Construction of a novel immune gene-based model for prognosis prediction of colorectal cancer

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Research article

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Abstract

Background

Colon adenocarcinoma (COAD) is the third leading cause of cancer-related death. Although surgical treatment and chemotherapy of COAD have made significant progress, its immunotherapy also has great potential, nowadays.

Methods

Gene expression profiles and clinical data of COAD patients were obtained from The Cancer Genome Atlas_Colon Adenocarcinoma (TCGA_COAD) and Gene Expression Omnibus (GEO) databases, which were further detected for immune-related genes. Immune-related genes were downloaded from Immunology Database and Analysis Portal (ImmPort). LASSO Cox regression analysis was utilized to analyze the immune-related prognostic signature. The prognostic value of the signature was validated by ROC curve. To further detected the potential pathway about immune-related genes in COAD patients, Gene Set Enrichment Analysis (GESA) was used to identify the most significant pathways.

Results

Finally, a total of 436 immune-related mRNA were identified. Eleven prognosis-related genes were selected to establish an immune-related prognostic signature, which divided patients into high and low risk groups. Several biological processes, such as immune response was enriched. Moreover, our prognosis model has better performance in predicting the 1-, 3-, 5- and 8-years overall survival (OS) for patients from the TCGA and GEO cohort. Also, the complicated signature obtained by combining immune-related signatures with clinical factors provides a more accurate OS predicting compared with individual molecular signatures.

Conclusion

We have established a prognostic signature consisting of 11 immune-related genes, which may be potential biomarkers for identifying COAD with a high risk of death. Then, the possibility including immunotherapy in personalized COAD treatment was evaluated.

1. Introduction

As one of the most common carcinoma worldwide, COAD, is the third-leading cause of cancer death and expected to reach more than 2.2 million new cases (with 1.1 million deaths) by 2030 [1–3]. In recent years, with the significant progress of surgical treatment and chemotherapy of COAD, immunotherapy also shows great potential for cancer treatment [1, 4]. It is generally believed that COAD is multifactorial

disease and the pathogenic factors of which include genetic factors, microenvironment factors and chronic inflammation [4]. Moreover, chronic inflammation caused by COAD could be seen in some tumor patients and this is directly related to the occurrence of colon cancer [4].

Tumor microenvironment (TME) consisting of the matrix, blood vessels, secreted factors, surrounding matrix and tumor cells, plays a vital role in the internal environment of tumor. Nowadays, more attention has been focused on the tumor microenvironment (TME). Meanwhile, immunotherapy has also achieved encouraging therapeutic effects in tumors [5, 6]. For example, Robert and Wolchok et al. found that the combination of ipilimumab and nivolumab in the treatment of metastatic melanoma increased its 3-year overall survival by five times [7, 8]. In addition, Marissa et al. found that a new strategy combining immunotherapy with adjuvant or neoadjuvant therapy could prolong the survival of nonsmall-cell lung cancer (NSCLC) patients undergoing surgery [9]. With the latest developments in tumor cytology and molecular biology, a deep understanding of TME is essential to reveal the underlying molecular mechanism of tumorigenesis and development [10]. Li et al. confirmed the non-cell autonomous role of retinoblastoma in the tumor microenvironment and linked the loss of retinoblastoma to immunosuppression [11]. Luca et al. proved that CCA cells exchange autocrine/paracrine signals with other cancer cells and infiltrating cell types that spread in the microenvironment [12]. Therefore, the exploration of new molecular mechanisms has excellent significance for formulating new immunotherapy strategies [13]. So far, however, the association between tumor-infiltrating immune cells (TIICs) and COAD remains unclear. Thus, it is vital to find effective biomarkers for early diagnosis and predicting the prognosis of COAD.

In recent years, the high-throughput platforms for gene expression are developing rapidly and being widely applied in many aspects, such as molecular classification, prognosis prediction, and new targeted drug discovery. TCGA and GEO are considered the most significant cancer database worldwide, containing massive information of gene expression profile of multiple cancer types. Here, we constructed a prognosis model based on 11 immune-related genes by series bioinformatics analysis. The key genes we identified are directly associated with the prognosis of COAD and maybe the candidate biomarkers for COAD.

2. Materials And Methods

2.1. Gene expression profile data

The independent COAD gene expression profiles, GSE17536, consisting of 177 COAD patients, was selected from the GEO database, whose platforms was GPL570 (Affymetrix Human Genome U133 Plus 2.0 Array). The gene transcriptome, clinical and survival information of COAD patients were obtained from TCGA database (https://portal.gdc.cancer.gov/; TCGA-COAD), which contains 470 COAD samples and 41 adjacent normal samples. Data preprocessing include background correction, data normalization, removing batch effects, combining normal and tumor group data, ID transform gene symbol, and probe supplemental missing value.

2.2. Identification of differentially expressed genes

The differentially expressed mRNA data were screened utilizing “limma” package, with threshold of log2 fold change (FC) > 1 and *p*-value < 0.05. Immune-related genes were obtained from the ImmPort (https://www.immport.org).

2.3. Construction of prognosis-model and nomogram.

On the basis of 436 immune-related mRNAs identified, the prognosis-related genes were primarily identified through "survival" package. In detail, 436 mRNA expression profile data were firstly combined with the survival information of COAD patients. Then, univariate cox analysis, LASSO regression and multivariate cox analysis were performed in sequential order to screen prognosis-related genes. The prognosis model was established with "Risk scores = $\Sigma coef * Exp(genes)$". The patients whose risk scores were above the median were defined as a high-risk group; otherwise, the low-risk group. ROC curve was used to evaluate the effectiveness of our prognosis model. Kaplan-Meier survival curve was used to compare the prognosis difference in high-risk group and low-risk group. P-value <0.05 was considered to be significant for the impact of OS. The nomogram was constructed based on the model established and clinical features. Calibration curves were used to measure the fitting degree of the actual OS and the predicted OS (one, three, five and eight years).

2.4. Clinical correlationanalysis

The association between clinical features (age, gender and each subset of TNM) and immune-related genes was analyzed by R software using the Wilcox test. The 7th edition of the TNM stage system 23 was adopted, and Mx was defined as unable to evaluate the presence or absence of distant metastasis.

2.5. Gene set enrichment analysis (GSEA) analysis

To further understand the underlying molecular mechanism and biological function of our model genes, GSEA was utilized with “c2.cp.kegg.v7.1.symbols.gmt” gene sets. In detail, the number of permutations was 1000; the metric for ranking genes was Signal2Noise. High risk group was regarded as experimental group and the low-risk group was seen as a reference group. FDR <0.25, and nominal P-value < 0.05 were used as the critical criterion.

2.6. *Cell Lines and reagents*

Four colon cancer cell clines, including SW620, LoVo, HCT116, RKO, and COLO205, and a normal colonic epithelial cell lines, HIEC-6, were utilized for the study. These cell lines were cultured in RMPI 1640 medium (Life Technologies, Gaithersburg, MD), with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere.

