

Bioinformatics Analysis of Immune Infiltration In Endometrial Carcinoma Tumors and its Relationship With Prognosis

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Abstract. Background/Aim: Endometrial carcinoma (EC) is the most common gynecological cancer, but lacks specific targetable markers. In order to explore the immune-related molecules that affect the progression and prognosis of EC, we analyzed the differential expression of genes in different histological grades of the disease. Materials and Methods: EC-related gene-expression data of different histological grades were downloaded from TCGA and GEO databases. The list of immune-related genes was obtained from the

ImmPort database. In order to identify differentially-expressed genes (DEGs), differential-expression analysis was performed. The intersection of DEGs and immune-related genes was termed immune-related differentially-expressed genes (IRDEGs). IRDEGs were enriched in cancer-related functional pathways by gene-correlation analysis and GSEA-enrichment analysis. The association of IRDEGs with immune-cell tumor infiltration and gene polymorphisms was analyzed using IRDEG mRNA and protein-expression data in EC from TCGA and THPA databases. Results: Three IRDEGs, TNFSF15, SEMA3E and TNFSF10, were involved in the analysis of the prognosis of EC patients. IRDEGs were not only related to clinical characteristics but could also affect the prognosis of patients. Gene-correlation and GSEA-enrichment analysis of IRDEGs showed that TNFSF15 and TNFSF10 were co-enriched in the IL2-STAT5 functional pathway. IRDEGs had a significant correlation with a variety of immune-cell types infiltrating EC tumors and were related to EC prognosis. IRDEG mRNA- and protein-expression levels were increased in EC compared to normal tissues. Conclusion: TNFSF15, SEMA3E and TNFSF10 may regulate the progression and prognosis of EC patients by affecting immune-cell infiltration of EC tumors.

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Key Words: Endometrial carcinoma, bioinformatics, gene databases, differential gene expression, histological grade, correlation, diagnosis, immune-cell infiltration, prognosis.

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Gynecological malignancies mainly include cervical cancer, endometrial carcinoma (EC) and ovarian cancer. EC is the most common gynecological cancer in the world (1, 2). It mainly occurs in post-menopausal women with an average age of 60 at diagnosis (3). At present, EC lacks specific targetable markers, and the treatment efficacy of advanced cancer is poor (4).

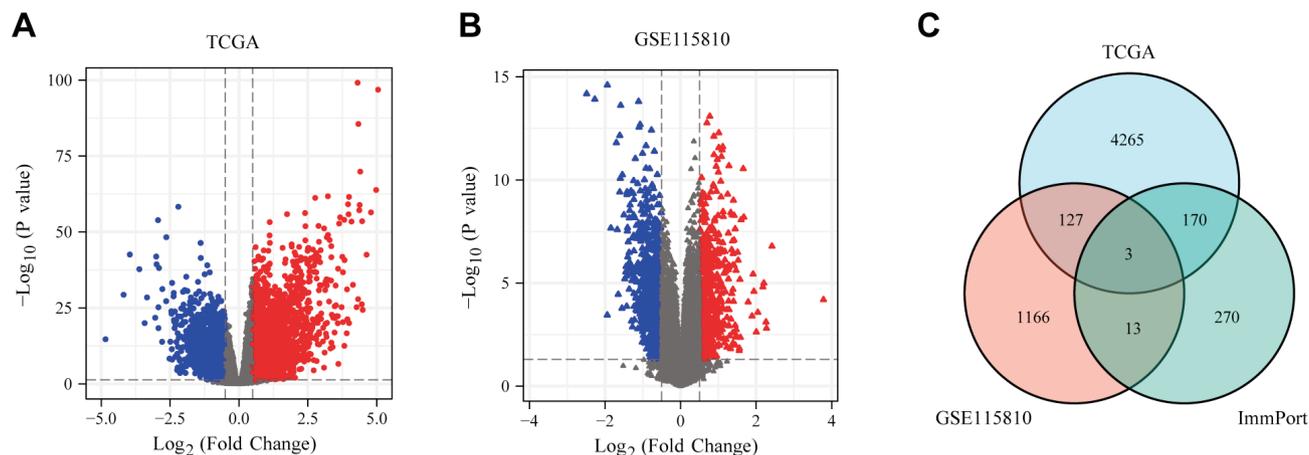


Figure 1. Screening for IRDEGs. (A) DEGs in TCGA database; (B) DEGs in the GSE115810 dataset; (C) Intersection of DEGs in TCGA and GSE115810 datasets and immune-related genes in the ImmPort database. IRDEGs: Immune-related differentially-expressed genes; DEGs: differentially expressed genes; TCGA, The Cancer Genome Atlas.

The goal of immunotherapy is to reverse the immune-escape mechanism of tumors and restore the local immune response to cancer cells (5, 6). The influence of immune-cell infiltration in tumors is very important, because the type of immune-cell infiltration in tumors mainly determines whether the balance tends to an anti-tumor or tumor-promotion immune response (7). Since the first PD-1/PD-L1 inhibitors were approved by the Food and Drug Administration (FDA) in 2014, many immune-checkpoint inhibitors have been used in clinical practice (8). Therefore, exploring the key immune-regulation genes and signaling pathways related to EC may clarify the critical molecular mechanisms of EC and improve the level of diagnosis and immunotherapy.

In the present study, The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO) and ImmPort databases were used to screen for immune-related differentially-expressed genes (IRDEGs) in EC, and identify their relationships with clinico-pathological parameters and prognosis. TIMER and cBioPortal databases were used to analyze the correlation between IRDEGs and immune-cell types present in EC tumors as well as gene polymorphisms, in order to provide a basis for the discovery of new immune targets for EC.

Materials and Methods

Data acquisition. All gene-expression profile data and clinical data of EC patients containing histological-grading information were downloaded and collated from TCGA database (<https://cancergenome.nih.gov/>). 541 EC samples were included, of which 218 samples were G1-G2 grade and 323 samples in G3 grade. We combined EC gene-expression data with histological information from the GEO database, GSE115810, including 18 samples of G1-G2 grade and 6 samples of G3 grade. The ImmPort

database (<https://www.immport.org/home>) was used to identify immune-related gene groups.

