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Research Article

PPI Identification of Immune-Related Biomarkers in Esophageal Cancer on the Basis of Gene Co-Expression Network

Shengjia Chen[1,†], Hongfu Zeng[1,†], Aiping Zhang[2], Jinlan Lin[1], Kai Zhu[1,*]

[1]Department of Thoracic Medical Oncology, Fujian Medical University Cancer Hospital, Fujian Cancer Hospital, Fuzhou, 350014, China.
[2]Department of Pharmacy, Fujian Medical University Cancer Hospital, Fujian Cancer Hospital, Fuzhou, 350014, China.

[*]*Corresponding author*: Kai Zhu, Department of Thoracic Medical Oncology, Fujian Medical University Cancer Hospital, Fujian Cancer Hospital, Fujian, China. Tel/ Fax: +86-13799988567, E-mail: zhukai7657@163.com

[†] These authors contributed equally

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Background: The mortality rate of esophageal cancer is on the continuous increase. Fortunately, with the development of immunotherapy, the prognosis and survival rate of patients with esophageal cancer have been improved gradually.

Objective: Immune markers have a crucial part in immunotherapy. Therefore, it is of great meaning to delve further into immune-related biomarkers of esophageal cancer for better treatment.

Materials and Methods: In this study, gene co-expression networks were established using weighted gene co-expression network analysis, thus forming gene modules with different clusters. The tumor immune microenvironment was assessed with the ESTIMATE algorithm.

Results: Analysis of the module Eigen gene -immune score trait indicated that the black module was markedly associated with immune score, with the top 80 genes regarding correlation ranking as the candidate hub gene set. Enrichment analysis revealed that genes within the black module were primarily enriched in tumor immune-related functions. To mine the hub genes that were closely connected with immunity, protein-protein interaction networks were constructed by STRING for genes within the black module, and genes with the interaction score top10 were retained. They were intersected with hub genes to finally obtain four hub genes: *CCR5, LCP2, PTPRC* and *TYROBP*. The samples were divided into high- and low-expression groups by the median expression of hub gene, and survival analysis was performed in combination with clinical information. The results revealed that the high-expression groups of genes *LCP2* and *PTPRC* had a poor prognosis. TIMER immune cell infiltration analysis revealed that the expression levels of the 4 hub genes were positively correlated with immune cell infiltration and negatively correlated with tumor purity. In addition, these 4 hub genes were correlated with the expression of immune checkpoint genes *CTLA-4* and *PDCD1* positively. Gene set enrichment analysis enrichment analysis demonstrated that there were differences in tumor immunity and cancer-related pathways between high and low expression of 4 hub genes.

Conclusion: Altogether, we identified four biomarkers that may have connection with tumor immunity, and speculated that these genes may influence patient prognosis by affecting pathways related to esophageal cancer immunity. This study will pave the way for the research of immune mechanisms of esophageal cancer and the analysis of patient's prognosis.

Keywords: Esophageal cancer; Immune score; Immune cell infiltration; Immune checkpoint; Protein-protein interaction; Weighted gene co-expression network analysis

1. Background

Esophageal cancer is a malignant tumor and is also a common digestive system tumor (1). The 5-year survival rate of esophageal cancer patients is only about 10% with insidious early clinical symptoms. Current strategies for treating esophageal cancer mainly consist of endoscopic mucosal resection (EMR), cisplatin (such as cisplatin combined with 5-FU, etc.), or other drugs, like fluorouracil or capecitabine, etc. (2-4). Though the 5-year survival rate increased from 10% to 15% ~ 40% after resection surgery, it is generally at a low level (5, 6). With the development of cancer immunotherapy technology, the prognosis and survival rate of patients with esophageal cancer have gradually improved. Esophageal squamous cell carcinoma (ESCC) is the main histological type of esophageal cancer. Kato K *et al.* (7) found that patients with advanced ESCC who underwent *PD-1* inhibitor nivolumab have a remarkably higher prognostic survival rate than those in the chemotherapy group, with a sharp reduction in adverse prognostic events. Y Baba *et al.* (8) discovered that tumor infiltrating lymphocytes (TILs) surrounding esophageal cancer cells are associated intimately with a favorable prognosis for patients. Therefore, in-depth study of esophageal cancer immune-related biomarkers is of great importance for cancer immunotherapy and prognosis.

The tumor microenvironment exerts an essential role in cancer development. Cancer develops and progresses concurrently with alterations in the surrounding stroma. Cancer cells can shape their microenvironment by secreting various cytokines, chemokines, and other factors (9). Pan *et al.* (10) analyzed the expression of *LAYN,* a hub gene regulating T cell function, in gastric cancer and colon adenocarcinoma, and revealed that the infiltration levels of CD4+ T cells, CD8+ T cells, macrophages, neutrophils and dendritic cells are higher in the high-expression group, with worse prognosis of patients. Y Ino *et al.* (11) revealed by immunohistochemical analysis combined with survival curves that high levels of infiltration of tumor-infiltrating pan-macrophages, M2, Neu and other immune cells are related to shorter prognostic survival in patients with pancreatic ductal carcinoma, while higher levels of infiltration of tumor-infiltrating CD4+ T cells and CD8+ T cells are associated with longer prognostic survival time in patients. TILs can influence the prognosis of patients with gastrointestinal cancers such as colorectal cancer (12, 13). Adile Orhan *et al.* (14) further revealed the prognostic value of subset of TILs for patients with pancreatic cancer, indicating that patients with high infiltration of CD8+ T cells and CD3+ T cells have increased prognostic overall survival (OS), while patients with high infiltration of FoxP3 + lymphocytes have worse OS. The above studies all illustrate that the tumor immune microenvironment has a close link with the prognosis of cancer patients.

Weighted gene co-expression network analysis (WGCNA) is a method utilized to analyze gene expression patterns of multiple samples. As a comprehensive R function, it can cluster genes through similar gene expression patterns to form modules, providing a basis for analyzing the connection between genes and specific characteristics. And it has an important role in identifying candidate biomarkers or therapeutic targets (15). Wan *et al.* (16) divided uveal melanoma genes into 21 modules by WGCNA and confirmed the hub genes of each module in combination with clinical information and enrichment analysis, ultimately revealing that *SLC17A7, NTRK2, ABTB1,* and *ADPRHL1* may serve as biomarkers affecting tumor recurrence. Yang *et al.* (17) found the correlation between modules and inflammatory response by WGCNA analysis of differentially expressed genes in glioblastoma multiform (GBM), combined with enrichment analysis, and revealed that up-regulated *NMI* may play a pivotal role in GBM development through inflammatory reaction. The above results demonstrate the reliability of WGCNA-based mining of cancer treatment biomarkers.

In this study, we analyzed the esophageal cancer gene expression data in TCGA database by WGCNA and constructed a gene co-expression network. Combined with ESTIMATE analysis, immune-related co-expressed gene modules in esophageal cancer were identified and immune-related hub genes were mined. Hub genes with higher interaction scores were mined by protein-protein interaction (PPI) network and intersected with hub genes to further screen immune-related hub genes in esophageal cancer. The expression of hub genes in tumor samples was analyzed, and the association with clinic pathological stages was analyzed, combined with survival analysis and enrichment analysis, so as to further clarify the correlation between hub genes that may be served as biomarkers and prognosis and immune pathways.

