified the co-expression genes module associated with EC through WGCNA based on TCGA-UCEC cohort. Secondly, we screened the intersection genes from module genes, IRGs, and DEGs for prognostic analysis and PPI network. Finally, we investigated the correlation between *ANGPT1* and immune cells. Our results indicated that *ANGPT1* was down-regulated in EC patients, while decreased expression of *ANGPT1* was associated with a better outcome in EC patients. Furthermore, Tregs were high-infiltrating in EC, and *ANGPT1* was negatively correlated with Tregs.

The gene *ANGPT1* encodes a secreted glycoprotein that belongs to the angiopoietin family (19), it binds to the endothelial cell membrane receptor tyrosine kinase w (also known as TEK) to contribute to tumor angiogenesis (20,21). In the present study, *ANGPT1* was shown to be low-expressed in EC tissues. Saito *et al.* also found that the expression of *ANGPT1* was lower in endometrial adenocarcinoma than in normal epithelium (15), which was consistent with our findings. Our results also demonstrated that *ANGPT1* was associated with poor prognosis, and the overexpression of *ANGPT1* resulted in reduced OS in EC patients,

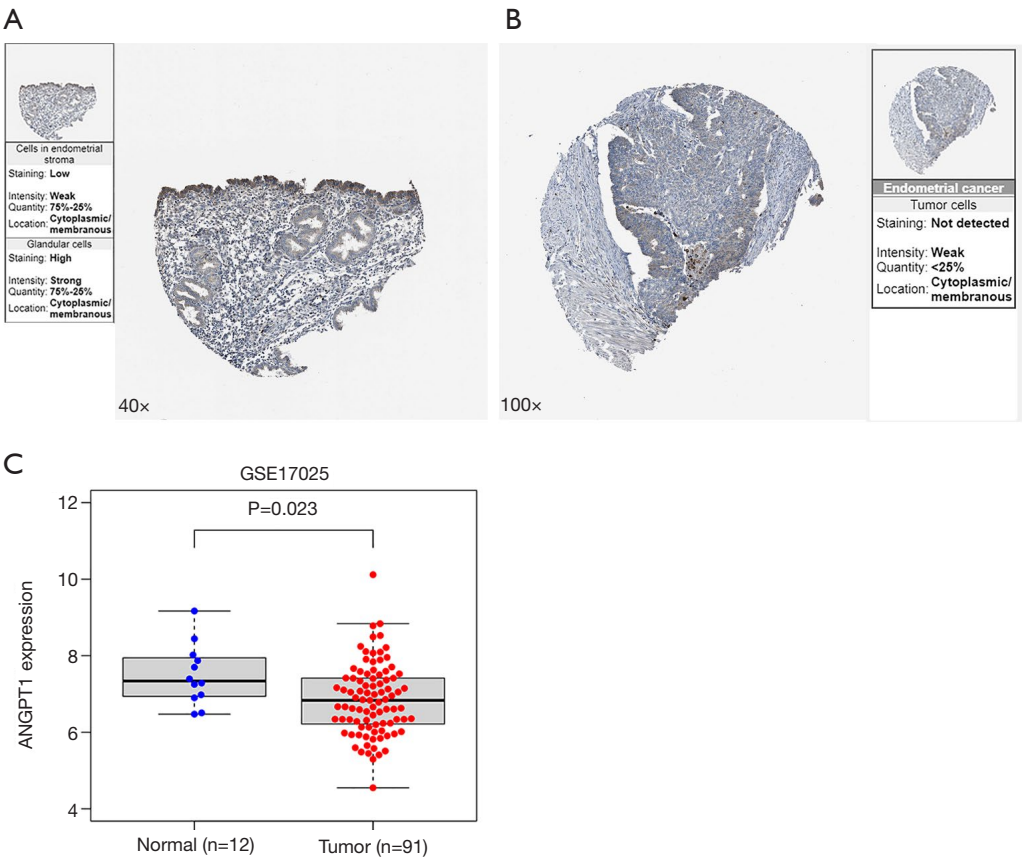

Figure 8 Expression of *ANGPT1* in EC and normal tissue (immunohistochemistry). Protein expression level in normal uterine tissue (40×) (A). Protein expression level in uterine carcinoma tissue (100×) (B). Expression level of *ANGPT1* in EC and normal tissue in the GSE17025 dataset (C). EC, endometrial carcinoma