Screening for IRDEGs. The differentially-expressed genes (DEGs) in G1-G2 and G3 grades of EC were analyzed with the “DESeq2” package of R software (V 4.0.4). The screening thresholds were \log_2 fold change (FC) $> |0.5|$ and p -value < 0.05 . The “ggplot2” package of R was used to visualize DEGs. In addition, the intersection of DEGs and immune-related genes in the ImmPort database was obtained with the “VennDiagram” program package.

The relationship between IRDEG and clinico-pathological parameters and prognosis of endometrial carcinoma. The clinical data of patients were obtained from TCGA database, and the relationship between IRDEGs was determined from the above-mentioned intersection and clinical stage, histological type and tumor invasion of EC patients. The survival data in TCGA database were used to analyze the relationship of IRDEGs to the prognosis of EC patients, including overall survival (OS), disease-specific survival (DSS) and platinum-free interval (PFI).

Gene Set Enrichment Analysis (GSEA). GSEA is a computational method that can be used to analyze the potential functional pathways of a single gene. First, we used TCGA database to analyze the IRDEGs, and then sorted the relevant genes as the input gene set, in order to determine their enrichment in different biological functions and signal pathways.

The “ClusterProfiler” package of R software, select “h.all.v7.2.symbols.gmt” was used as the annotation gene set, and selected 1000 replacement times. In the results, a normalized enrichment score (NES) > 1 and $\text{adj}p < 0.05$ were considered to be statistically significant.

Relationship between IRDEGs and immune-cell infiltration in EC tumors. The “GSVA” package of R software was used to analyze the relationship between IRDEGs and immune cells infiltrating EC

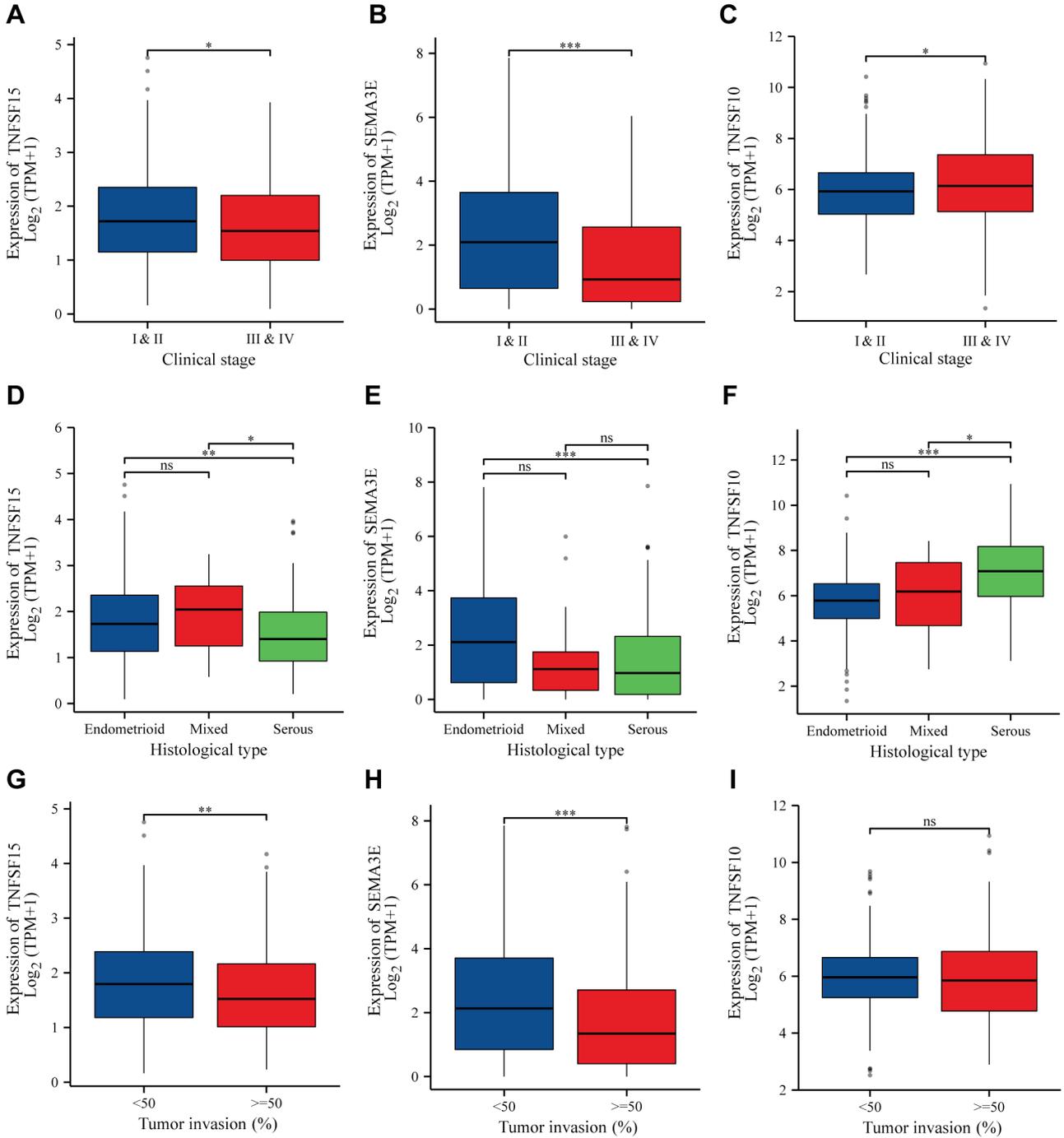


Figure 2. Relationship between IRDEGs and clinico-pathological parameters of EC. (A-C) IRDEGs correlation with clinical stage; (D-F) with histological type; and (G-I) with tumor invasion. IRDEGs: immune-related differential-expressed genes; EC: endometrial carcinoma.

tumors. The “ssGSEA” algorithm built into the “GSVA” package was used as the immune-infiltration algorithm. TIMER2.0 (<http://timer.cistrome.org/>) was used to provide comprehensive analysis and visualization of EC tumor-infiltrating immune cells, which was used to study the association between tumor immune infiltration and genetic or clinical characteristics (9). We further

analyzed the relationship between IRDEGs and immune cells in EC tumors; and between immune cells in EC tumors and EC prognosis using the TIMER2.0 database.