This study may provide new therapeutic targets for immunotherapy of esophageal cancer.

2. Objective

Immune markers have a crucial part in immunotherapy. Therefore, it is of great meaning to delve further into immune-related biomarkers of esophageal cancer for better treatment.

3. Materials and Methods

3.1. Data Sources

The transcript mRNA expression dataset TCGA-ESCA and clinical information of human esophageal cancer were obtained from TCGA database (https://portal.gdc.cancer.gov/), and the data were comprised of 160 tumor samples and 11 normal samples, and the transcript data format was FPKM. The top 25% of genes ranked in the median absolute deviation (MAD) of tumor samples after sample filtering were selected as the study subjects.

3.2. Immune Microenvironment Analysis

The tumor microenvironment includes not only tumor cells, but also tumor-related epithelial cells, immune cells, stromal cells, and vascular cells. Among them, stromal cells and immune cells constitute the main non-tumor components, and their expression levels can be judged by the nature of transcripts to further determine the tumor purity in tumor tissues, which is of great value for diagnosis and prognostic evaluation of tumors. In this study, R package "estimate"(18) was utilized to analyze the transcription of genes in tumor samples and obtain four indicators related to cellular immunity, namely, Immune Score, Stromal Score, ESTIMATE Score and Tumor Purity.

3.3. Construction of Gene Co-Expression Network

To screen immune-related hub genes in esophageal cancer, we constructed a weighted co-expression network based on TCGA-ESCA gene expression data using the R package "WGCNA"(15), Firstly, the data were preprocessed and the genes with expression level of 0 in 80% of the samples in TCGA-ESCA were excluded, and the top 25% of the genes with MAD were retained. The sample cluster tree was constructed using hierarchical clustering to eliminate outlier samples, and finally, 4,882 genes in 160 samples were used as the subjects for subsequent analysis (15). A similarity matrix was constructed using Pearson correlation test, and a power function was used to transform the correlation matrix into an adjacency matrix so that the connections among genes in the network were more in line with the scale-free network distribution. The scale-free topological criterion was defined as the correlation between module average connectivity (k) and p (k) of 0.85, and the optimal soft threshold β (power) was screened to construct a scale-free topological network and transform the adjacency matrix into a topological overlap matrix (TOM). Finally, hierarchical clustering was performed on TOM to merge highly similar gene modules (setting the module minimum number of genes to be 50 and the anisotropy threshold to be 0.25).

3.4. Target Module Acquisition and Gene Functional Analysis

For the selection of the gene modules with the highest correlation with tumor immune characteristics, Immune Score, Stromal Score, ESTIMATE Score and Tumor Purity obtained by ESTIMATE algorithm were used as clinical traits (https://r-forge.r-project.org/projects/estimate/). The correlation between these traits and module eigengene (ME) was analyzed using Pearson correlation, and finally, modules prominently associated with immune traits were selected for subsequent analysis. Functional enrichment analysis of the genes within the modules was performed using the R package "clusterProfiler" (19) with the screening criteria: p value<0.05 and q value<0.05.

3.5. Identification of Hub Genes

The Gene significance (correlation between modules and clinical traits), as well as Module Membership (correlation between genes within modules and module eigenvalues), was calculated respectively. With cor.geneModuleMembership >0.8 and cor.geneTraitSignificance >0.8 for candidate hub genes within the threshold screening module composed candidate hub gene set. For all genes within the selected modules in STRING (http://string-db.org/cgi/input.pl), the website built a PPI network to screen hub genes using an interaction score >0.4 as a screening threshold (https://doi.org/10.1093/nar/gkg034). The obtained candidate hub gene set was overlapped with the top 10 genes obtained from the PPI network screening.