which might be associated with tumor microenvironment immune cells. Recent studies have shown that some anti-angiogenic therapies have immunomodulatory effects in both peripheral and tumor microenvironments (16,22). Antiangiogenic drugs tyrosine kinase inhibitors (TKIs) can target vascular endothelial growth factor (VEGF) and its receptors to inhibition of the angiopoietin/TIE2 pathway to delay the growth and development of tumors (23). Finke *et al.* and Grenga *et al.* found that the TKIs sunitinib and sorafenib altered the immune landscape and reduced the number or function of immunosuppressive cells (16,24). Furthermore, an anti-angiogenic TKIs combined vaccine can enhance the anti-tumor activity, which may be due to the activation of lymphocytes and myelocytes by the tumor microenvironment, normalization of blood vessels, decrease of tumor cell density, and improvement of vascular perfusion and oxygenation (25). A phase II trial which aimed to investigate the efficacy of trebananib (a TKI inhibitor) in recurrent/persistent EC indicated that the objective response rate (ORR) was 43.7% (26). Despite the limited efficacy, this showed the potential of TKI inhibitors in the treatment of EC. Since *ANGPT1* is the 1 of the regulators of angiogenesis for EC, it is a potential therapeutic target for EC patients.

Enrichment function analysis of *ANGPT1* showed that it is an essential part for leukocyte migration, epithelial cell proliferation, membrane regulation, growth factor binding, Ras signaling pathway, PI3K-Akt signaling pathway, Rap1 signaling pathway, MAPK signaling pathway, cytokine-cytokine receptor interaction, and so on. The enrichment functions results indicated that *ANGPT1* participates in in various cancer processes. Activated leukocyte cell adhesion molecule is an adverse biomarker for endometrioid EC via promoting cancer cell migration, invasion, and metastasis (27). This indicates that leukocyte cells have a significant role in progression for endometrioid EC.