Relationship between IRDEGs and gene polymorphisms. The cBioPortal for Cancer Genomics (<http://cbioportal.org>) provides a

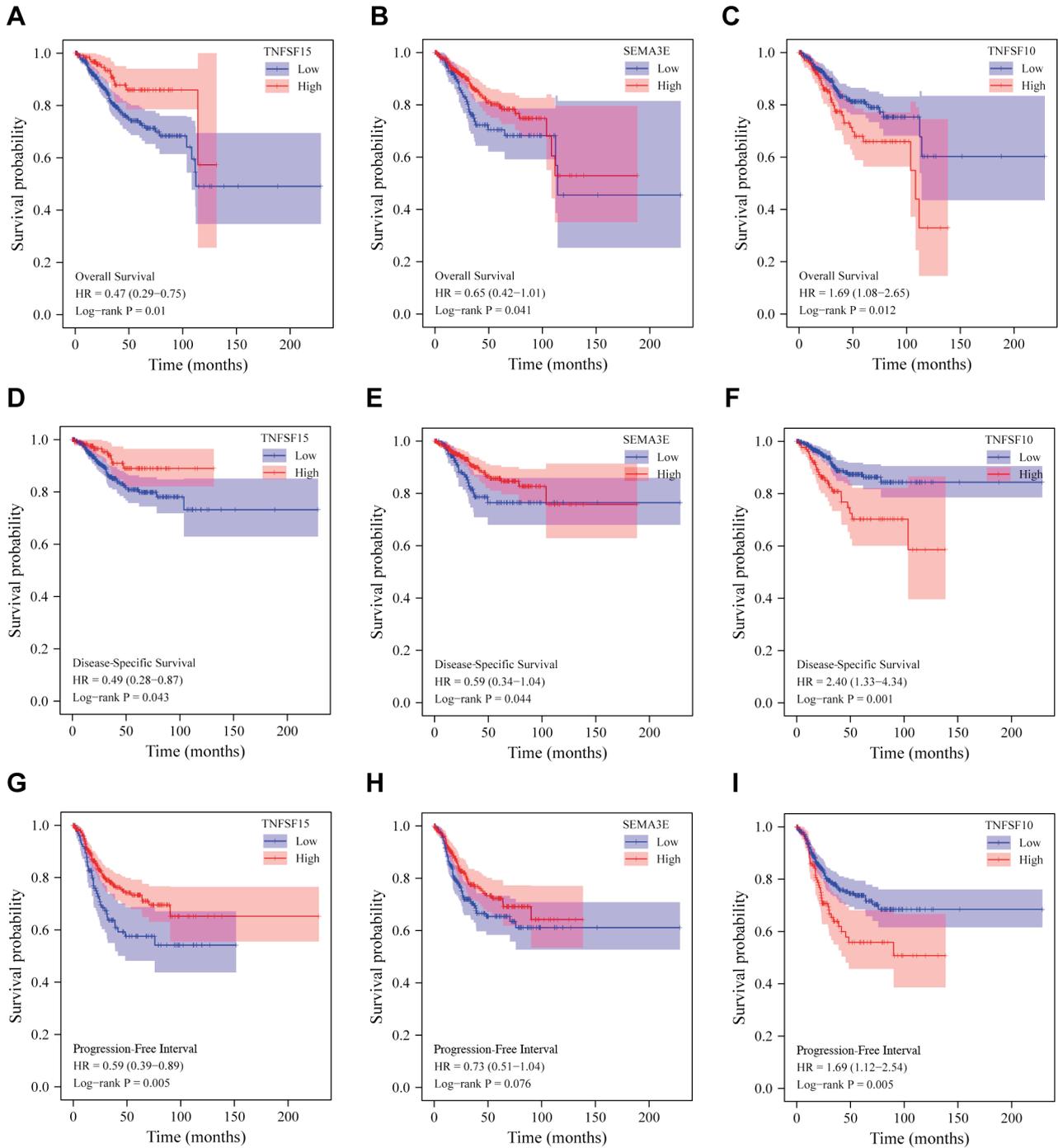


Figure 3. Relationship between IREDGs and prognosis of EC patients. (A, D, G) TNFSF15; (B, E, H) SEMA3E; (C, F, I) TNFSF10. IRDEGs: Immune-related differential-expressed genes; EC: endometrial carcinoma.

Web resource for exploring, identifying, and analyzing multi-dimensional cancer-genomics data (10). The cBioPortal database was used to analyze the relationship between IRDEGs and gene polymorphisms.

Analysis of mRNA and protein expression levels of IRDEGs in EC. TCGA database was used to further analyze the differential expression of IRDEG mRNA in EC. The human-protein expression atlas (THPA) (<https://www.protein.atlas.org/>) provides the