2.7.*Quantitative reverse transcription polymerase chain reaction*

Total RNA was extracted from the HIEC-6, SW620, LoVo, HCT116, RKO, and COLO205 cell lines utilizing TRIzol reagent (Life Technologies). Total RNA was used as a template to synthesize complementary DNA (cDNA) utilizing PrimeScript RT Reagent Kit. Then, qRT-PCR was conducted with SYBR Premix Ex Taq (Takara Bio Inc.). The primer sequences used for real-time PCR were listed in the Table S1. Last, the ABI 7900 system (Applied Biosystems) was used to perform qRT-PCR assays.

2.8. *Cell proliferation assay*

The Cell Counting Kit-8 (CCK-8) assay (Vazyme Biotech Co.,Ltd) was used to assess the cell proliferation. SW620 and LoVo cells were seeded into 96-well plates (5×10^3 cells/plate). 10 microlitres (ml) of CCK-8 was added to each well of the plate at the several times: 0 h, 24 h, 48 h, 72 h and 96 h. Then, optical density (OD) was measured at 1-4 days at a wavelength of 450 nm.

2.9. Colony formation assay

SW620 and LoVo cells were plated into 6-well plates (1 × 103 cells/plate) and cultured for 2-3 weeks. Then, cells were fixed with 10% formaldehyde for 15 minutes and stained with 1% crystal violet for 30 s prior to compute the number of colonies.

2.10. Cell invasion and migration assay

Cell invasion and migration was performed with transwell plates (24-well insert, 8 μm pore size; BD Biosciences, Bedford, MA, USA), respectively. For cell invasion assay, filters needed covered with 100 μL of Matrigel (1:5 dilution; BD Biosciences). Then, 5×10^4 SW620 and LoVo cells were seeded into the upper chamber with serum-free RPMI-1640. And, 600 μl RPMI-1640 medium with 10% FBS was added to the bottom chamber. After 36h of incubation , the chamber was fixed with 4% paraformaldehyde for 15 min and stained with 1% crystal violet for 30S. Last, magnification microscope was utilized to count the number of cells in the bottom chamber.

2.11. Statistical analysis

In this research, the experiments were carried out in triplicate, and the data were expressed as the mean ± standard deviation.

3. Results

3.1. Gene expression of patients in dataset

We obtained gene expression level and clinical information from TCGA_COAD dataset, including 514 COAD and 41 non-tumorous sample. Totally, 7698 DEGs was finally identified (***Figure 1A***). The intersection of DEGs and immune-related genes identified 580 genes and 436 in which were mRNA (***Figure 1B***). 438 overlapped mRNAs were utilized to make a heatmap which showed gene expression patterns of DEGs between different clinical subgroup (including TNM, stage, gender, age and fustat).

3.2. Screening and verification of DEG related to prognosis

LASSO regression that could make the optimal lambda value from the minimum partial likelihood deviance with tenfold cross-validation was utilized to identify the DEGs significantly correlated with the prognosis of COAD patients (***Figure 2A-B***). Ultimately, 11 genes in DEGs (C8G, AEN, FGF9, GAL, STC2, UCN, UTS2, IL1RL2, MC1R, OXTR and TNFRSF13C) were screened for their significant association with OS (***Figure 2C***).

3.3. Establishment and validation of RFS prediction model

Based on these 11 genes, a model for predicting the OS of COAD was constructed. Each patient received a risk score according to the model (***Table 1***). Based on the median value of the risk score, we divided the patients into high-risk and low-risk groups. The result of Kaplan-Meier curves indicated that the patients in the high-risk group might have a poor prognosis compared with low-risk group (***Figure 2D***). To further evaluate the predictive ability of our prognosis model, the survival status and riskscores of each COAD patient in the TCGA cohort were shown in ***Figure 3A*** and the patients in GSE17536 were shown in ***Figure 3C***. The ROC curve revealed that our model had good sensitivity and specificity in predicting OS (TCGA cohort: Five years, AUC = 0.718; Three years, AUC = 0.779; One years, AUC = 0.806) (***Figure 3B***). Simultaneously, the validation set GSE17563 was also demonstrated satisfactory performance (GSE17536 cohort: Five years, AUC = 0.846; Three years, AUC = 0.830; One years, AUC = 0.837), as expected.

3.4. The construction of nomogram and calibration.

A nomogram based on the predictive model and clinical features, for example, the TNM stage, clinical stage, gender and age, was established for the better application in clinical (***Figure 4A***). The nomogram showed great effectiveness and stability when assessed with calibrations (One year, Gray = ideal; Three years, Gray = ideal; Five years, Gray = ideal; Eight years, Gray = ideal) (***Figure 4B***).

3.5. Independence and effectiveness of our prognosis models

Furthermore, to investigate the independence and effectiveness of prognostic models and clinicopathological factors, we utilized multivariate Cox analysis to assure whether the prognostic value of the prognosis model was independent of all other clinical characteristics such as age, gender and TNM stage. The result revealed the satisfactory independence of our model, indicating the potential of our model in clinical application (***Figure 5A***). Meanwhile, the result of correlation analysis showed that the patients in the high-risk group might have an adverse N and M classification, which may be one reason for its poor prognosis (***Figure 5B***).

3.6. Differential pathways between high-risk group and low-risk group

GSEA was performed to detect the potential biological process between the low-risk group and the high-risk group. As seen in ***Figure 6***, the high-risk phenotype of 11 immune-related genes, homologous

recombination, glyoxylate and dicarboxylate metabolism, glycine serine, threonine metabolism was enriched in. The downregulated pathway included glycan biosynthesis, vasopressin regulated water reabsorption, regulation of autophagy.

3.7. UCN was screened and correlated with immune cells

To find the most valuable genes among the model-associated genes, we built a protein protein interaction (PPI) network and selected Top25 genes (***Figure 7A***). We overlapped the Top25 genes with 11 model-related genes and screened out UCN (***Figure 7B***). Correlation analysis of UCN with TNM stage showed that UCN expression was positively correlated with N stage and M stage (***Figure 7C***). UCN were up-regulated in COAD and correlated with poor OS (***Figure 7D, E***). By gene set variation analysis (GSVA), the up-regulated of UCN was correlated with activation of coagulation, xenobiotic metabolism, WNT beta-catenin signaling, and estrogen response late pathways (***Figure 8A***). Regarding immune correlation-analysis, UCN expression was positively correlated with the content of NK CD56 bright cells and Th17 cells, and negatively correlated with the content of immune cells such as Tgd, macrophages, T helper cells, and Th2 cells (***Figure 8B, C***).

3.8. Expression and functional validation of UCN in COAD

Since the WNT beta-catenin signaling pathway was linked to the progression of COAD, we verified the expression and function of UCN in COAD. The expression of UCN in COAD was higher than that in paried non-tumor (***Figure 9A***). Based on the expression of colon cancer cell lines, the UCN was significantly up-regulated in several colon cancer cell lines, especially SW620 and LoVo (***Figure 9B***). We performed transfection with SiRNA (NC and SiUCN) in SW620 and LoVo, respectively, to knock down the expression level of UCN. The function of UCN in colon cancer was verified by CCK8, plate cloning, and transwell (***Figure 9C-F***). The results showed that knockdown of UCN inhibited the proliferation, migration and invasion of SW620 and LoVo (***Figure 9C-F***).