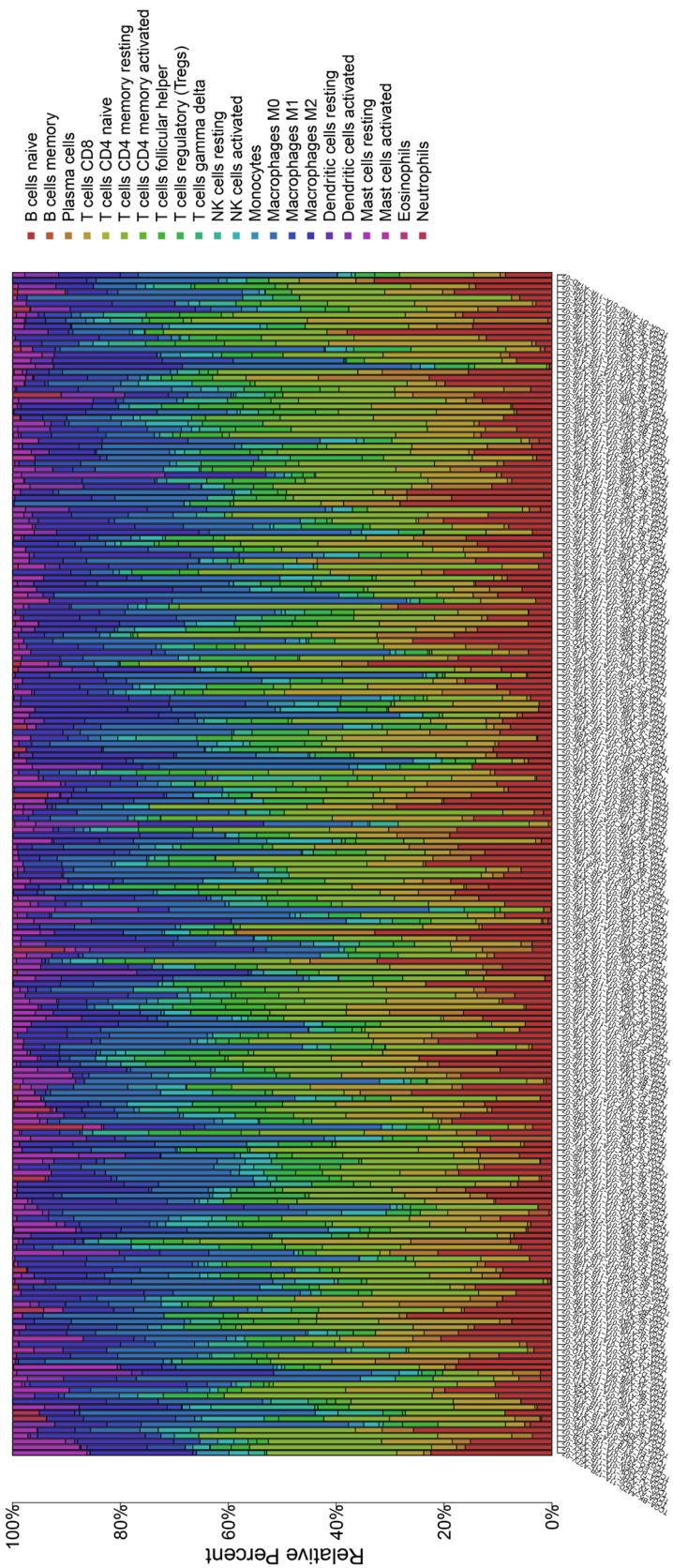

Figure 9 The immune infiltration analysis of EC. The relative abundance of 22 immune cells in 203 EC samples, X-axis represents individual sample, Y-axis represents relative abundance of immune cells. Individual color represents individual immune cells. EC, endometrial carcinoma.