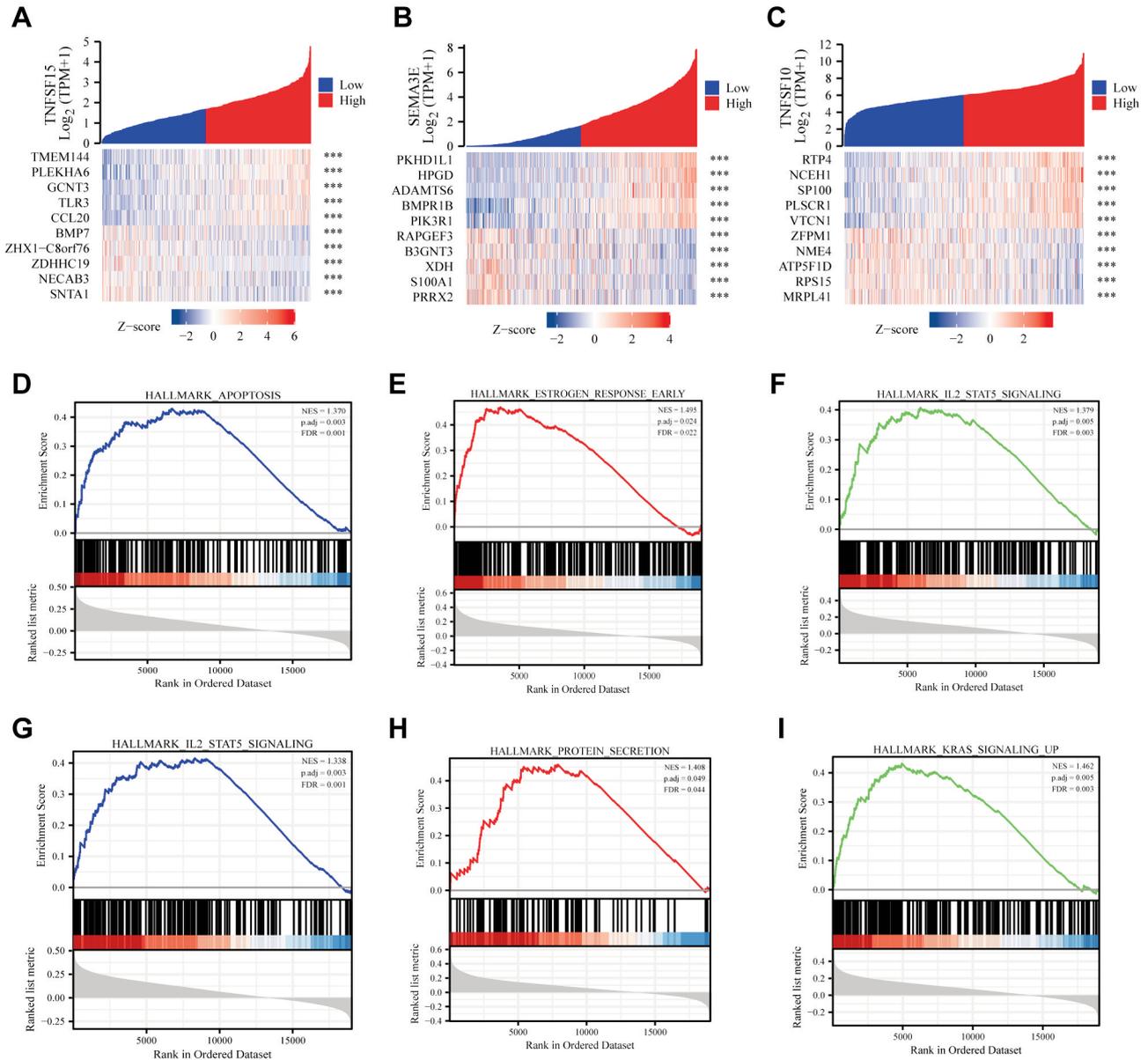


Figure 4. Gene correlation analysis of IRDEGs and enrichment analysis of GSEA. (A-C) The top 10 genes significantly related to TNFSF15, SEMA3E and TNFSF10, respectively; Two functional pathways significantly related to TNFSF15 (D-E); SEMA3E (F-G); and TNFSF10 (H-I), respectively. IRDEGs: Immune-related differential-expressed genes; GSEA: Gene Set Enrichment Analysis.

expression maps of more than 24000 human proteins in various pathological and normal tissues. Through this database, the protein-level expression of IRDEGs in EC and normal tissues was analyzed.

Results

Identification of IRDEGs in EC. Differential gene-expression was investigated in 218 cases of G1-G2 and 313 cases of G3 EC in the TCGA database. Based on log2FC and *p*-values, 4,565 differentially-expressed mRNAs were obtained,

including 2,822 up-regulated and 1,743 down-regulated DEGs (Figure 1A). Similarly, according to threshold screening, 1,309 DEGs were obtained from the GSE89102 dataset, of which 608 were up-regulated and 701 were down-regulated (Figure 1B). In the data from TCGA and GSE89102, there are 130 common DEGs, of which 3 genes are IRDEGs: TNFSF15, SEMA3E and TNFSF10 (Figure 1C).

Correlation of IRDEGs with clinico-pathological parameters of EC. By analyzing the relationship between IRDEGs and EC