4. Discussion

COAD, in the world, was a malignant tumor with high morbidity and mortality. However, the current research on COAD had not found effective methods to prevent the occurrence of colon cancer and reduce the incidence of colon cancer. When COAD patients enter the advanced stage of the tumor, their 5-year survival rate was extremely low. In fact, evidence of close relationship between the tumor microenvironment and tumor development has increased dramatically, the discovery of effective immune-related molecules is of great significance for the treatment of COAD [14–16]. Based on these immune-related molecules, the establishment of a diagnostic and prognostic model that could accurately predict the status and survival time of COAD patients also played an important role.

Here, we had gained a better understanding of the etiology and pathogenesis of COAD by bioinformatics analysis. Results got from the basic research of molecular biology have proved that the occurrence and development of COAD was a multi-stage and multi-factor process. In this investigation, we determined

reliable prognostic markers based on tumor immune-related genes and their prognostic values have been verified in multiple independent cohorts. The immune-related genes that we identified through the TCGA database including 11 genes, C8G, AEN, FGF9, GAL, STC2, UCN, UTS2, IL1RL2, MC1R, OXTR and TNFRSF13C. They could be used as effective biomarkers and targets for immunotherapy of COAD patients. Besides, we also identified the underlying biology pathway of low-risk and high-risk groups, which might demonstrate the vital role of tumor immune microenvironment in the occurrence and development of COAD.

Finally, we screened 11 immune-related genes through the TCGA database, and these genes were all down-regulated in COAD cohort. Although remarkable progress of COAD treatment has been made in the past decade, surgical treatment could not achieve satisfactory results and mostly be supplemented with radiotherapy or chemotherapy in advanced patients [2]. Therefore, it was meaningful for our reliable prognosis model based on immune-related genes in the TCGA-COAD cohort.

All these 11 genes were significantly related to the OS of COAD patients and predictably, immune-related gene markers had high applicability and stability in predicting the prognosis of patients with COAD. We further evaluated the correlation between these 11 genes with clinical parameters to explore the clinical value of these immune-related gene. The results showed that these 11 genes were closely related to the prognosis and TNM. A lot of evidence had shown that innate and adaptive immune systems might play a non-negligible role in the occurrence and development of diseases, especially in cancer[4, 10, 13, 17–23]. Immunotherapy might be active for some cancer patient; however, previous research has shown that breast cancer and melanoma may be more suitable for immunotherapy than COAD [23, 24]. Further, we used the model established by immune-related genes to divide patients into high-risk groups and low-risk groups, and performed GSEA analysis to find enrichment pathways. The results showed that these immune-related genes were actively involved in processes such as homologous recombination, glyoxylate and dicarboxylate metabolism and glycine serine and threonine metabolism, o glycan biosynthesis, vasopressin regulated water reabsorption and regulation of autophagy, and played an important role in the metabolic process of tumor occurrence and development. Liu et al. established a FaDu cell line with specific radiation resistance, and found that homologous recombination enhanced the radiation resistance of hypopharyngeal carcinoma[25]. Pan et al. found that the activation of innate immune receptors, including Toll-like receptors (TLR) and nucleotide oligomerization domain (NOD)-like receptors (NLR), could up-regulate innate immune-mediated autophagy [26]. Jiang and Lu et al. found that TLR2 stimulates autophagy by activating the c-Jun amino terminal kinase (JNK) and the extracellular signal-regulated kinase (ERK) signaling pathways, thereby enhancing the host's innate immune response [27, 28]. Cytokines and their receptors involved in tumor metabolism could affect tumor cells in the tumor microenvironment by changing their metabolites[10, 18, 20, 21, 29]. They could inhibit or promote tumorigenesis and development in a specific condition and have also been proven effective in cancer treatment. By exploring the role of cytokines in the immune response and tumor metabolism, future studies might discover the therapeutic potential of immune-related proteins in COAD immunotherapy.

In conclusion, despite the fact that few studies focused on the association between immune-related genes and the prognosis of COAD patients, they had excellent research value. Our study still has some limitations. Firstly, it has not yet been tested on the function and mechanism of the genes after screening. Secondly, since there are no inhibitors of these immune-related genes, the verification of the correlation between these immune-related signals and the response to tumor immunotherapy is hard to achieve.

Declarations

Authors' contributions

Competing interests

The authors declare no competing interests.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