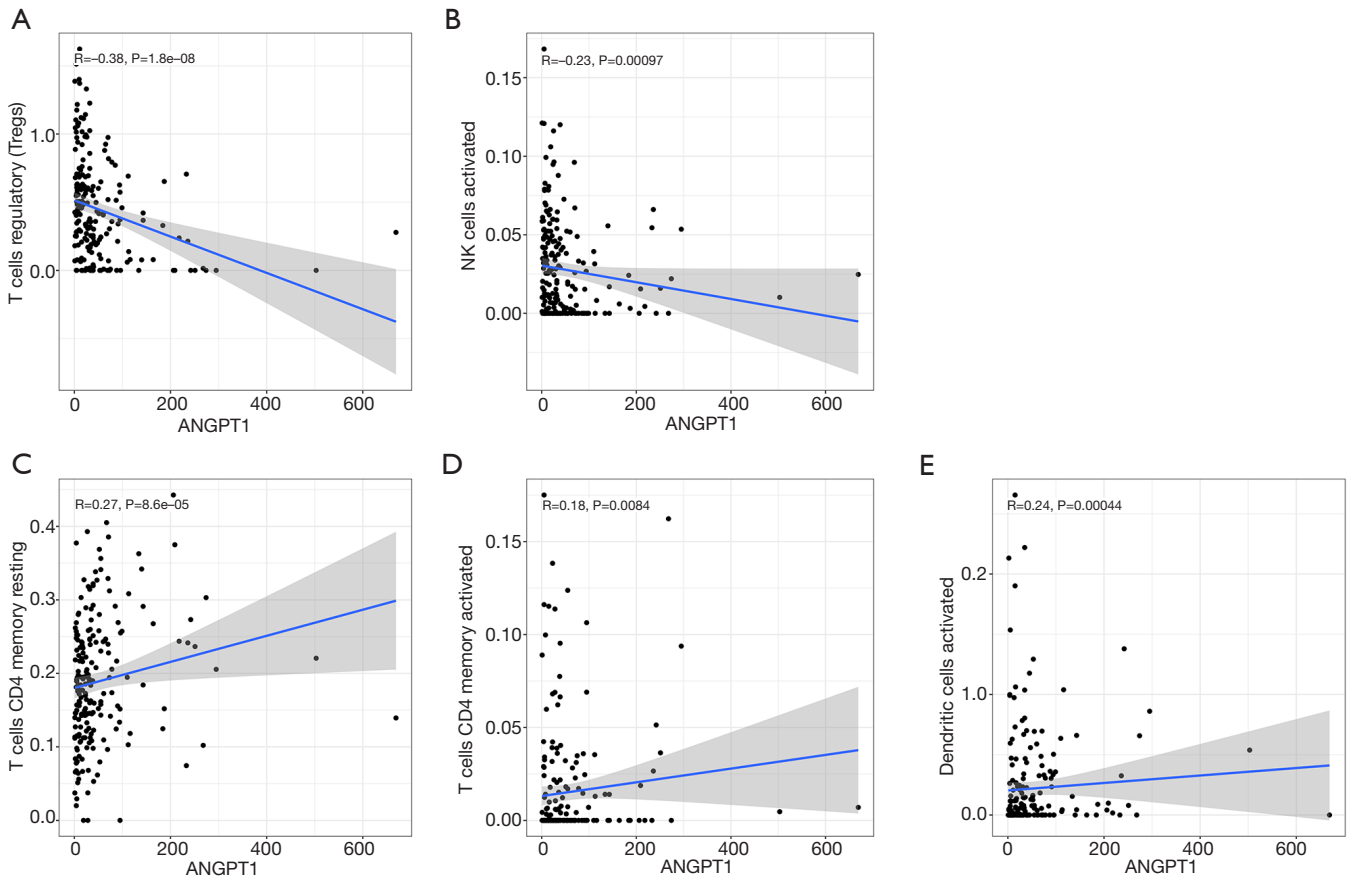

Figure 10 The correlation between *ANGPT1* and immune cells (Spearman). Tregs (A); activated NK cells (B); resting memory CD4 T cells (C); activated memory CD4T cells (D); activated DCs (E). Tregs, T cells regulatory; NK, natural killer; CD4, cluster of differentiation 4; DC, dendritic cells.

Leukocyte cells have also been shown to include numbers of immune cells, which also indicated that the immune cells infiltration into TME may have a deep influence on EC patients. Regrettably, the association between *ANGPT1* and immune cell infiltrations of EC remains unclear. The *ANGPT1* is also an important regulator of epithelial cell proliferation; it is well known that EC is an epithelial cancer, and cell proliferation and growth are critical processes for tumor development. Cancer cells with a higher ability of proliferation and growth may indicate poorer outcomes. Several studies have investigated the inhibition of epithelial EC cell proliferation and growth via microRNA-29c or silencing the gene expression (28,29). Further, *ANGPT1* also has an essential role in several signaling pathways in EC. Numerous studies have supported that the Ras signaling pathway, PI3K-Akt signaling pathway, Rap1 signaling pathway, and MAPK signaling pathway were important pathways in tumorigenesis, drug resistance, and progression of EC (30-34). In addition to several cancer pathways, *ANGPT1* is also a significant regulator of cytokine-cytokine receptor interaction, and the PPI network showed that *ANGPT1* interacted with CXCL12, FGF2, HGF, PDGFRA, and so on; most of them were the cytokine or cytokine receptor, as well as the regulator for the immune system (35-39). It has been shown that CXCL12 and FGF2 are adverse markers for UCEC via promotion of the proliferation, migration, and invasion of cancer cells (40,41). The interaction of *ANGPT1* and other genes showed that *ANGPT1* may be a significant regulator of the TME for EC.

In exploring the expression levels of *ANGPT1* and its prognostic role for EC patients, we found that *ANGPT1* was down-regulated in EC, and the lower expression of *ANGPT1* was associated with longer lifespan in TCGA