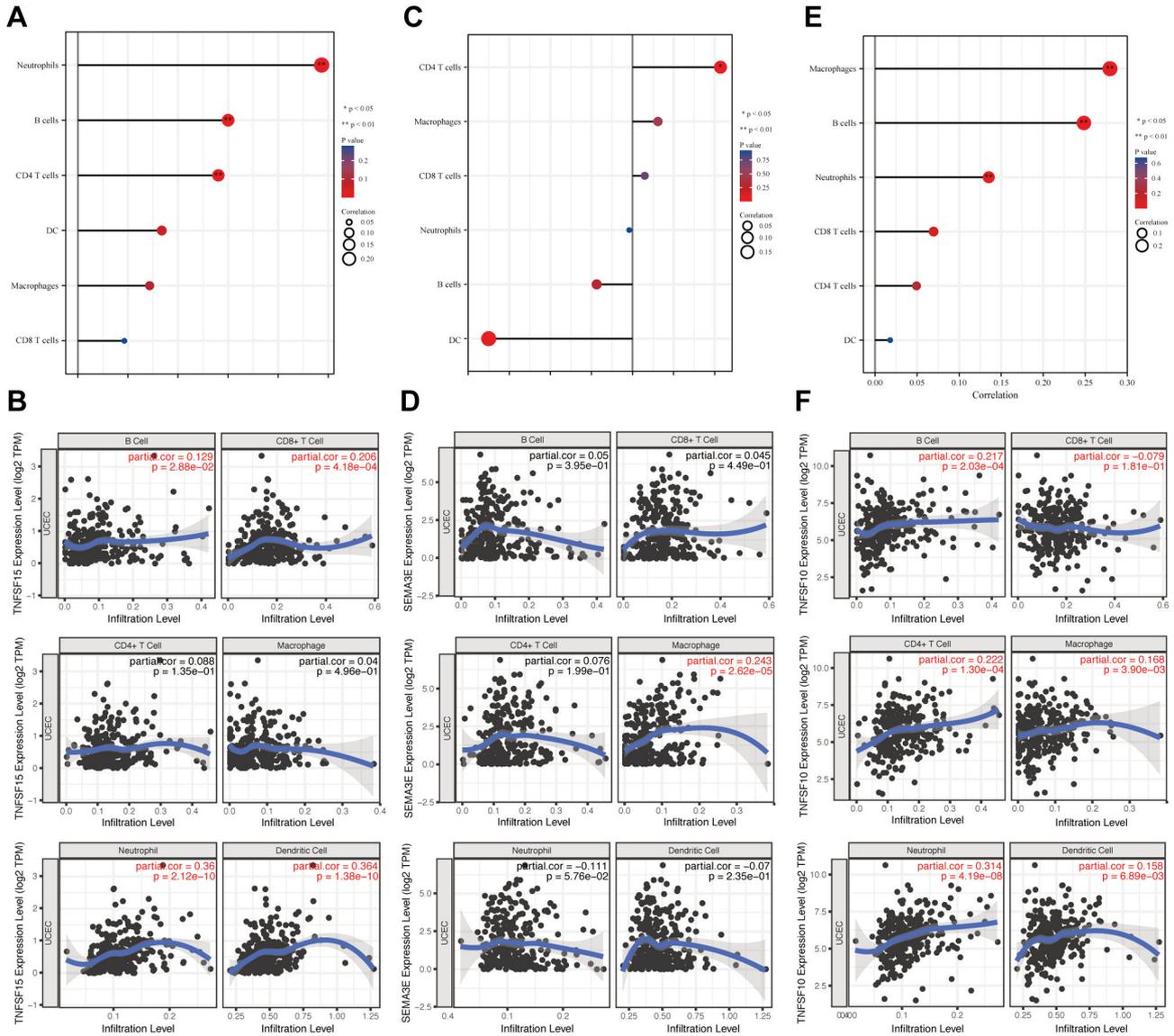


Figure 5. Correlation analysis between IRDEGs and immune-cell infiltration in EC tumors. (A, C, E) "ssGSEA" algorithm; (B, D, F) TIMER2.0 database. IRDEGs: Immune-related differential-expressed genes; ssGSEA: single sample gene set enrichment analysis.

clinico-pathological parameters, it was found that *TNFSF15* and *SEMA3E* were significantly correlated with clinical stage, histological type, and tumor invasion; *TNFSF10* was significantly correlated with clinical stage and histological type, but not with tumor invasion (Figure 2).

IRDEGs correlate with EC prognosis. Kaplan-Meier survival analysis showed that *TNFSF15*, *SEMA3E* and *TNFSF10* correlated with the overall survival (OS) of EC patients. The higher the expression of *TNFSF15* and *SEMA3E*, the longer the survival of patients (Figure 3A-B). In contrast, the higher

the expression of *TNFSF10*, the worse the prognosis of patients (Figure 3C). In addition, both *TNFSF15* and *TNFSF10* correlated with the DSS and PFI of EC patients, while *SEMA3E* only correlated with the DSS of EC patients and has no significant effect on PFI (Figure 3E-I).

Gene set enrichment analysis (GSEA). Through gene correlation analysis of IRDEGs, it was found that the top five genes most positively related to *TNFSF15* are *TMEM144*, *PLEKHA6*, *GCNT3*, *TLR3* and *CCL20*, respectively. The top five genes most negatively related to *TNFSF15* are *BMP7*, *ZHX1-C8orf76*,

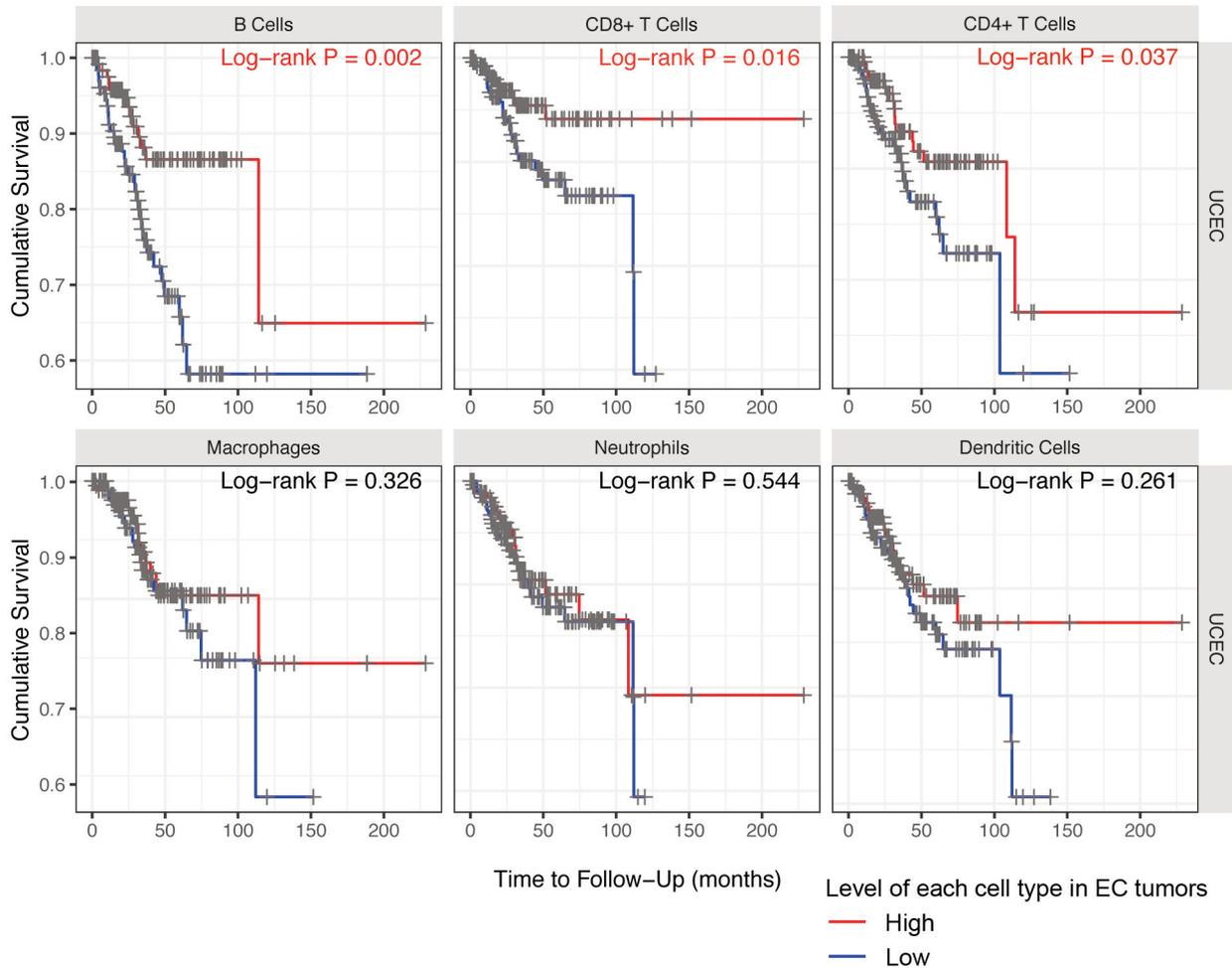


Figure 6. Correlation between immune-cell infiltration in EC tumors and prognosis of EC. EC: endometrial carcinoma.