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Figures

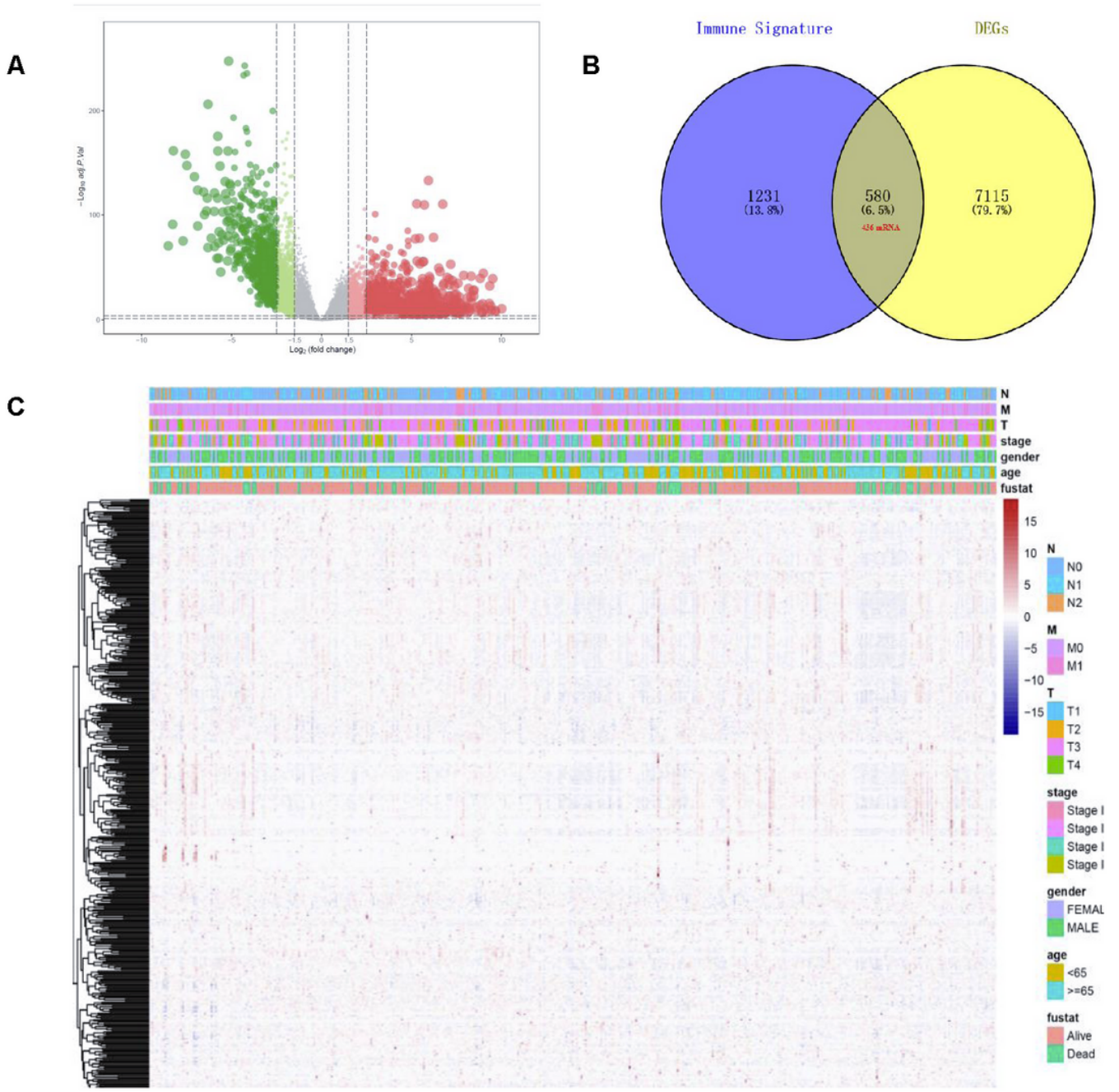

Figure 1

Comparison of differential expression of immune-related genes in colon cancer. A: Volcano plot visualizing the differential expression genes between colon cancer and normal tissue. B: Venn diagram analysis of mRNA resulting from comparison of downregulated genes and Immune signature genes. C: Heatmap analysis of the immune-related genes between different clinical and pathological groups.

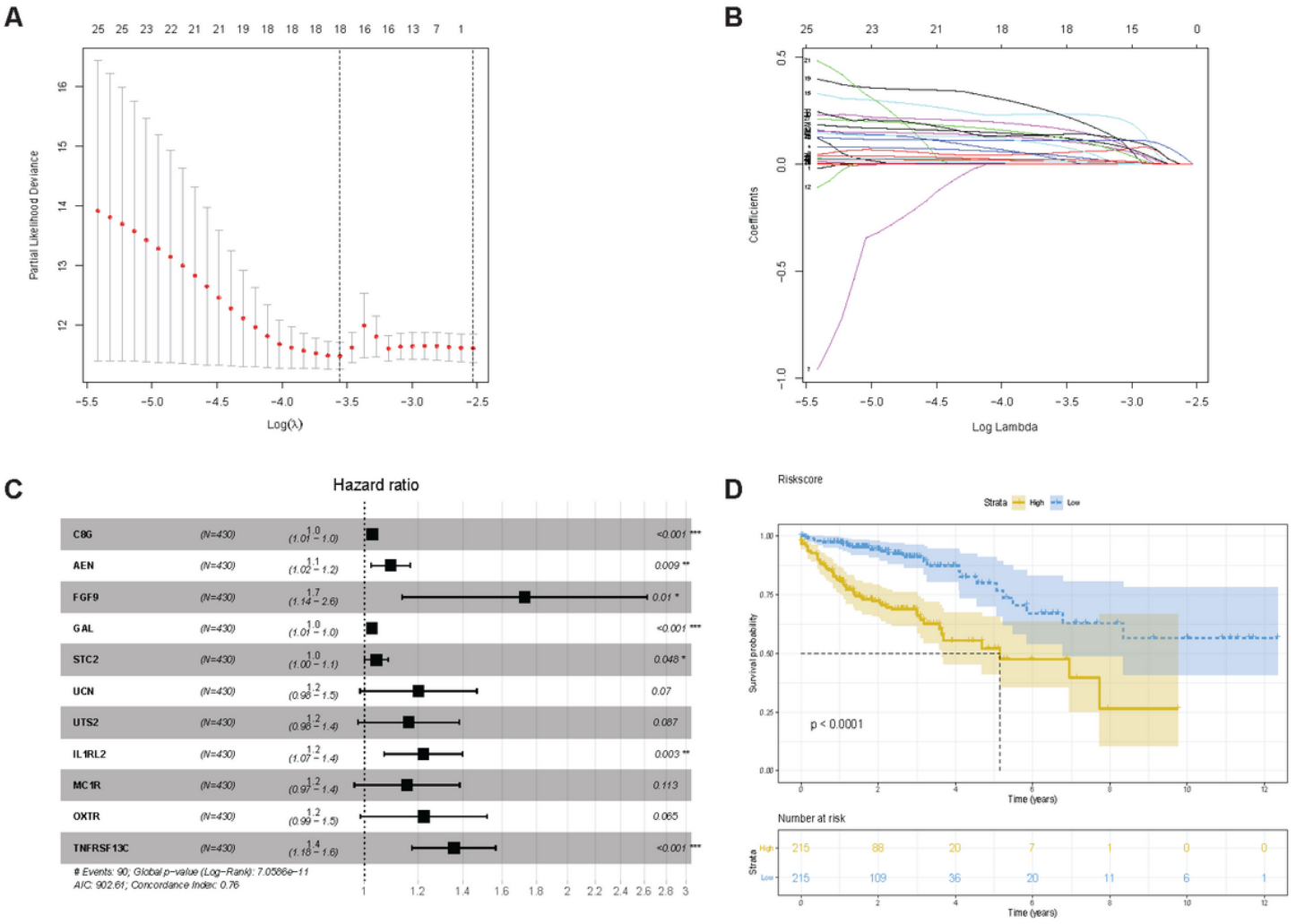

Figure 2

Identification of 11 significantly prognostic genes in colon cancer. A: LASSO regression obtained 11 prognostic genes utilizing minimum lambda value. B: LASSO coefficients profiles of 11 11 prognostic genes. C: Univariate Cox regression analysis of 11 prognostic genes. D: Kaplan–Meier survival analysis between low expression and high expression group.