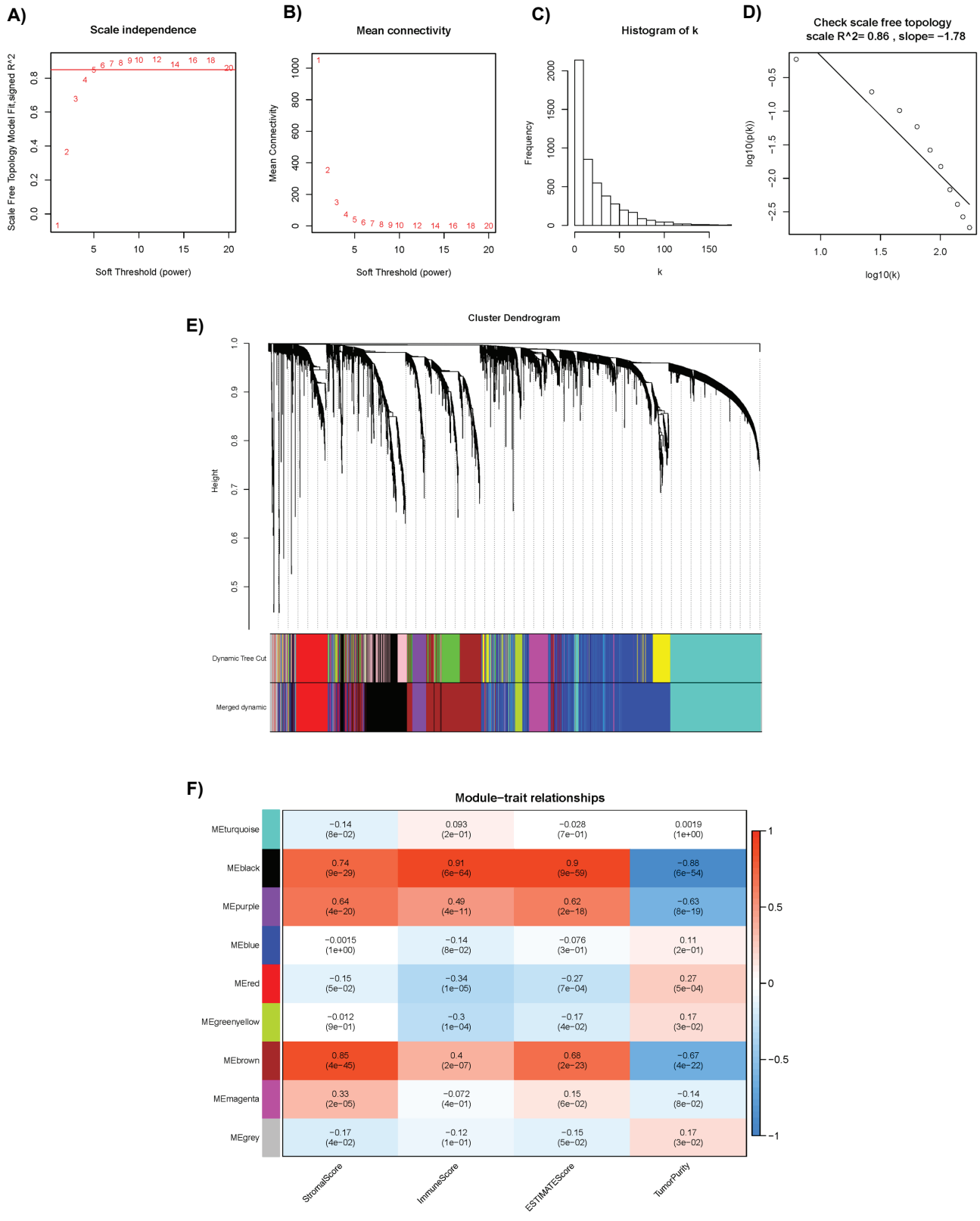

Figure 1. Weighted gene co-expression network analysis. A) Correlation between scale-free topological model fit and soft threshold (β), **B**) Correlation of average connectivity with each soft threshold (β), **C**) Histogram of connectivity distribution at β=6, **D**) Scale-free topological test at β=6, **E**) Dendrogram of all co-expressed genes based on dissimilarity measure (1-TOM) clustering. Different colors represent different gene modules, F) Heatmap of the correlation between ME of each module and Stromal Score, ESTIMATE Score, Immune Score, and Tumor Purity.

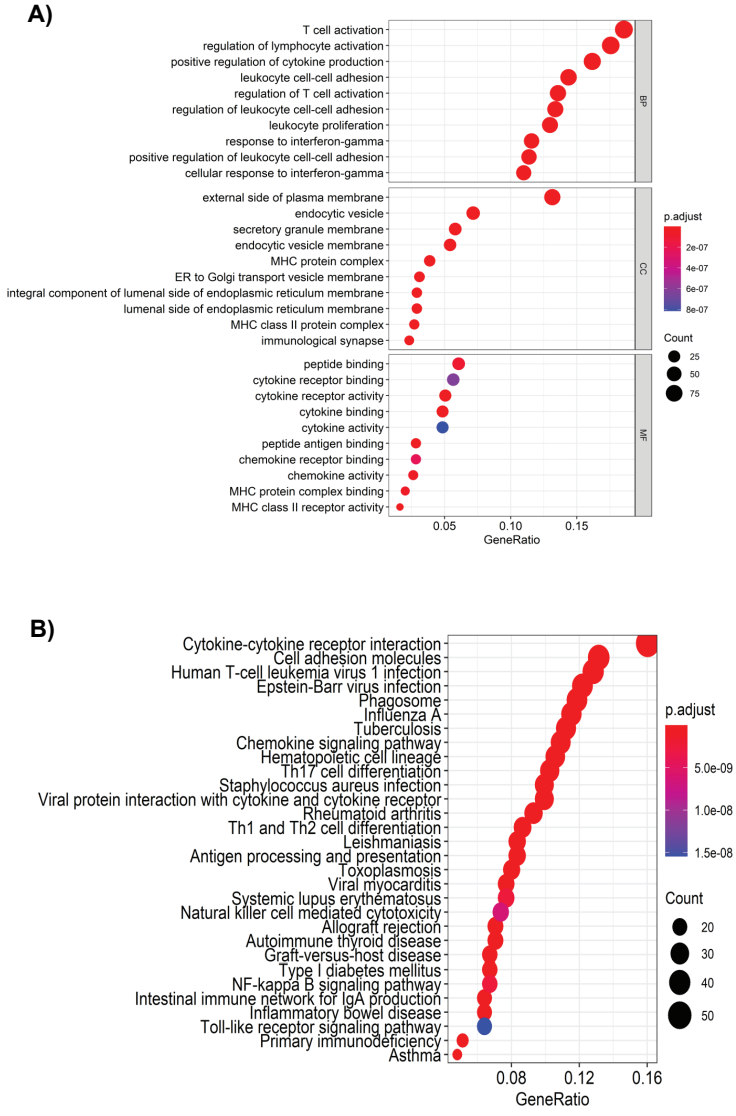

Figure 2. Enrichment analyses of MEs within the black module. A) Bubble plot of GO functional enrichment analysis of MEs within black module, with dot size indicating the number of gene enrichments; **B**) Bubble plot of KEGG pathway analysis of MEs within black module, with dot size indicating the number of gene enrichments. The smaller the *P* value, the redder the dot color tends to be.

3.6. Validation of Hub Genes

To validate the association between hub gene and clinical and prognostic outcomes, patients were first divided into high- and low-expression groups of hub genes by the optimal classification threshold of the R package "survival" based on the expression level of hub gene, followed by Kaplan-Meier survival analysis (20). The results were then visualized by the R package "suvminer" (https://cran.r-project.org/web/packages/survminer/survminer.pdf) (21). Also, to reveal the association of hub gene with tumor immunity, based on the TIMER database (https: //cistrome.shinyapps.io/timer/), the content of six immune cells (B cell, CD4+ T cell, CD8+ T cell, Neutrophil, Macrophage, Dendritic cell) and the expression data of immune checkpoint (*CTLA4, PDCD1*) genes in each sample of esophageal cancer were obtained. And the correlation between the high- and low-expression of each hub gene and the content of immune cells was assessed by TIMER (https://cran.r-project.org/web/packages/timeR/timeR.

pdf). Finally, gene set enrichment analysis (GSEA) was performed by R package "clusterProfiler"(19), and the results were visualized by R package "enrichplot" (22) (https://bioconductor.org/packages/devel/bioc/manuals/enrichplot/man/enrichplot.pdf).

4. Results

4.1. Esophageal Cancer Gene Co-Expression Network Construction and Immune-Related Module Screening

The samples and genes that were not up to the standard in TCGA-ESCA dataset were eliminated, and finally, 160 esophageal cancer samples and 4,882 genes were retained for constructing weighted gene co-expression networks. β (power)=6 (scale - free R2=0.86) was chosen as the soft threshold to construct a scale-free network **(Fig. 1A-1D).** Totally nine gene modules were finally obtained **(Fig. 1E, Table 1)**. Stromal Score, Immune Score, ESTIMATE Score and Tumor Purity of LCGA-ESCA dataset samples were obtained as clinical traits based on ESTIMATE analysis. A Pearson correlation analysis of all MEs with clinical traits revealed that the black module showed notable correlations with Stromal Score (r=0.74, *p*=9e − 29), Immune Score (r=0.91, *p*=6e−64), ESTIMATE Score (r=0.9, *p*=9e−59) and TumorPurity (r=−0.88, *p*=6e−54) **(Fig. 1F)**. Therefore, the black module was utilized for subsequent studies.