ZDHHC19, *NECAB3* and *SNTA1*, respectively (Figure 4A). The top five genes most positively related to *SEMA3E* are *PKHD111*, *HPGD*, *ADAMTS6*, *BMPRI1B* and *PIK3R1*, respectively. The top five genes with greatest negative correlation to *SEMA3E*, are respectively: *RAPEF3*, *B3GNT3*, *XDH*, *S100A1* and *PRRX2* (Figure 4B). The top five genes most positively related to *TNFSF10* are *RTP4*, *NCEH1*, *SP100*, *PLSCR1* and *VTCN1*, respectively. The top five most negatively correlated genes related to *TNFSF10* are *ZFPM1*, *NME4*, *ATP5F1D*, *RPS15* and *MRPL41*, respectively (Figure 4C).

GSEA enrichment analysis of all the above-related genes as input gene sets showed that *TNFSF15* is significantly related to apoptosis and the IL2-STAT5 signaling pathway. *SEMA3E* is significantly related to an early estrogen-response and protein-secretion function. *TNFSF10* is significantly related to IL2-STAT5 and the KRAS-signaling pathways (Figure 4D-I).

IRDEGs are associated with immune-cell infiltration in EC tumors. The abundance of six types of immune cells in EC tumors in the high- and low-expression groups of *IRDEGs* was further compared. Combined with the results of the “ssGSEA” algorithm and TIMER2.0 database, it was found that neutrophils and B-cells in the tumors in the *TNFSF15* high-expression group were higher than those in the low-expression group, and the difference was statistically significant (Figure 5A-B). *TNFSF10* is mainly positively correlated with the extent of macrophages, B cells and neutrophils in EC tumors (Figure 5E-F). *SEMA3E* was not found to be significantly related to immune-cell types in EC tumors from the intersection of the two modes (Figure 5C-D).

By analyzing the relationship between six types of immune cells in EC tumors and the prognosis of EC patients, we found that the higher the infiltration degree of B-cells, CD8+

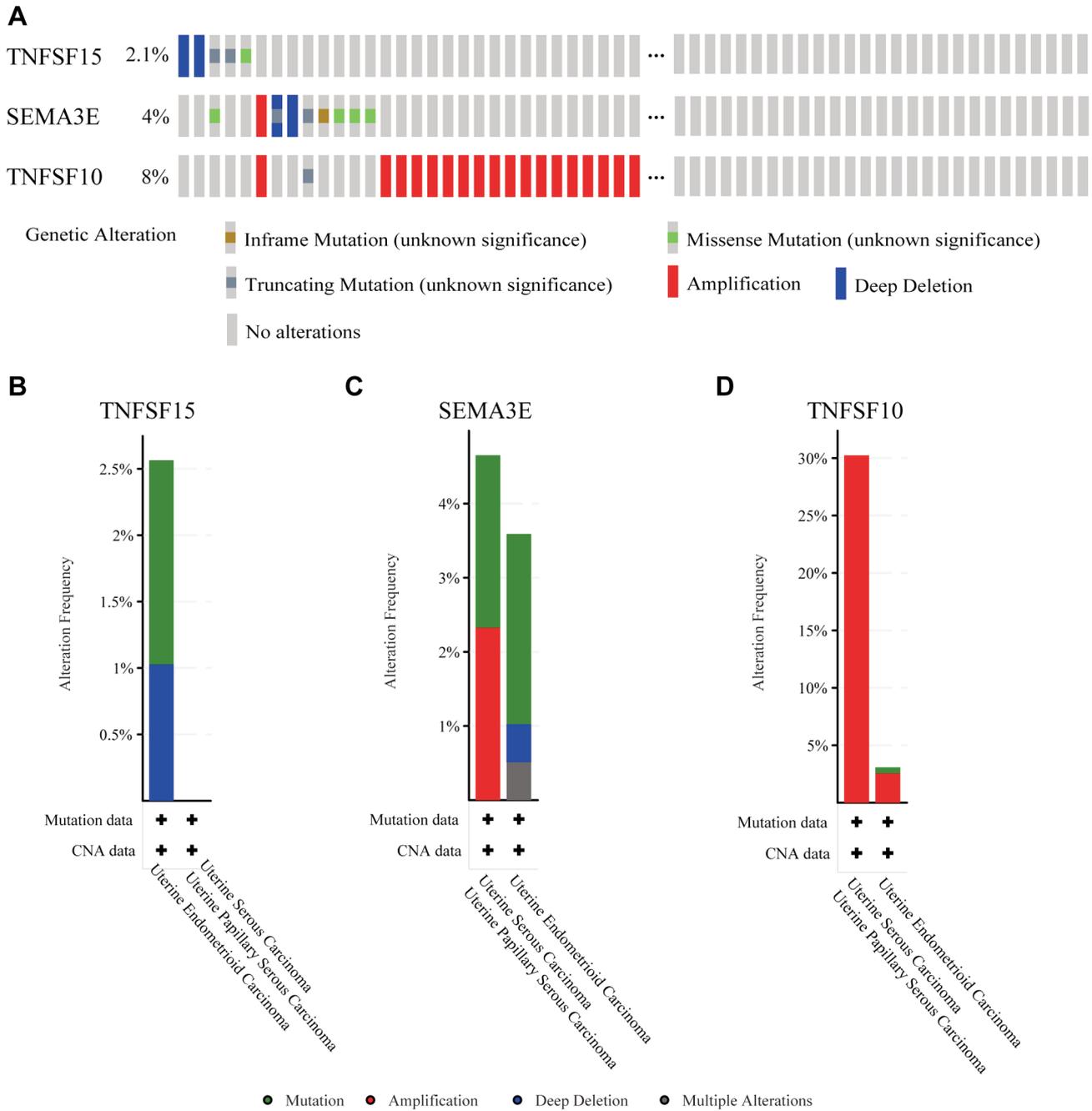


Figure 7. Gene-polymorphism analysis of IRDEGs in EC. (A) Mutation rate of *TNFSF15*, *SEMA3E* and *TNFSF10* genes, (B-D) Tumor types with a greater number of gene mutations in *TNFSF15*, *SEMA3E* and *TNFSF10* genes, respectively. IRDEGs: Immune-related differential-expressed genes.