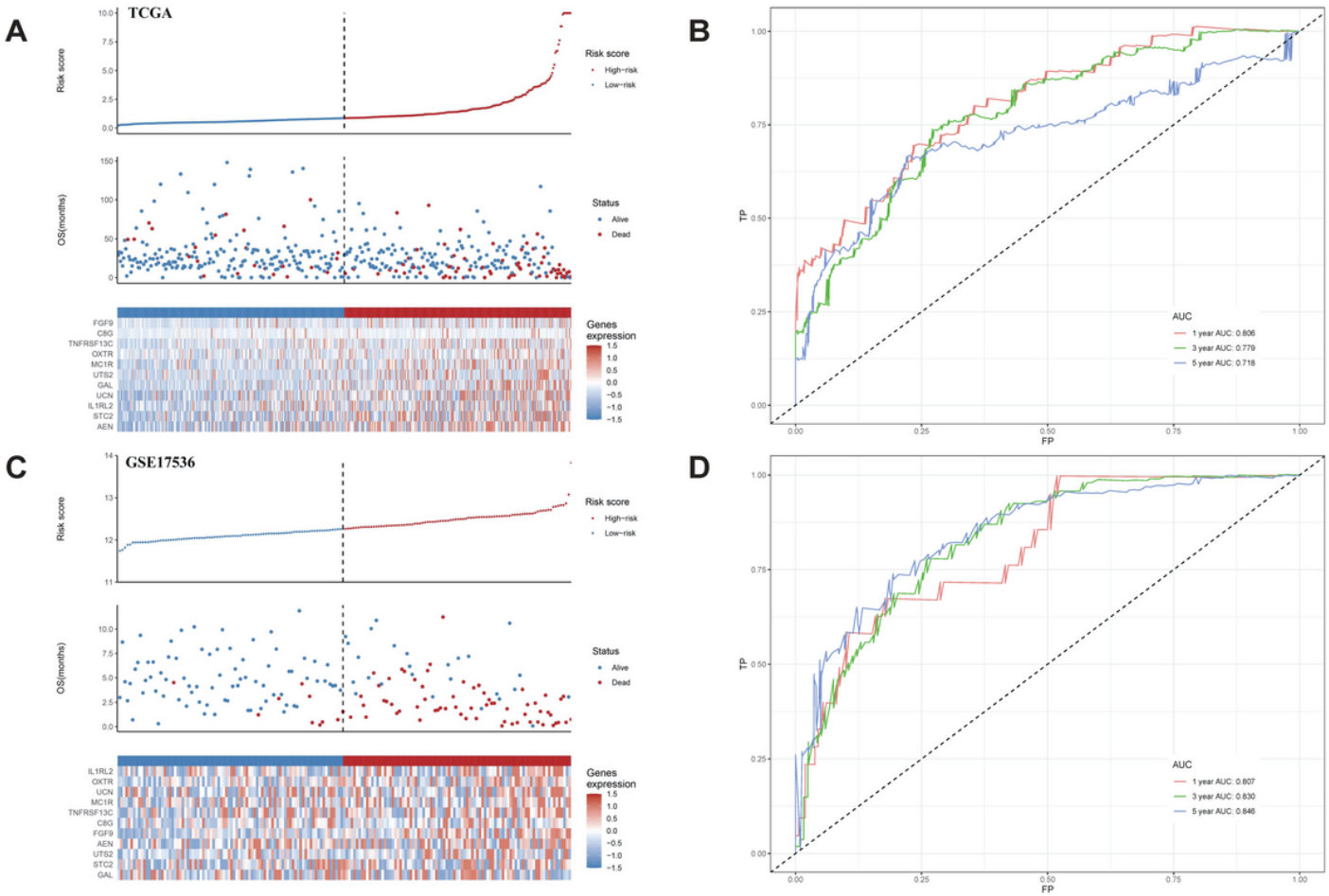

Figure 3

Verify the prognostic markers of colon 5cancer. A: TCGA cohort: the risk score of each colon cancer increased from blue to red, survival time of each colon cancer and heatmap of the 11 genes immune-related signature. B: Time-dependent ROC curves of OS for the 11-gene immune-related signature score in the TCGA cohort at 1-, 3-, and 5-year period. C: GEO cohort: the risk score of each colon cancer increased from blue to red, survival time of each colon cancer and heatmap of the 11 genes immune-related signature. D: Time-dependent ROC curves of OS for the 11-gene immune-related signature score in the GEO cohort at 1-, 3-, and 5-year period.

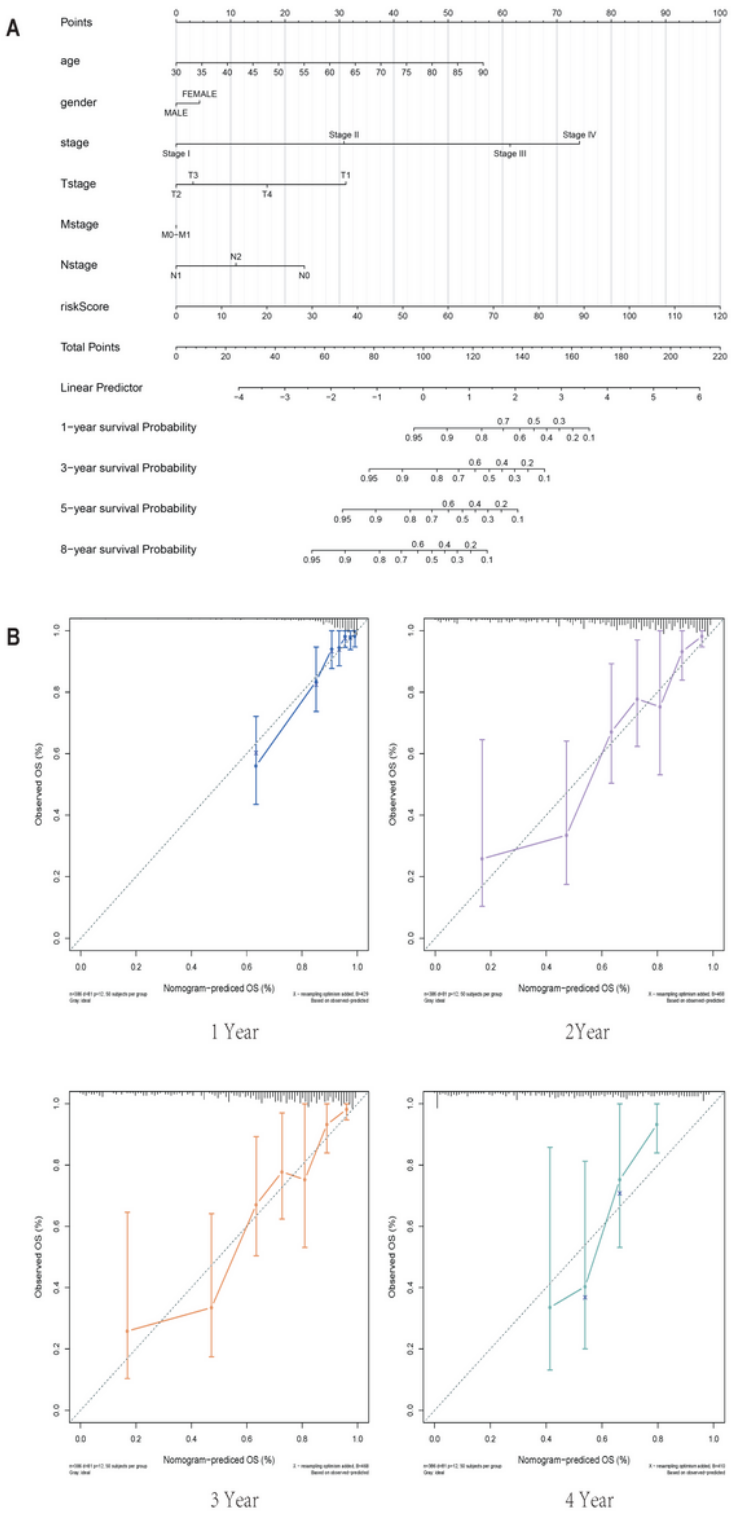

Figure 4

Mining independent prognostic parameters and establishing prognostic models. A: Nomogram integrated 11 gene-based risk score, TNM stage, linear predictor, grade and age. B: Calibration chart showed that the predicted OS fits well with the actual OS.