Table 1. Gene modules and gene number

Gene modules	Gene number
black	537
blue	1407
brown	908
greenyellow	94
grey	98
magenta	216
purple	174
red	332
turquoise	1116

4.2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analyses of Module Genes

GO biological function enrichment analysis of 537 genes within the black module displayed that they were mainly enriched in biological processes, such as T cell activation, positive regulation of cytokine production, leukocyte cell-cell adhesion, regulation of lymphocyte activation, regulation of T cell activation, regulation of leukocyte cell-cell adhesion, leukocyte proliferation and other GO terms related to T cell activation, lymphocyte regulation and leukocyte proliferation. They were mainly enriched in cellular components, such as external side of plasma membrane, endocytic vesicle membrane, endocytic vesicle, secretory granule membrane, and other functions related to endoplasmic reticulum (ER) components. They were mainly enriched in molecular functions, such as peptide binding, cytokine receptor activity, cytokine receptor binding, cytokine binding, MHC protein complex binding and other GO term related to cytokines and MHC **(Fig. 2A)**. KEGG pathway enrichment result implied that genes were mainly involved in immune and cancer-related pathways such as Cytokine – cytokine receptor interaction, Chemokine signaling pathway, Human T−cell leukemia virus 1 infection, Th1 and Th2 cell differentiation, Th17 cell differentiation, **(Fig. 2B)**. In summary, the genes within the black module are mainly related to immune-related biological functions and pathways such as immune cell activation, regulation, proliferation and immune signal activation and transduction.

4.3. Identification of Hub Genes

The genes ranked in the top 80 correlation **(Fig. 3A)** were screened as candidates with the highest correlation with immunity based on the correlation between genes in the black gene module and Immune Score. A PPI network was constructed using STRING for all genes in the black module, and the top10 genes with the highest connectivity were selected as the hub genes in the PPI network with an interaction score >0.4 as the threshold **(Fig. 3B)**. The 80 genes with high immune correlation were intersected with 10 hub genes in the PPI network, and finally, four hub genes, *CCR5, LCP2, PTPRC,* and *TYROBP*, were screened **(Fig. 3C)**.

4.4. Hub Genes Are Associated with Tumor Immune Microenvironment

The expression levels of 4 hub genes in immune-related esophageal cancer samples were considerably up-regulated compared with normal samples **(Fig. 4A-4D)**, while we analyzed the expression of four hub genes

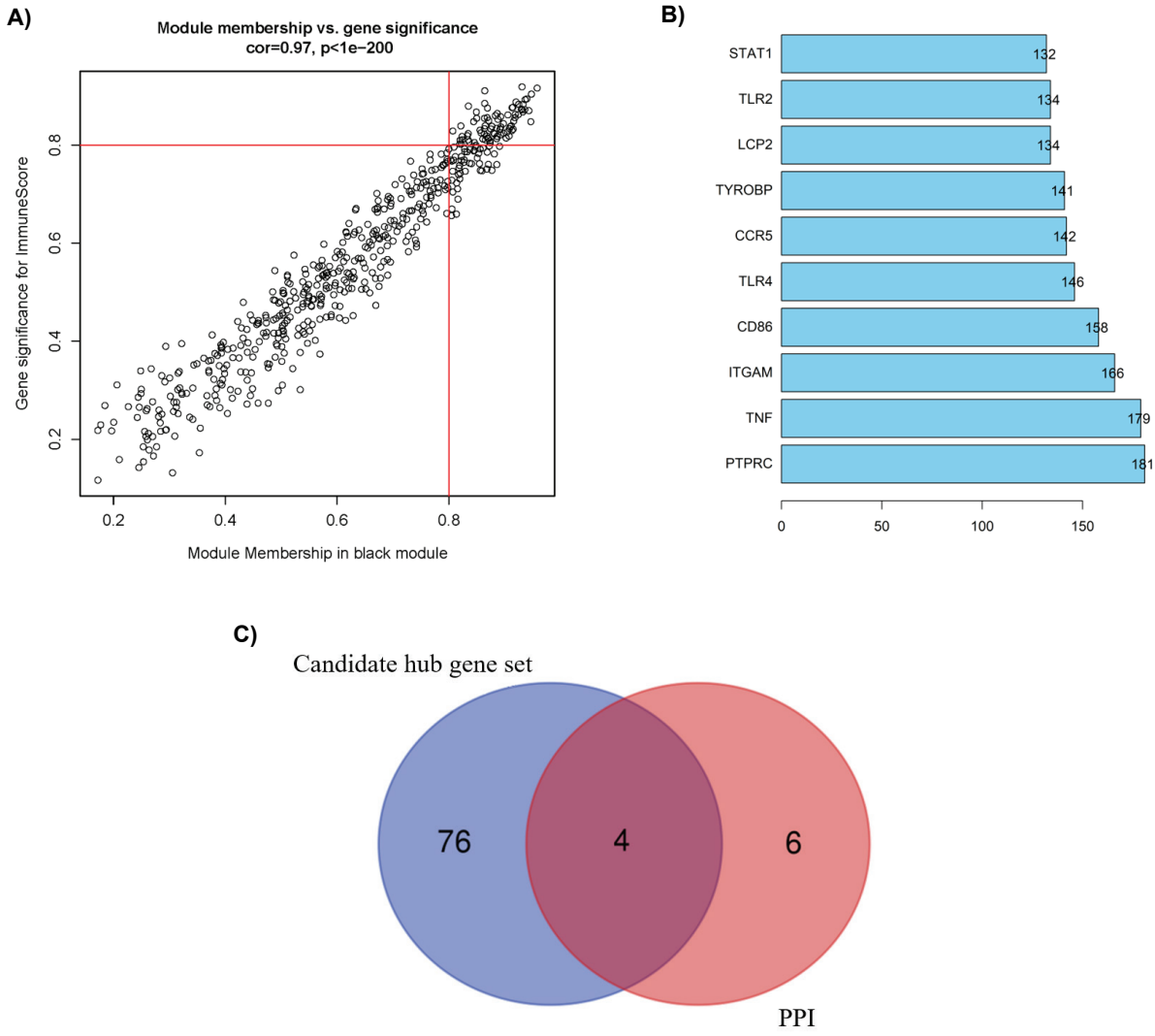

Figure 3. Screening of hub genes associated with immunity in esophageal cancer. A) 80 genes highly associated with esophageal cancer immunity were screened from the black module. The abscissa represents the correlation coefficient between gene and module traits, and the ordinate represents the correlation coefficient between gene and immunity score; **B**) Top10 gene with the highest connectivity based on PPI network; **C**) Venn diagram indicating immune-related hub genes; intersection of 80 candidate hub genes with PPI degree top10 genes.

in different stages **(Fig. 4E)**. The results revealed that the expression of gene *TYROBP* showed an enormous increase from stage I to stage III, but tended to be stable in stage IV; the expression of the remaining three genes generally increased with stags, but did not show a remarkable difference. In addition, survival analysis indicated that high expression of *LCP2* and *PTPRC* was remarkably negatively correlated with prognosis (p<0.05), which may indicate that these two genes have a greater impact on prognosis **(Supplementary Fig. 1A-1D)**. The above results showed that the four genes showed a certain correlation trend with stage, and the expression of *LCP2* and *PTPRC* was detrimental to the prognosis of patients with esophageal cancer, thus indicating that hub gene has the potential to be a clinical indicator to predict tumor development.

To validate the involvement of the four hub genes in tumor immune regulation, the contents of six immune cells and the expression levels of the immune checkpoint genes *CTLA4* and *PDCD1* in each sample of esophageal cancer were obtained through the TIMER website, and the correlation between the four hub genes and immune

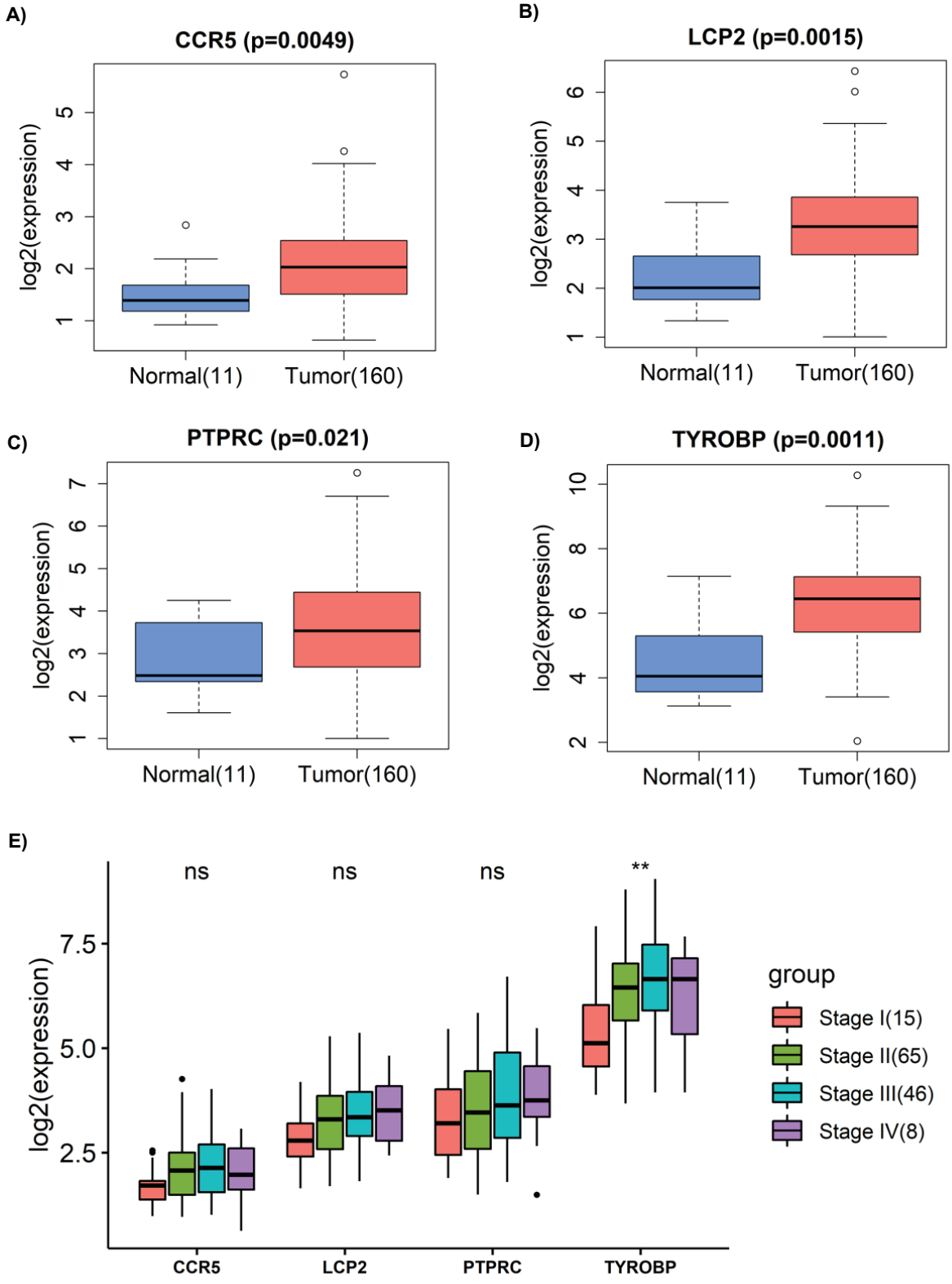

Figure 4. Expression of four hub genes in esophageal cancer samples from TCGA-ESCA dataset. A-D) Differential expression of *CCR5, LCP2, PTPRC, TYROBP* in esophageal cancer samples compared with normal samples; **E**) Box plots of hub gene expression in samples with different stages. The *p* values represent the results of one-way analysis of variance for different pathological stages. ** $p<0.01$, ns: no significant difference.

cell infiltration level was assessed. The results showed that with the increase of the infiltration level of 6 kinds of immune cells, the expression of the four hub genes showed an increasing trend, indicating a notable positive correlation, while the expression of the four hub genes showed a remarkable negative correlation with tumor purity **(Supplementary Fig.2A-2D)**.

Additionally, the expression of the four genes was also markedly positively correlated with the immune checkpoint genes *CTLA4* and *PDCD1* expression, and the expression levels of the two were basically linear **(Supplementary Fig. 3A-3B)**. The 4 hub genes were associated with tumor immunity, and may exist as biomarkers involved in tumor immune regulation.

4.5. GSEA Results

GSEA results indicated that the four hub genes in high- and low-expression group gene sets mainly differed in immune or cancer-related pathways such as JAK-STAT signaling pathway, NF-kappa B signaling pathway, T cell receptor signaling pathway, PI3K-Akt signaling pathway, TNF signaling pathway, Th1 and Th2 cell differentiation, Th17 cell differentiation, TNF signaling pathway and Toll-like receptor signaling pathway **(Fig. 5A-5D)**. In summary, the high- and low-expression groups of hub genes *CCR5, LCP2, PTPRC* and *TYROBP* are different in pathways related to esophageal cancer immunity, which may also be a risk factor for adverse prognosis of patients with high expression of hub genes.

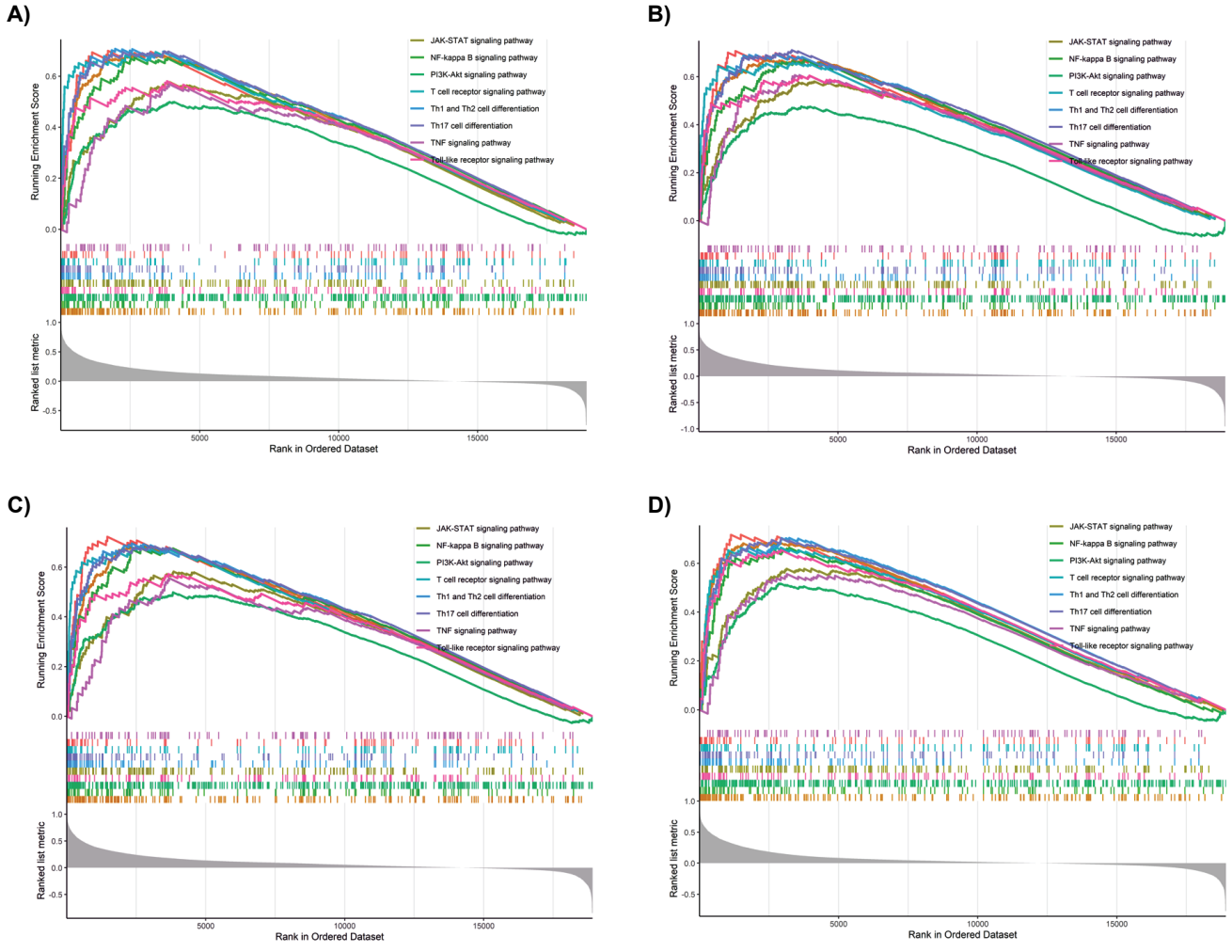

Figure 5. The KEGG pathways of the four hub genes in high- and low-expression groups analyzed based on GSEA. A-D) KEGG pathway enrichment analysis of *CCR5, LCP2, PTPRC* and *TYROBP* respectively in high- and low-expression groups.

5. Discussion

At present, there are few studies on the immune mechanism and prognostic molecular markers of esophageal cancer, and its pathogenesis is not yet understood. Due to the insidious early symptoms, patients with esophageal cancer are often diagnosed at an advanced stage (23, 24). It is known that Zhang *et al.* (25) constructed a gene co-expression network by WGCNA based on TCGA esophageal cancer gene expression data and performed the correlation analysis between module genes with multiple survival data, indicating the correlation between each esophageal cancer-related gene module and patient prognosis. The esophageal cancer biomarkers *PTAFR* and *FGR* were further mined, which provided an important reference for the prognosis and treatment of this disease. Herein, we focused on immune-related genes in esophageal cancer and explored key biomarkers to provide data support for immunotherapy of esophageal cancer. We screened the immune-related genes by WGCNA and ESTIMATE analysis, and further mined four hub genes in combination with PPI network analysis, followed by correlation analysis between gene expression and pathological stage, survival analysis and functional enrichment analysis, further revealing that the four genes may be potential biomarkers in the immunotherapy of esophageal cancer.

The expression of the four hub genes *CCR5, LCP2, PTPRC* and *TYROBP* mined in this study was positively correlated with stage. And survival analysis showed that high expression of each hub gene was an adverse factor for prognosis. Among them, *CCR5* is a G-protein-coupled receptor that modulates the trafficking and effector function of T lymphocytes, immature dendritic cells, and macrophages, (26) and is also a novel therapeutic target for cancer therapy. Asim *et al.* (27) found that in animal models, liver metastasis of colorectal cancer is significantly inhibited in the group receiving *CCR5*-targeted therapy, and circulatory and tumor-associated *CCR5* and its ligands are differentially expressed compared with the normal group. The study illustrated the association of *CCR5* with cell proliferation and migration, and targeting this receptor interferes with cell cycle-related signaling cascades. *LCP2*, a member of the *SLP-76* adaptor protein family, regulates immunoreceptor signaling and is also required for integrin signaling in neutrophils and platelets (28). Studies have shown that *LCP2* is positively correlated with the expression of multiple immune checkpoints and the degree of CD8+T cell infiltration in cutaneous melanoma, and can be a prognostic biomarker in patients receiving anti-*PD1* immunotherapy (29). *PTPRC* en-codes protein tyrosine kinase (*CD45*), which presents in all cells of lymphoid origin and acts as a receptor signal for B and T cells, and its expression reduces *PD-1* phosphorylation, thereby regulating cellular immune processes (30-32). *TYROBP* can be overexpressed in a variety of cancers and has been implicated in tumor progression, encoding a protein that is a transmembrane signaling polypeptide located on the surface of a variety of immune cells (33, 34) and is often a negative factor in cancer prognosis. For example, Cheray *et al.* (35) have shown that *TYROBP* is associated with tumorigenesis and invasiveness of glioblastoma; Jiang *et al.* (36) noted that the expression of *TYROBP* is remarkably negatively correlated with the prognosis of patients with gastric cancer.

Combined with the published literature and our findings, we hypothesize that the four hub genes in this study are closely related to immune signaling pathways in esophageal cancer, which may be new targets for esophageal cancer immunotherapy.

To further explore the immune regulatory mechanism of hub genes, this study uncovered that the expression levels of four hub genes were positively correlated with the expression levels of *CTLA4* and *PDCD1*. Both *CTLA4* and *PDCD1* expression products CTLA4 and *PD-1* are common immunosuppressive factors, of which *CTLA-4* negatively regulates T cell activation in multiple ways, such as competitively binding to common ligands B7-1 and B7-2 of *CD28* that regulates T cell activation, or inducing the development of Treg cells with immunosuppressive effect (37). Further studies showed that the anti-tumor immune mechanism of *CTLA4* is not limited to the inhibition of T cell activation, and silencing or antibody blockade of *CTLA4* in B-cell lymphoma also inhibits tumor growth in an immune microenvironment lacking T cells (38). Similar to *CTLA4*, *PD-1* has received much attention because of its inhibitory effect on immune cell activation, which affects the normal biological function of immune cells and also affects the prognosis of patients. From the perspective of immunosuppressive mechanism, PD1 activated by ligand *PD-L1* is generally considered to inhibit cell activation by inhibiting T cell receptor (*TCR*) signaling, while recent studies have shown that *CD28* receptor is preferentially dephosphorylated in

cell systems in response to *PD-1* activation by *PD-L1* compared with *TCR*, demonstrating that *PD-1* inhibits T cell function by inhibiting *CD28* signaling (39, 40). From a prognostic perspective, high *PD-1* expression has been demonstrated to be associated with poor prognosis in patients with many types of cancer, such as cervical adenocarcinoma, small cell carcinoma, gastric cancer (41-43). In summary, the four high hub gene expression groups in this study may activate the tumor immune escape mechanism, so that immune cells are unable to normally exert their function and kill tumor cells, which in turn causes a poor prognosis.

The results of GSEA demonstrated that there were differences in metabolic pathways such as JAK-STAT signaling pathway, PI3K-Akt signaling pathway, T cell receptor signaling pathway, NF-kappa B signaling pathway, Th1 and Th2 cell differentiation, Th17 cell differentiation, Toll-like receptor signaling pathway, and TNF signaling pathway among the 4 hub genes. JAK-STAT is a recently discovered pathway involved in cell proliferation, differentiation, hematopoiesis and immune regulation, which phosphorylates transcriptional activators through JAK kinases, dimerizes and transports them into the nucleus through the nuclear membrane to regulate gene expression (44). Similar to this mechanism, NF-kB factors associated with the NF-kappa B pathway are regulated by the inhibitory protein IKba, and phosphorylated NF-kB promotes the process by coordinating inflammatory cells and antioxidant response elements to regulate gene expression (45, 46). Activated PKB can directly phosphorylate apoptotic proteins, which is beneficial to inhibit the activation of apoptosis-related pathways (47). Therefore, the hub genes in this study may worsen the prognosis by affecting immune-related metabolic pathways, and the regulation of this process may involve the transfer of cytokines and the phosphorylation of immune proteins, therefore, the specific mechanism needs further experimental verification.

To sum up, four potential immune-related biomarkers of esophageal tumors were screened by gene co-expression network combined with PPI interaction in this study, which provided a reliable theoretical basis for the study of immune mechanism of esophageal cancer based on clinical survival analysis, immune cell infiltration level and pathway enrichment analysis. However, this study is a pure bioinformatics study without any experimental proof, so further molecular and cellular experiments are required to support the results of this study.

References

1. Rice TW, Gress DM, Patil DT, Hofstetter WL, Kelsen DP, Blackstone EH. Cancer of the esophagus and esophagogastric junction-Major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA*: a cancer journal for clinicians. 2017;**67**(4):304-317. doi: 10.3322/caac.21399
2. Wang KK, Prasad G, Tian J. Endoscopic mucosal resection and endoscopic submucosal dissection in esophageal and gastric cancers. *Curr Opin Gastroenterol.* 2010;**26**(5):453-458. doi: 10.1097/mog.0b013e32833e4712
3. Fatehi Hassanabad A, Wong JVS. Statins as Potential Therapeutics for Esophageal Cancer. *J Gastrointest Cancer*. 2021;**52**(3):833-838. doi: 10.1007/s12029-021-00648-4
4. Ross P, Nicolson M, Cunningham D, Valle J, Seymour M, Harper P, *et al*. Prospective randomized trial comparing mitomycin, cisplatin, and protracted venous-infusion fluorouracil (PVI 5-FU) With epirubicin, cisplatin, and PVI 5-FU in advanced esophagogastric cancer. *J Clin Oncol*. 2002;**20**(8):1996-2004. doi: 10.1200/jco.2002.08.105
5. Huang FL, Yu SJ. Esophageal cancer: Risk factors, genetic association, and treatment. *Asian J Surg*. 2018;**41**(3):210-215.
6. Alsop BR, Sharma P. Esophageal Cancer. *Gastroenterol Clin North Am*. 2016;**45**(3):399-412.
7. Kato K, Cho BC, Takahashi M, Okada M, Lin CY, Chin K, *et al*. Nivolumab versus chemotherapy in patients with advanced oesophageal squamous cell carcinoma refractory or intolerant to previous chemotherapy (ATTRACTION-3): a multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol*. 2019;**20**(11):1506-1517. doi: 10.1016/s1470-2045(19)30626-6
8. Baba Y, Yagi T, Kosumi K, Okadome K, Nomoto D, Eto K, *et al*. Morphological lymphocytic reaction, patient prognosis and PD-1 expression after surgical resection for oesophageal cancer. *Br J Surg*. 2019;**106**(10):1352-1361. doi: 10.1002/bjs.11301
9. Hinshaw DC, Shevde LA. The Tumor Microenvironment Innately Modulates Cancer Progression. *Cancer Res*. 2019;**79**(18):4557-4566. doi: 10.1158/0008-5472.can-18-3962
10. Pan JH, Zhou H, Cooper L, Huang JL, Zhu SB, Zhao XX, *et al*. LAYN Is a Prognostic Biomarker and Correlated With Immune Infiltrates in Gastric and Colon Cancers. *Front Immunol*. 2019;**10**:6.
11. Ino Y, Yamazaki-Itoh R, Shimada K, Iwasaki M, Kosuge T, Kanai Y, *et al*. Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer. *Br J Cancer*. 2013;**108**(4):914-923. doi: 10.1038/bjc.2013.32
12. Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discov*. 2019;**18**(3):197-218. doi: 10.1038/s41573-018-0007-y
13. Van der Woude LL, Gorris MAJ, Halilovic A, Figdor CG, de Vries IJM. Migrating into the Tumor: a Roadmap for T Cells. *Trends Cancer*. 2017;**3**(11):797-808. doi: 10.1016/j.trecan.2017.09.006
14. Orhan A, Vogelsang RP, Andersen MB, Madsen MT, Holmich ER, Raskov H, *et al*. The prognostic value of tumour-infiltrating