T cells and CD4+ T cells, in EC tumors, the better the prognosis of patients (Figure 6).

Analyzing the mutations in IRDEGs, demonstrated that the mutation rates of *TNFSF15*, *SEMA3E* and *TNFSF10* genes were 2.1%, 4% and 8% in EC, respectively. The mutation types of the *TNFSF15* gene in EC are deep deletions, truncating mutations, and missense mutations. The mutation

types of the *SEMA3E* gene in EC are amplifications, deep deletions, truncating mutations, inframe mutations and missense mutations, while most of the mutation types of the *TNFSF10* gene in EC are gene amplifications, and only one case had a truncating mutation (Figure 7A).

In addition, we also found that patients with uterine endometrioid carcinoma were more likely to have

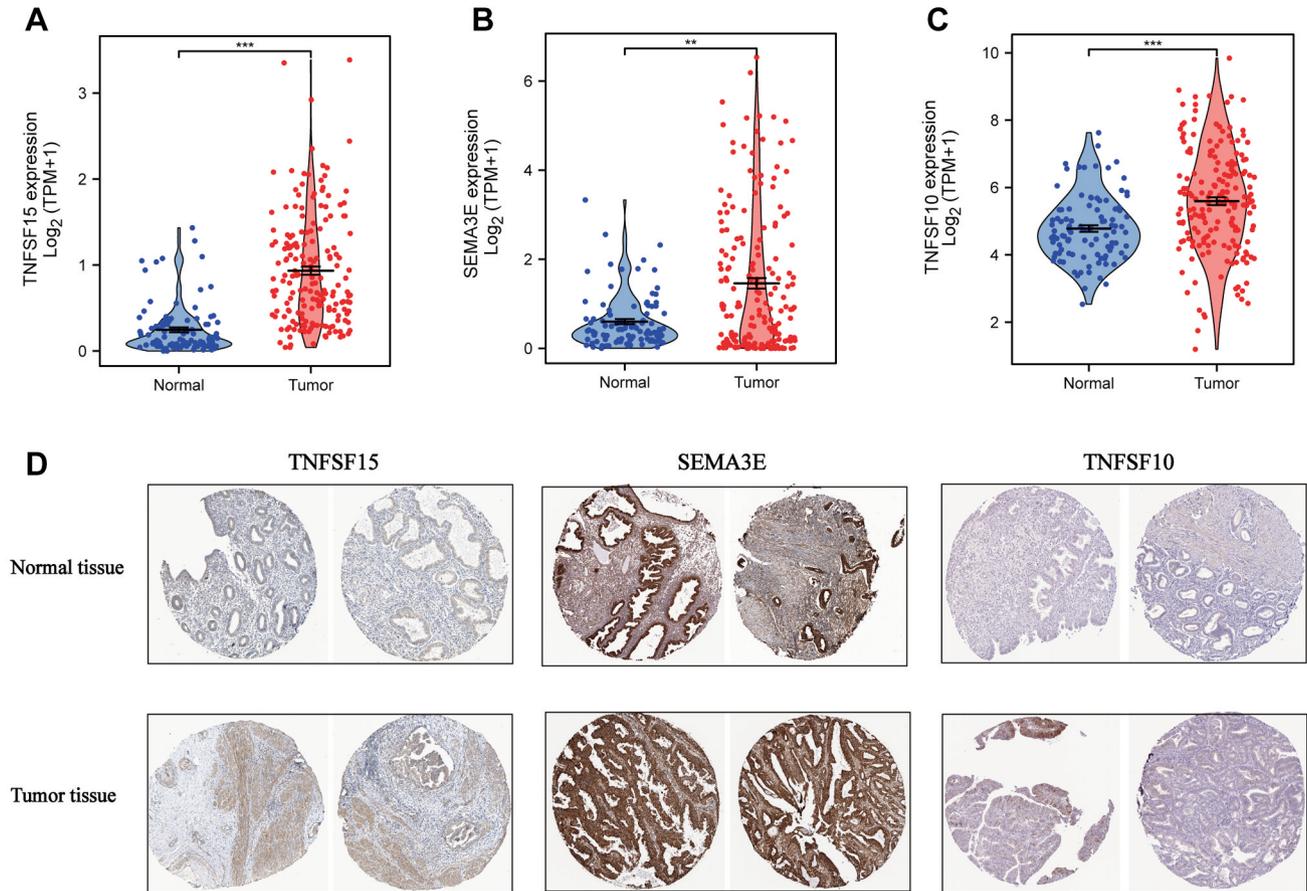


Figure 8. mRNA and protein expression of IRDEGs in EC. (A-C) mRNA expression of IRDEGs in TCGA database, (D) protein expression of IRDEGs in THPA database. IRDEGs, Immune-related differential-expressed genes; EC, endometrial carcinoma; TCGA, The Cancer Genome Atlas; THPA, The Human Protein Atlas.

TNFSF15 mutations. However, *SEMA3E* and *TNFSF10* had mutations in uterine endometrioid carcinoma, uterine serous carcinoma, and uterine papillary serous carcinoma (Figure 7B-D).

IRDEGs mRNA and protein expression in EC and normal tissue. TNFSF15, SEMA3E and TNFSF10 mRNA expression in EC is significantly higher than in normal tissues (Figure 8A-C). THPA database was used to analyze the expression of TNFSF15, SEMA3E and TNFSF10 proteins, and the results were consistent with mRNA expression: The protein expression of TNFSF15, SEMA3E and TNFSF10 proteins are increased in EC compared to normal tissue (Figure 8D).