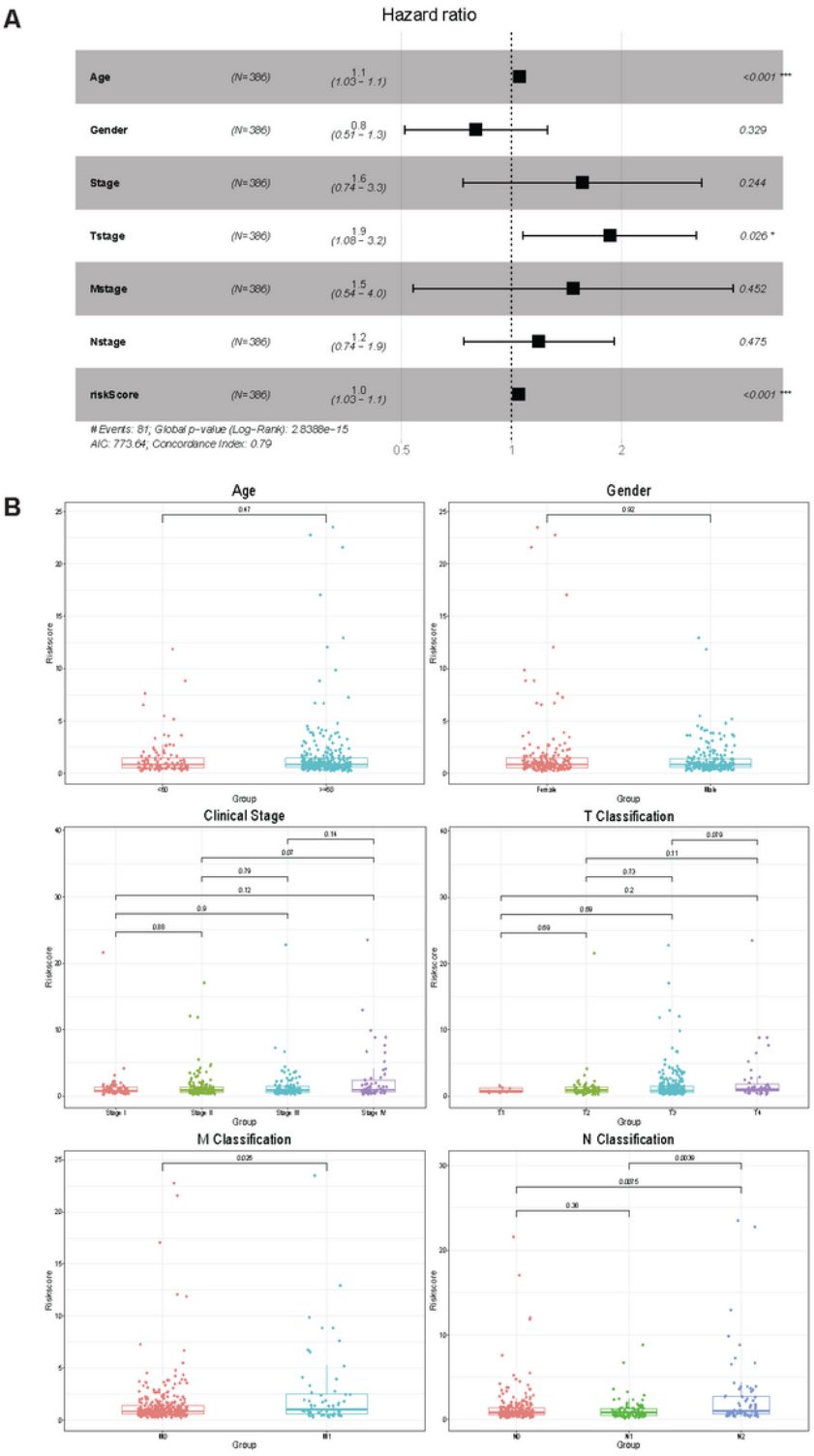

Figure 5

The independence and validity of the prognostic model and clinical factors. A: Multivariate correlation analysis of prognosis model. B: The NM staging of the high-risk group has significant clinical significance.