lymphocytes in pancreatic cancer: a systematic review and meta-analysis. *Eur J Cancer*. 2020;**132**:71-84. doi: 10.1016/j.ejca.2020.03.013
15. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*. 2008;**9**:559. doi: 10.1186/1471-2105-9-559
16. Wan Q, Tang J, Han Y, Wang D. Co-expression modules construction by WGCNA and identify potential prognostic markers of uveal melanoma. *Exp Eye Res*. 2018;**166**:13-20. doi: 10.1016/j.exer.2017.10.007
17. Yang Q, Wang R, Wei B, Peng C, Wang L, Hu G, *et al*. Candidate Biomarkers and Molecular Mechanism Investigation for Glioblastoma Multiforme Utilizing WGCNA. *Biomed Res Int*. 2018;**2018**:4246703. doi: 10.1155/2018/4246703
18. Yoshihara K, Shahmoradgoli M, Martinez E, Vegesna R, Kim H, Torres-Garcia W, *et al*. Inferring tumour purity and stromal and immune cell admixture from expression data. *Nat Commun*. 2013;**4**:2612. doi: 10.1038/ncomms3612
19. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*. 2012;**16**(5):284-728. doi: 10.1089/omi.2011.0118
20. Borgan Ø. Modeling Survival Data: Extending the Cox Model. Terry M. Therneau and Patricia M. Grambsch, Springer-Verlag, New York, 2000. No. of pages: xiii + 350. Price: $69.95. ISBN 0-387-98784-3. *Statistics in Medicine*. 2001;**20**(13):2053-2054. doi: 10.1002/sim.956
21. Zhao Y, Zhang J, Wang S, Jiang Q, Xu K. Identification and Validation of a Nine-Gene Amino Acid Metabolism-Related Risk Signature in HCC. *Front Cell Dev Biol*. 2021;**9**:731790. doi: 10.3389/fcell.2021.731790
22. Cao Y, Tang W, Tang W. Immune cell infiltration characteristics and related core genes in lupus nephritis: results from bioinformatic analysis. *BMC Immunol*. 2019;**20**(1):37. doi: 10.1186/s12865-019-0316-x
23. Watanabe M, Otake R, Kozuki R, Toihata T, Takahashi K, Okamura A, *et al*. Recent progress in multidisciplinary treatment for patients with esophageal cancer. *Surg Today*. 2020;**50**(1):12-20. doi: 10.1007/s00595-019-01878-7
24. Bharat A, Crabtree T. Management of advanced-stage operable esophageal cancer. *Surg Clin North Am*. 2012;**92**(5):1179-1197. doi: 10.1016/j.suc.2012.07.012
25. Zhang C, Sun Q. Weighted gene co-expression network analysis of gene modules for the prognosis of esophageal cancer. *J Huazhong Univ Sci Technolog Med Sci*. 2017;**37**(3):319-325. doi: 10.1007/s11596-017-1734-8
26. Oppermann M. Chemokine receptor CCR5: insights into structure, function, and regulation. *Cell Signal*. 2004;**16**(11):1201-1310. doi: 10.1016/j.cellsig.2004.04.007
27. Pervaiz A, Zepp M, Georges R, Bergmann F, Mahmood S, Faiza S, *et al*. Antineoplastic effects of targeting CCR5 and its therapeutic potential for colorectal cancer liver metastasis. *J Cancer Res Clin Oncol*. 2021;**147**(1):73-91. doi: 10.1007/s00432-020-03382-9
28. Barr VA, Sherman E, Yi J, Akpan I, Rouquette-Jazdanian AK, Samelson LE. Development of nanoscale structure in LAT-based signaling complexes. *J Cell Sci*. 2016;**129**(24):4548-4562. doi: 10.1242/jcs.194886
29. Wang Z, Peng M. A novel prognostic biomarker LCP2 correlates with metastatic melanoma-infiltrating CD8(+) T cells. *Sci Rep*. 2021;**11**(1):9164. doi: 10.1038/s41598-021-88676-9
30. Porcu M, Kleppe M, Gianfelici V, Geerdens E, De Keersmaecker K, Tartaglia M, *et al*. Mutation of the receptor tyrosine phosphatase PTPRC (CD45) in T-cell acute lymphoblastic leukemia. *Blood*. 2012;**119**(19):4476-4479. doi: 10.1182/blood-2011-09-379958
31. Fernandes RA, Su L, Nishiga Y, Ren J, Bhuiyan AM, Cheng N, *et al*. Immune receptor inhibition through enforced phosphatase recruitment. *Nature*. 2020;**586**(7831):779-784. doi: 10.1038/s41586-020-2851-2
32. Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. *Nat Rev Mol Cell Biol*. 2006;**7**(11):833-846. doi: 10.1038/nrm2039
33. Lanier LL, Corliss B, Wu J, Phillips JH. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity*. 1998;**8**(6):693-701. doi: 10.1016/s1074-7613(00)80574-9
34. Dietrich J, Cella M, Seiffert M, Buhring HJ, Colonna M. Cutting edge: signal-regulatory protein beta 1 is a DAP12-associated activating receptor expressed in myeloid cells. *J Immunol*. 2000;**164**(1):9-12. doi: 10.4049/jimmunol.164.1.9
35. Cheray M, Bessette B, Lacroix A, Melin C, Jawhari S, Pinet S, *et al*. KLRC3, a Natural Killer receptor gene, is a key factor involved in glioblastoma tumourigenesis and aggressiveness. *J Cell Mol Med*. 2017;**21**(2):244-253. doi: *10.1111/jcmm.12960*
36. Jiang J, Ding Y, Wu M, Lyu X, Wang H, Chen Y, *et al*. Identification of TYROBP and C1QB as Two Novel Key Genes With Prognostic Value in Gastric Cancer by Network Analysis. *Front Oncol*. 2020;**10**:1765. doi: 10.3389/fonc.2020.01765
37. Hosseini A, Gharibi T, Marofi F, Babaloo Z, Baradaran B. CTLA-4: From mechanism to autoimmune therapy. *Int Immunopharmacol*. 2020;**80**:106221. doi: 10.1016/j.intimp.2020.106221
38. Herrmann A, Lahtz C, Nagao T, Song JY, Chan WC, Lee H, *et al*. CTLA4 Promotes Tyk2-STAT3-Dependent B-cell Oncogenicity. *Cancer Res*. 2017;**77**(18):5118-4128. doi: 10.1158/0008-5472.can-16-0342
39. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, *et al*. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science*. 2017;**355**(6332):1428-1433. doi: 10.1126/science.aaf1292
40. Kamphorst AO, Wieland A, Nasti T, Yang S, Zhang R, Barber DL, *et al*. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. *Science*. 2017;**355**(6332):1423-1427. doi: 10.1126/science.aaf0683
41. Ishikawa M, Nakayama K, Nakamura K, Yamashita H, Ishibashi T, Minamoto T, *et al*. High PD-1 expression level is associated with an unfavorable prognosis in patients with cervical adenocarcinoma. *Arch Gynecol Obstet*. 2020;**302**(1):209-218. doi: 10.1007/s00404-020-05589-0
42. Mazzaschi G, Madeddu D, Falco A, Bocchialini G, Goldoni M, Sogni F, *et al*. Low PD-1 Expression in Cytotoxic CD8(+) Tumor-Infiltrating Lymphocytes Confers an Immune-Privileged Tissue Microenvironment in NSCLC with a Prognostic and Predictive Value. *Clin Cancer Res*. 2018;**24**(2):407-419. doi: 10.1158/1078-0432.ccr-17-2156
43. Kono Y, Saito H, Miyauchi W, Shimizu S, Murakami Y, Shishido Y, *et al*. Increased PD-1-positive macrophages in the tissue of gastric cancer are closely associated with poor prognosis in gastric cancer patients. *BMC Cancer*. 2020;**20**(1):175. doi: 10.1186/s12885-020-6629-6
44. Xin P, Xu X, Deng C, Liu S, Wang Y, Zhou X, *et al*. The role of JAK/STAT signaling pathway and its inhibitors in diseases. *Int Immunopharmacol*. 2020;**80**:106210. doi: 10.1016/j.intimp.

2020.106210
45. Ahmed SM, Luo L, Namani A, Wang XJ, Tang X. Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochim Biophys Acta Mol Basis Dis*. 2017;**1863**(2):585-597. doi: 10.1016/j.bbadis.2016.11.005
46. Qiu L, Liu J, Wang Z, Chen S, Hu W, Huang Q, *et al*. ZGDHu-1 promotes apoptosis of mantle cell lymphoma cells. *Oncotarget*. 2017;**8**(7):11659-71165. doi: 10.18632/oncotarget.14274
47. Cantley LC. The phosphoinositide 3-kinase pathway. *Science*. 2002;**296**(5573):1655-1657. doi: 10.1126/science.296.5573.1655