Discussion

EC is a common malignancy of the female reproductive system, and its incidence rate is rising (11). In addition, although some patients with EC can be successfully treated by hysterectomy

after early diagnosis, younger patients may need to retain fertility, while others are too old to tolerate surgery or its side effects, so routine surgery is not the best option (12, 13). At present, many studies have shown that there is a significant relationship between host immunity and solid tumors (14), but its complex interaction relationship still needs to be further explored. Gene-expression and immune-related gene libraries provide rich information for exploring genomic characteristics of tumor progression (15). Immune-related biomarkers that can predict prognosis and that can be used in clinical treatment are needed. In the present study, IRDEGs with differential expression in EC grade G3, compared with grades G1-G2, were screened through TCGA, GEO and ImmPort databases, and their relationship with clinico-pathological parameters and prognosis of EC was further analyzed. Gene-expression correlation and GSEA-enrichment analysis to identify the potential functional pathways of IRDEGs and analyze their association with six immune-cell types in EC tumors and gene polymorphisms, provide important information for clarifying

the molecular basis of EC development and identifying potential immuno-therapeutic targets.

TNFSF15, *SEMA3E*, and *TNFSF10* were identified by analyzing EC IRDEGs. *TNFSF15* is an endogenous angiogenesis-inhibitor and a key component of the negative-control mechanism that plays a role in normal ovaries, but is missing in ovarian cancer (16). *SEMA3E* is highly expressed in metastatic cancer cells, which can drive the invasive and metastatic spread of human cancer cells in mice (17), and can also predict lymph-node metastasis of tongue squamous-cell carcinoma (18). In addition, *SEMA3E* is highly expressed in human high-grade ovarian EC, and through the Plexin-D1 receptor, enhances cell migration and the epithelial-mesenchymal transformation (19). It was reported that *TNFSF10* mediates apoptosis of EC cells by binding with death receptors, which may be an effective target for the treatment of EC and other female reproductive cancer (20), which indirectly confirms the results of the present study.

Gene-correlation analysis of IRDEGs showed that *TMEM144*, *PLEKHA6*, *GCNT3*, *TLR3* and *CCL20* are most positively correlated with *TNFSF15* expression, respectively. The top genes-most negatively-correlating with *TNFSF15* are *BMP7*, *ZHX1-C8orf76*, *ZDHHC19*, *NECAB3* and *SNTA1*, respectively. *CCL20* and *TNFSF15* are involved in osteoblast biology (21). However, whether these two molecules interact in cancer is still unclear, which needs to be further explored. Both *BMP7* and *TNFSF15* can stimulate epithelial-mesenchymal transformation of intestinal fibrosis associated with chronic colitis (22). At present, the other 8 genes have not been reported to be associated with *TNFSF15*, which is worthy of further research. More studies are needed on the top 10 genes significantly related to *SEMA3E* and *TNFSF10*. GSEA-enrichment analysis showed that *TNFSF15* and *TNFSF10* are enriched in the IL2-STAT5 signal pathway. *TNFSF15* promotes T-cell-mediated immune function by enhancing the differentiation and pathogenicity of IL9-producing T-cells through IL-2- and STAT5-dependent mechanisms (23), which directly indicates that the TNFSF family plays an important role in tumor immunity. *TNFSF15* and *TNFSF10* are also enriched in apoptosis and KRAS-signaling pathways, respectively. The TNFSF family is closely related to cancer-cell apoptosis (24-26). Silencing KRAS in pancreatic-ductal-adenocarcinoma cells increased *TNFSF10*-induced apoptosis (27).

The immune microenvironment, including, but not limited to tumor-infiltrating lymphocytes, regulatory T-cells and tumor-related macrophages, can affect the progression of solid tumors, such as breast cancer, ovarian cancer and kidney cancer (28). The immune microenvironment, comprising cancer cells, stromal cells and immune cells, is considered the “fertile soil” for malignant transformation (29), but there are also many immune factors that can promote or inhibit tumor growth (30). The present study clarified that *TNFSF15* and *TNFSF10*, both members of the TNFSF family, can drive B-

cells and CD8+ T-cells to regulate the immunogenicity of EC and affect its prognosis. *SEMA3E* can affect macrophages to influence the progression of EC. TNFSF family members regulate cell differentiation, survival, and programmed death, but their most critical functions are related to the immune system. Both innate and adaptive immune cells are controlled by members of the TNFSF family, and are crucial for coordinating the various mechanisms of co-stimulation or co-inhibition that drive the immune response (31), which is basically consistent with the present findings. In addition, *SEMA3E* also regulates a variety of immune-cell types to control systemic inflammatory responses (32-34). The present study found that these three genes have various types of mutations in EC, which may be important to its pathogenicity.

The present study found that *TNFSF15*, *SEMA3E* and *TNFSF10* can not only affect the prognosis of EC patients, but also correlate with a variety of immune-cell types infiltrating EC tumors that may be able to participate in the regulation of tumor immune-response or escape, thus also affecting the prognosis of patients. *TNFSF15* promotes macrophage differentiation and polarization to the M1 phenotype to inhibit tumor growth (35). *SEMA3E* is a biomarker of sensitivity of melanoma immune-checkpoint therapy (36). *TNFSF10* plays a crucial role in regulating multiple interactions between cancer cells and the tumor microenvironment (37), which indirectly confirms the present results, but further research is necessary.

Conclusion

In conclusion, the present study, using TCGA, GEO and ImmPort databases, has shown three IRDEGs, *TNFSF15*, *SEMA3E* and *TNFSF10*, have altered expression in EC at different histological grades. These three IRDEGs can significantly affect the prognosis of patients with EC. These three genes may also regulate the progression and prognosis of EC by affecting tumor immune-cell infiltration. This provides a novel immune target and direction for EC research to improve the outcome of this recalcitrant disease.

Conflicts of Interest

The Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

Y.L. and S.L. designed this experiment. Y.L., L.B., C.L. and P.L. analyzed data. S.L. and Z.T. explained the experimental results. Y.L. and S.L. prepared these figures. Y.L., S.L. and P.L. wrote this manuscript. R.M.H, G.Z., and B.Z. contributed to manuscript editing. R.M.H revised the manuscript. All Authors participated in reading and discussing the manuscript.

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