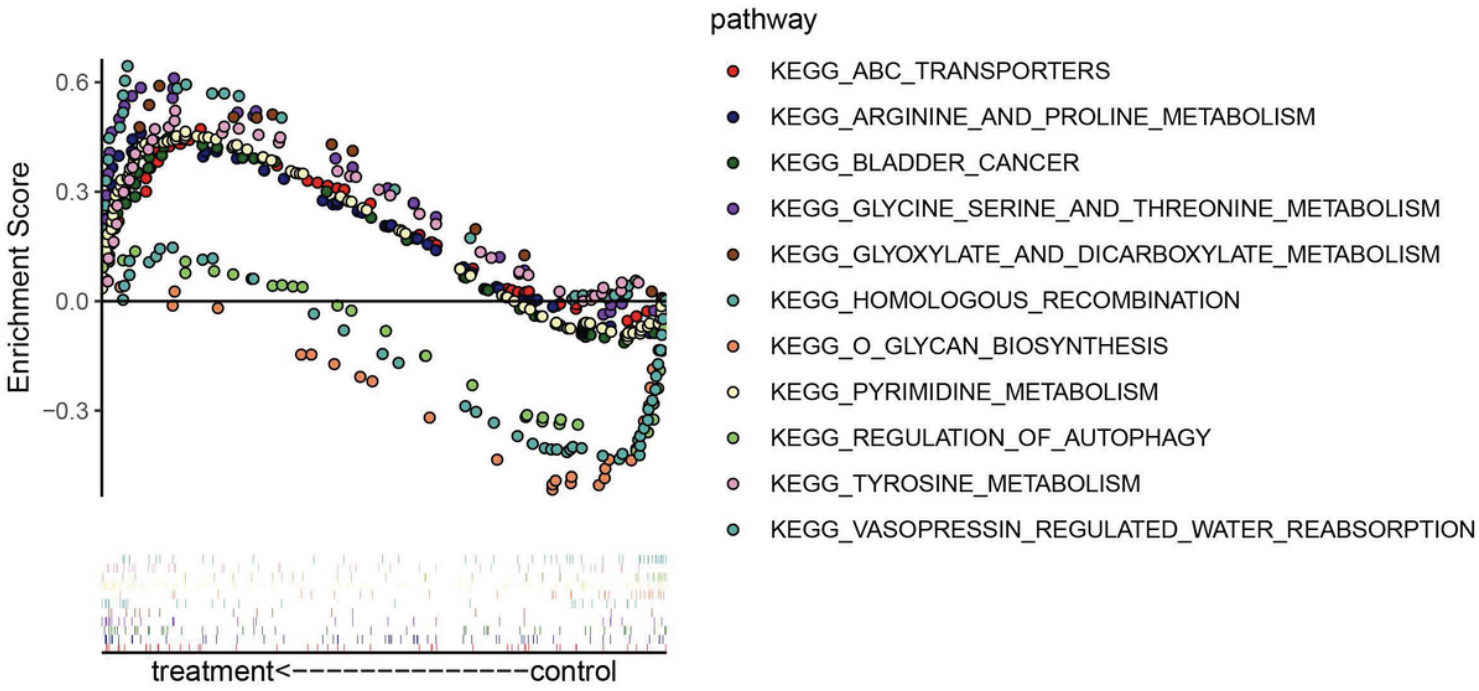

Figure 6

GSEA associated with the 11 genes expression. Up-regulation and down-regulation pathways of colon cancer patients in the high-risk and low-risk groups.

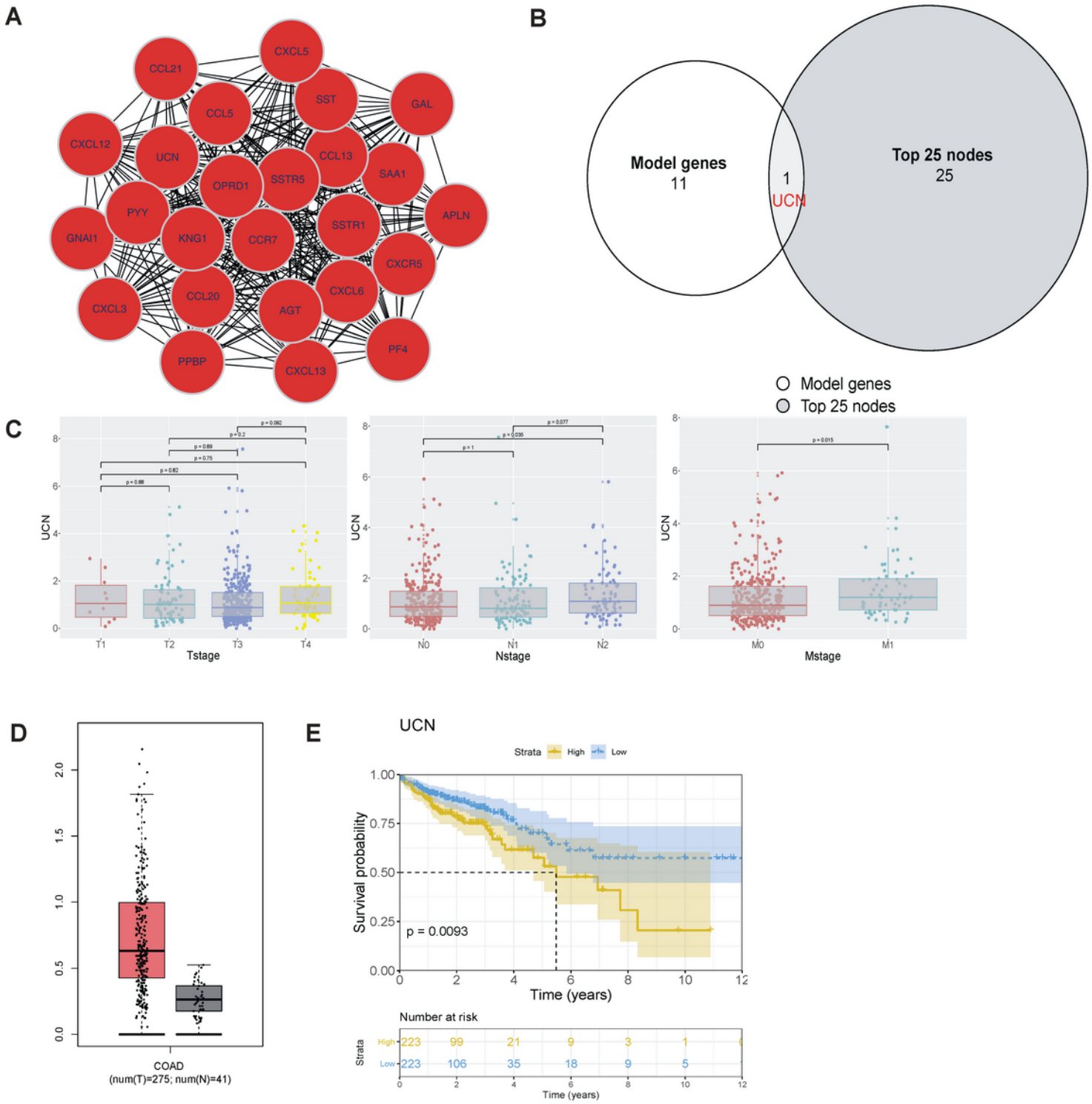

Figure 7

UCN was screened and associated with a poor prognosis for COAD. A: Top 25 nodes genes of PPI network. B: UCN was screened by overlapping between modeling related genes and Top25nodes genes. C: UCN expression was positively correlated with N stage and M stage. D: UCN were up-regulated in COAD and correlated with poor OS.