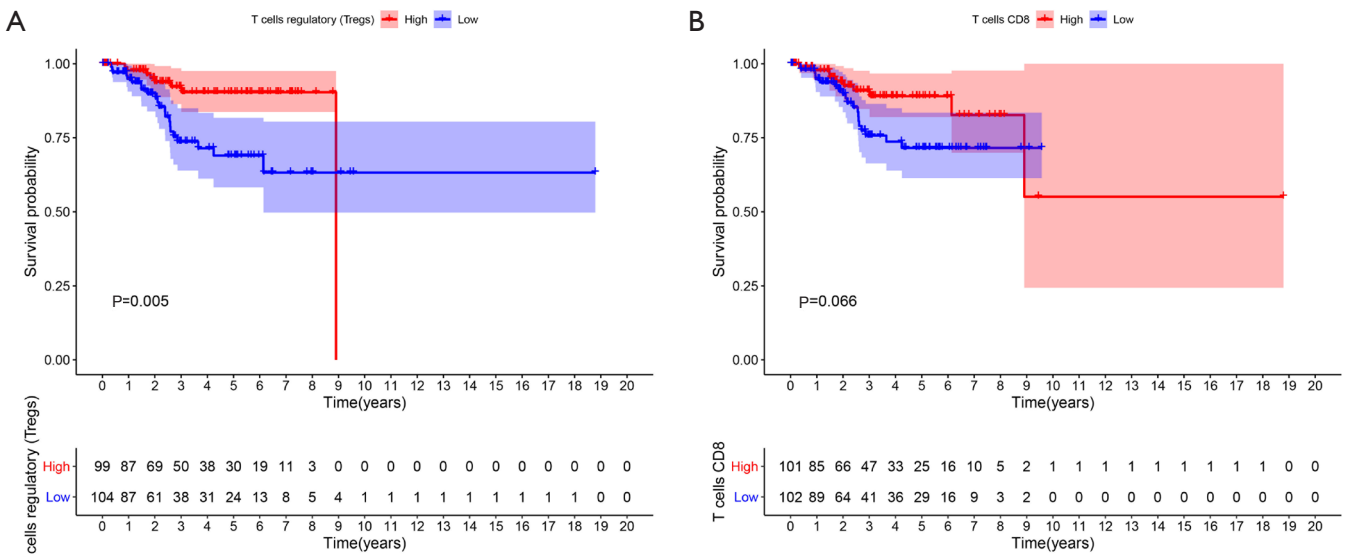

Figure 11 Kaplan-Meier survival analysis of immune cells in EC patients. Tregs (A); T cells CD8 (B). EC, endometrial carcinoma; Tregs, T cells regulatory; CD8, cluster of differentiation 8.

database analysis. This was validated via the expression levels of *ANGPT1* in the GSE17025 dataset. Nevertheless, *ANGPT1* is not only low-expressed in EC, but also in epithelial ovarian cancer and bladder cancer (42,43). However, the role of *ANGPT1* in EC remains unclear. Flores-Pérez *et al.* showed that *ANGPT1* and *TGFBR2* were positive regulators of angiogenesis in breast cancer (44), indicating that *ANGPT1* acted as an adverse marker for breast cancer patients. Despite of its role in breast cancer, *ANGPT1* seemed to act as a tumor suppressor in lung cancer (45).

To date, with the better understanding of the TME in cancer, there is no doubt that TME plays a pivotal role in tumor occurrence, progression, drug resistance, and recurrence for EC (46-48). Since the enrichment functions and PPI network showed that *ANGPT1* has a significant role or interaction in immune cell, cytokine, and cytokine receptor, we also investigated the relationship between *ANGPT1* and the immune cell infiltration for EC. The results showed that *ANGPT1* was negatively correlated with Tregs and activated NK cells. Witkiewicz *et al.* showed that there were high levels of NK cells but decreased Tregs in complex atypical endometrial hyperplasia and well-differentiated carcinoma treated with progestins (49). Further, a study by Degos *et al.* indicated that the TME can reshape NK cell phenotype and function to promote tumor progression (50); this finding showed us that NK cells can be changed in TME and promote the tumor progression, which contrasted the prior theory that NK cells only act protectively for cancer patients. The association between immune cell infiltration and its effect on the prognosis of EC patients showed that high infiltration of Tregs and CD8 T cells lengthens the OS of EC patients. Meanwhile, *ANGPT1* was positively correlated with the resting memory of CD4 T cells, activated memory CD4 T cells, and activated DCs. Zhang *et al.* supported that the higher the expression of tumor-infiltrating immune cell (TIC) of CD4+ cells, the better outcome of EC patients (51). There have been DCs detected in the TME of EC as well, and the S100- and HLA-DR-positive DCs may act as tumor suppressors (52). From these findings, *ANGPT1* is positively correlated with protection from immune cells infiltrate, but negatively correlated with the promoting tumor progression TICs in the TME of EC; thus *ANGPT1* is a favorable IRG for EC patients.

Conclusions

It was shown that *ANGPT1* was downregulated in EC patients, and its low expression indicated a better prognosis of EC patients. The enrichment functions of *ANGPT1* showed that it is an essential regulator for immune response for EC. Analysis of the association between *ANGPT1* and TICs showed that it has a positive correlation with protect

immune cells infiltrate such as CD4 T cells, activated memory CD4 T cells, and activated DCs, but is negatively correlated with the promoting tumor progression TICs such as such as Tregs and activated NK cells in the TME of EC, thus *ANGPT1* is an adverse IRG for EC patients.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://dx.doi.org/10.21037/tcr-21-671

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/tcr-21-671). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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