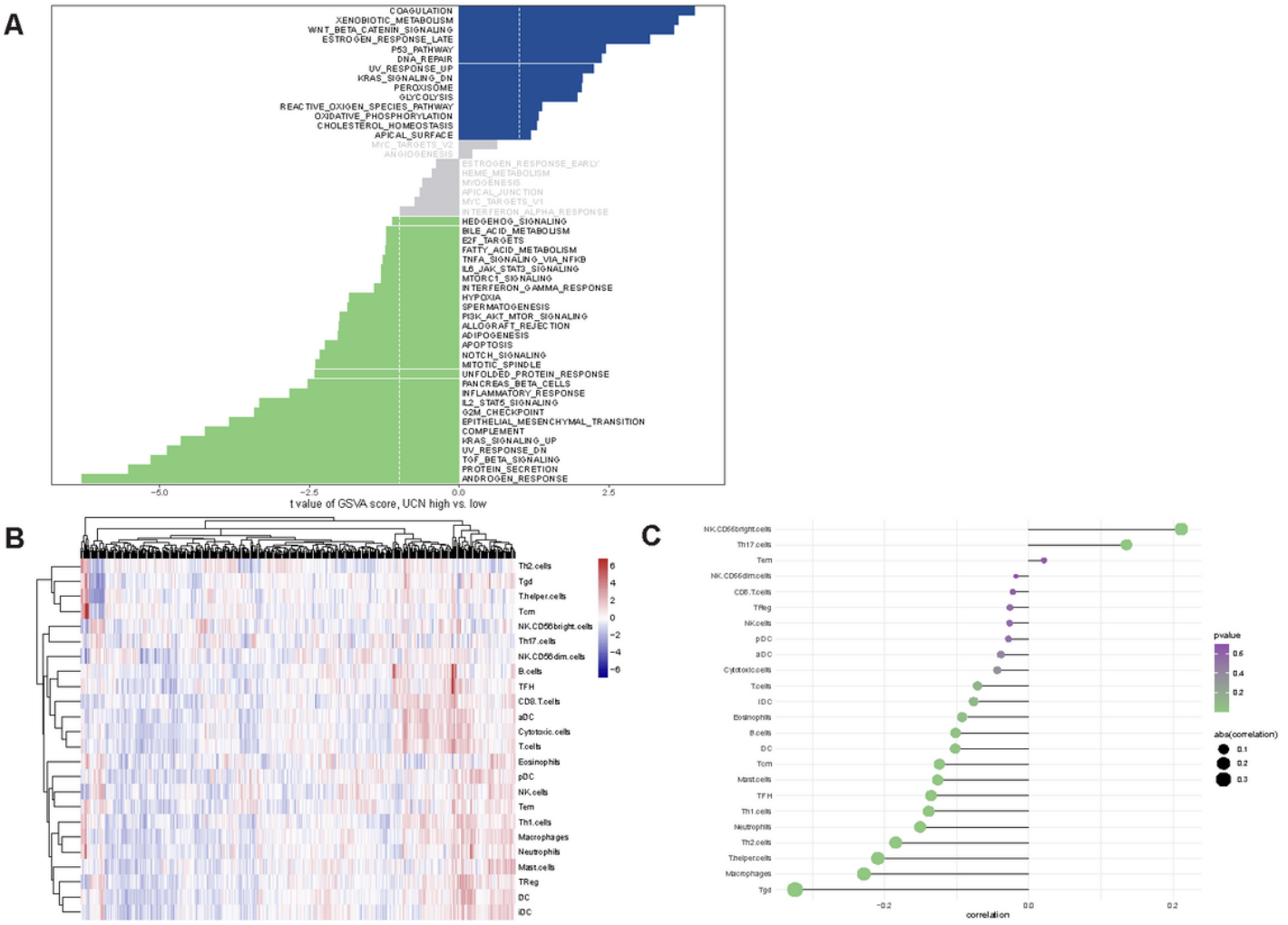

Figure 8

UCN-related pathways and relevance to immune cells. A: UCN was associated with a variety of pathways in COAD, such as coagulation, xenobiotic metabolism, WNT beta-catenin signaling, and estrogen response late pathways. B-C: UCN was associated with a variety of immune cells in COAD.

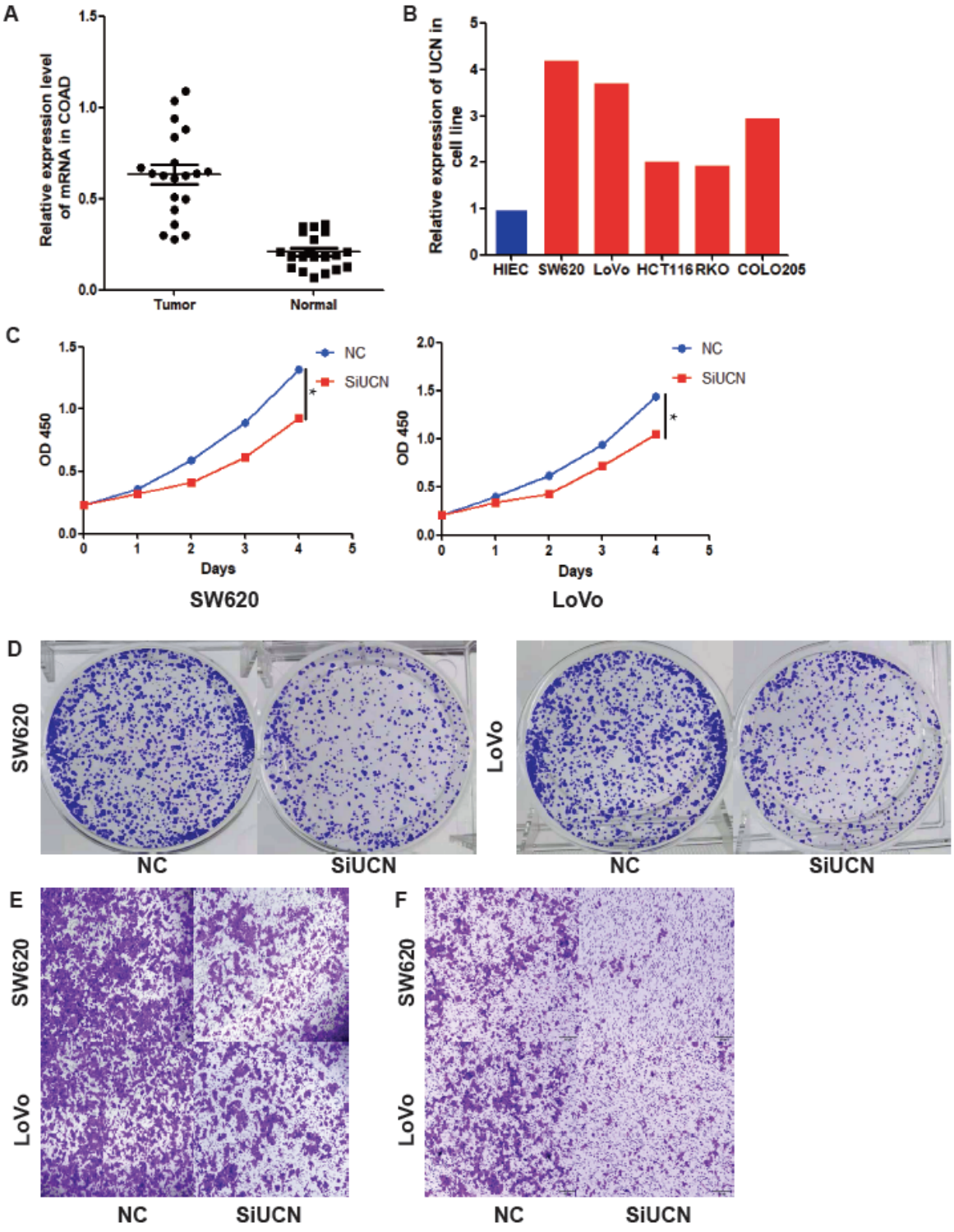

Figure 9

Expression and function of UCN in COAD. A: UCN expression was upregulated in COAD. B: UCN expression was upregulated in COAD cell lines. C: In CCK8, knockdown of UCN resulted in inhibition of proliferation of SW620 and LoVo. D: In the plate clones, knockdown of UCN resulted in inhibition of proliferation of SW620 and LoVo. E: In transwell, the knockdown of UCN leads to the suppression of the

migration of SW620 and LoVo. F: In transwell, the knockdown of UCN leads to the suppression of the invasion of SW620 and LoVo.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx