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A novel nomogram associated with regulatory T cells infiltration by weighted gene co-expression network analysis for predicting survival in patients with colon cancer

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Abstract. – OBJECTIVE: Traditional diagnostic strategies are unable to accurately discriminate between patients with poor and satisfied prognosis in colon cancer. Therefore, it is urgently recommended to identify new biomarkers in favor of better selection of patients at higher risk of recurrence or poor outcomes, with the aim of early intervention or avoiding overtreatment.

MATERIALS AND METHODS: The weighted gene correlation network analysis (WGCNA), together with the proportion of tumor infiltrating immune cells, were employed to screen the key module related to immune infiltration. Using these genes among the key module, a predictive signature was generated *via* LASSO and multi-Cox regression method. Moreover, a novel nomogram was further developed by combining important clinical parameters and the predictive signature.

RESULTS: Genes among the green module, indicating the highest correlation with regulatory T cells (Tregs), were incorporated into the establishment of predictive model. Then, a Tregs-related risk signature (TRRS) consisting of four genes *(NRG1, TEX11, OVOL3 and FCRL2)* was established, which performed well in predicting the mortality risk of colon cancer in both internal and external validation groups (p=0.004 for TCGA training set, p=0.016 for TCGA testing set and p=0.03 for GSE39582 dataset). Combining TNM stage and age, we developed a nomogram for 1-, 3-, 5-year OS, presenting a more reliable predictive performance in survival based on the receiver operating characteristic (ROC) curves and calibration curves (3-year AUC: 0.83 and 0.74 in the TCGA and GEO database, respectively).

CONCLUSIONS: We constructed a four-gene signature for predicting the prognosis of patients with colon cancer, and further developed the nomogram together with TNM stage and age to improve the predictive efficacy.

Key Words:
Colon cancer, Regulatory T cells, Nomogram, Tumor-infiltrating immune cell, Weighted gene correlation network analysis.

Abbreviations

TCGA: the cancer genome atlas; GEO: Gene Expression Omnibus; WGCNA: weighted gene correlation network analysis; MSI: microsatellite instability; TRRS: Tregs-related risk signature; LASSO: least absolute shrinkage and selection operator; Treg: regulatory T cell; AUC: area under the ROC curves; ROC: receiver operating characteristic curves; IHC: immunohistochemistry; TPM: transcripts per million; DEGs: differentially expressed genes; MEs: module Eigengenes; MAF: mutation annotation format; TMB: tumor mutation load; OS: overall survival.

Introduction

Colon cancer is one of the most common causes of cancer-related mortality in the world, with a high incidence rate or a high mortality rate in developed or developing countries[1]. Due to the breakthroughs in surgery, chemoradiotherapy and targeted therapy, the prognosis of patients with colon cancer has improved, but it remains a great challenge for oncologists to screen high-risk patients with potential relapse or distant metastasis[2]. Previous studies[3-7] identified several molecular biomarkers such as KRAS, BRAF, circulating tumor DNA and microsatellite instability (MSI) for predicting the prognosis and response to drug therapy. However, a single diagnostic indicator often fails to meet the practical needs for predicting risk stratification. The compound model composed of several prognostic biomarkers, compensating for the shortage of single indicator, has been reported[8].

Tumor-infiltrating immune cells play a vital role in accelerating tumor growth and causing therapeutic resistance[9,10]. The proportion of intratumor immune cells varies with the disease stage[11]. It has been reported[12] that evaluation of tumor-infiltrating cells is capable of predicting the relapse

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and mortality of patients. Conventional approaches of assessing immune fractions, including immunohistochemistry (IHC) or flow cytometry, may not be representative of the entire tumor immune infiltration and do not provide the landscape of microenvironment due to the small number of specific immune markers that can be measured simultaneously. In this scenario, researchers[13] suggested that the development of computational methods to quantify the number of tumor immune cells from RNA-seq data can offer an alternative, continuously mean for large-scale analysis of the immune landscape. For instance, QuanTIseq, a computational method for the quantification of immune-cell fractions from blood or tumor samples, is often employed to deconvolute types of tumor-infiltrating immune cells from RNA-Seq data[14].

Weighted gene co-expression network analysis (WGCNA), capable of screening highly correlated gene modules, is widely employed to filter candidate biomarkers or therapeutic targets[15]. This study utilized QuanTIseq to quantify immune cells, investigated immune-related features based on the key module *via* WGCNA, and thus determined pivotal genes to develop a genetic label for colon cancer by LASSO and Cox regression analysis. We also constructed a novel prognostic nomogram in combination of clinical features to provide more powerful and precise prognostic assessment model for colon cancer patients.

Materials and Methods

Data Acquisition

FPKM values of gene expression profiles (n=437) and corresponding clinical data of patients with colon cancer, were derived from TCGA database (February 2022), including 398 tumor cases and 39 normal samples. Furthermore, cases with the corresponding clinical data (n=363) were randomized into training (n=218) and internal testing (n=145) groups. GSE39582 cohort with its corresponding clinical information was downloaded from GEO database as independent external validation group. The study did not need the approval from the ethics committee because all data were open access in the TCGA or GEO database. The detailed flow diagram is shown in Figure 1.

Identification of Differentially Expressed Genes

We removed these genes with abnormal expression levels (FPKM value < 0.05) and converted other genes to transcripts per million (TPM) expression data. The differentially expressed genes (DEGs) were screened out by using "Limma" (Linear Models for Microarray Data, Bioconductor, Roswell Park Comprehensive Cancer Center, NY, USA) package based on the criteria of $|\log_2(\text{fold change})|>1$ and $p<0.05$.

Deconvoluting Tumor-infiltrating Immune Cells

QuanTIseq is a deconvolution mean for quantifying the fractions and densities of 10 immune cell types, including B cells, classically activated macrophages (M1), alternatively activated macrophages (M2), monocytes, neutrophils, natural killer (NK) cells, nonregulatory $CD4^+$ T cells, $CD8^+$ T cells, regulatory $CD4^+$ (Treg) cells and dendritic cells, as well as the proportion of uncharacterized cells (such as cancer cells in bulk solid tumors)[16,17]. We obtained the "absolute score" of tumor-infiltrating immune cells calculated by QuanTIseq from TIMER online (http://timer.cistrome.org/)[18].

Construction of Co-expression Network and Selection of Hub Module Related to Immune Cells

WGCNA algorithm was performed to determine important modules highly correlated with tumor-infiltrating immune cells of colon cancer[19]. The co-expression network was constructed by Pearson's coefficients for gene pairs. Parameter β was then defined and used to construct a weighted proximity matrix that matched the gene distribution. Genes with similar expression levels were merged into one category. We clustered these genes with the minimum size to build dynamic trees. Module Eigengenes (MEs) and heatmaps were used to characterize modules with the highest correlation coefficients. Together with the proportion of immune cells, hub gene modules related to infiltrating immune cells were identified.

Construction of Risk Signature and Evaluation of Identification Performance

RNA-seq data regarding the hub module was first permutated 1,000 times *via* LASSO regression analysis to obtain stable prognostic genes, which were further entered into multivariate Cox regression analysis (forward and backward) to judge their prognostic significance and to establish the risk signature. We calculated the comprehensive risk score *via* regression coefficients and

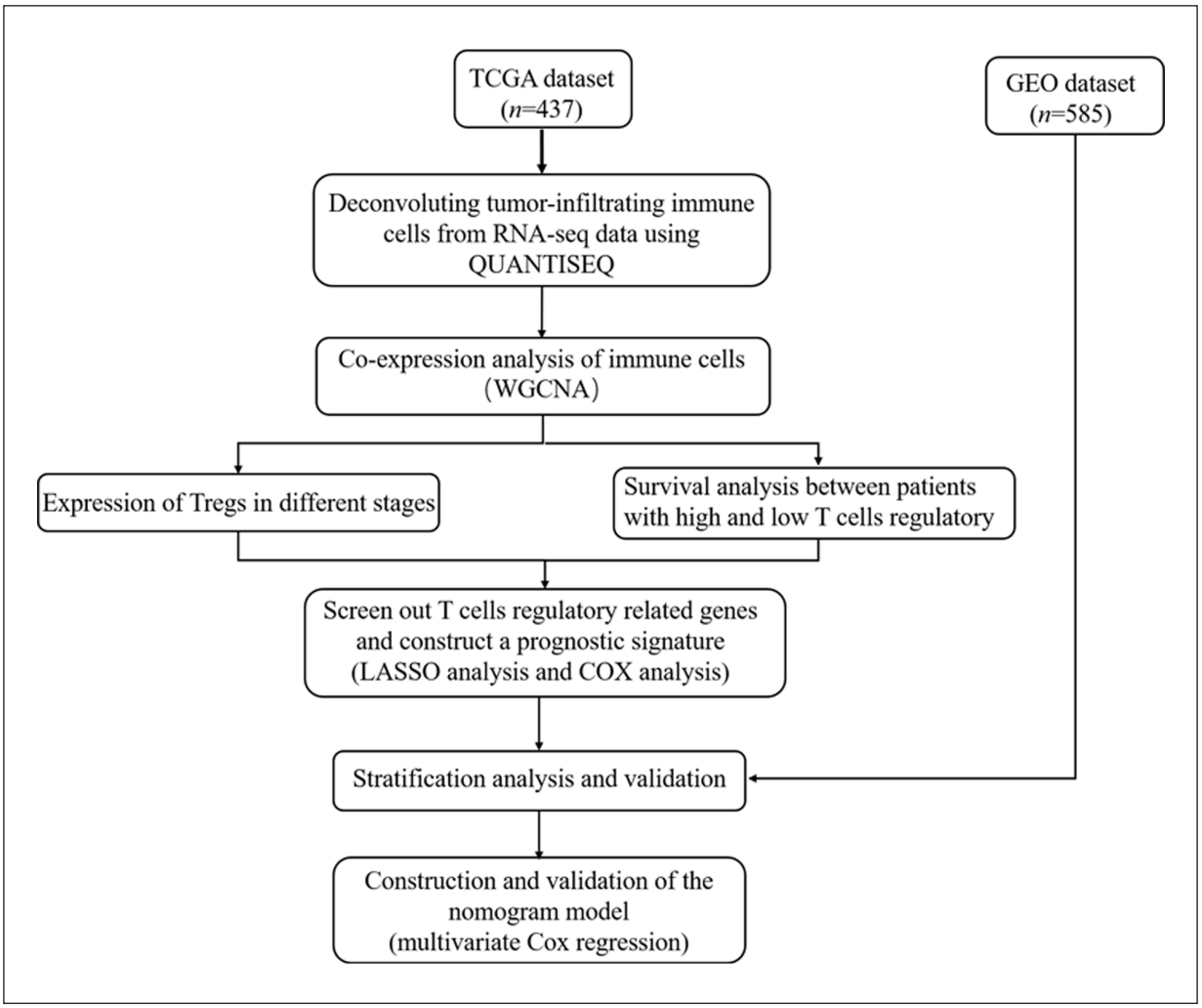

Figure 1. Flowchart of the study.

gene expression levels (β value×gene expression level). Patients were divided into the high- and low- risk groups by the median value of riskscore**.** We investigated the predictive capability of the risk signature by Kaplan-Meier survival curves and time-dependent receiver operating characteristic (ROC) curves in TCGA and GEO groups[20].

Mutation Patterns in Distinct Risk Subgroups

Somatic mutation data of colon cancer, stored in mutation annotation format, were obtained from TCGA. The "Maftools" (Bioconductor, Roswell Park Comprehensive Cancer Center, NY, USA) R package was used to analyze the mutation patterns of patients in different risk status, and the top 15 genes were listed. Tumor mutation load (TMB) is defined as the total number of somatic gene coding errors, base substitutions, gene insertion or deletion per million bases. The study calculated difference of TMB between the high- and low-risk groups.

Construction of a Nomogram and its Prediction Value

Nomogram integrates multiple variables to visualize risk model for predicting the OS[21]. To screen the independent prognostic factor, the constructed riskscore, together with clinical variables, were all incorporated into the multivariate Cox analysis. Based on the result of multivariate analysis, selected biomarkers and significant clinical factors ($p<0.05$) were employed to construct a distinctive nomogram for colon cancer patients in the TCGA group. A comprehensive riskscore of an individual could be calculated according to

the point of each variable, and then his/her probability of the 1-, 3- and 5-OS labels in the diagram. Moreover, in order to ensure validity of nomogram, calibration plots (bootstrapping) and area under the ROC curves (AUCs) were performed and calculated in the TCGA and external GEO cohorts, respectively.

Statistical Analysis

All statistical analyses were conducted using R statistical program (version 3.6.1, The R Foundation for Statistical Computing, Vienna, Austria). Student's *t*-test was used to compare between the normally distributed variables in the two groups, and Wilcoxon test was used to calculate the continuous variables. When $p<0.05$, results were considered as statistical different.

Results

Construction of Weighted Co-Expression Network

Based on the criteria of $p<0.05$ and $|\log2(FC)|>1$, we obtained 4,582 DEGs which were then utilized to construct a co-expression network by the integrated algorithms of WGCNA and QuanTIseq. Firstly, combining with 10 tumor-infiltrating immune cells mentioned above, we clustered 398 samples and regarded the value of $\beta=4$ as the threshold (Figure 2A-2B). Moreover, a dynamic clustering tree had been drawn (Figure 2C), and 7 modules associated with tumor-infiltrating immune cells were generated based on paired genes (Figure 2D). Among these modules, the green module, including 393 DEGs, exhibited the highest correlation coefficient with T regulatory cells (Tregs) compared to others (Figure 2E).

Tregs in Colon Carcinoma

As illustrated in Figure 3A, the proportion of Tregs gradually decreased with the development of TNM stage, and the proportion of infiltrating Tregs in stage IV subset was remarkably lower than that in stage I, stage II and stage III. Interestingly, the similar tendency was also observed in the expression of *FOXP3* and *BLIMP1* (*PRDM1*) among stepwise stages (Figure 3B-3C). In addition, Kaplan-Meier curves indicated that the patients with higher infiltrating Tregs or higher expression of *FOXP3* and *BLIMP1* underwent prolonged survival time (Figure 3D-3F).

The Tregs-related Risk Signature (TRRS) Determined by Hub Module and Its Predictive Capability

A total of four genes (*NRG1*, *TEX11*, *OVOL3*, *FCRL2*), derived from 393 DEGs *via* Lasso and multivariate Cox regression analysis in TCGA training set, were enrolled into the establishment of the Tregs-related risk signature (TRRS) (Figure 4A-4C). These four genes were weighted by their relative coefficient, including *FCRL2* (HR: 3.74, $p<0.001$), *TEX11* (HR: 0.34, $p=0.027$), *NRG1* (HR: 0.44, $p=0.162$) and *OVOL3* (HR: 2.65, $p=0.017$). Based on TRRS, each patient was scored by the following formula (-0.82×*NRG1* expression + -1.08×*TEX11* expression + 0.97×*OVOL3* expression + 1.32×*FCRL2* expression). Then, patients with colon cancer were stratified into high- and low-risk groups using the median cutoff of riskscore. Kaplan-Meier survival curves indicated that patients in the high-risk group had shorter survival time than those in the low-risk group, whether in the TCGA training and testing cohorts or GEO cohorts (Figure 4D-4E). In the TCGA training cohort, the AUCs for 1-, 3-, and 5-year OS were 0.67, 0.66, and 0.65, respectively; in the TCGA testing cohort, it was 0.73, 0.69, and 0.62, respectively; and in the GEO cohort, it was 0.57, 0.58, and 0.56, respectively (Figure 4G-4I). The distribution of patients' risk score, survival status and expression heatmaps of prognostic genes in these three cohorts were shown in Figure S1. All results showed the qualified performance of this TRRS.

Mutation Landscape associated with TRRS

TMB is related to overall survival and treatment response to immunotherapy. The total mutation rate in high-risk group was higher than that in the low-risk group (96.9% *vs.* 94.0%). We listed the top 15 genes with highest mutation frequency, among which *KRAS*, *BRAF*, *PIK3CA* and *MUC16* had a higher mutation rate in the high-risk group (Figure 5A-5B). For another, compared to low-risk group, TMB was significantly higher in high-risk group ($p=0.025$, Figure 5C), suggesting a better response to immunotherapy.

Nomogram Construction Combined with Clinical Characteristics and TRRS

Kaplan-Meier survival curves indicated that patients with increased expression of *NRG1* and *TEX11* had longer OS, while patients with decreased expression of *OVOL3* and *FCRL2* had longer OS (Figure 6A-6D). In addition, *NRG1*, *TEX11* and *FCRL2* were strongly positively relat-

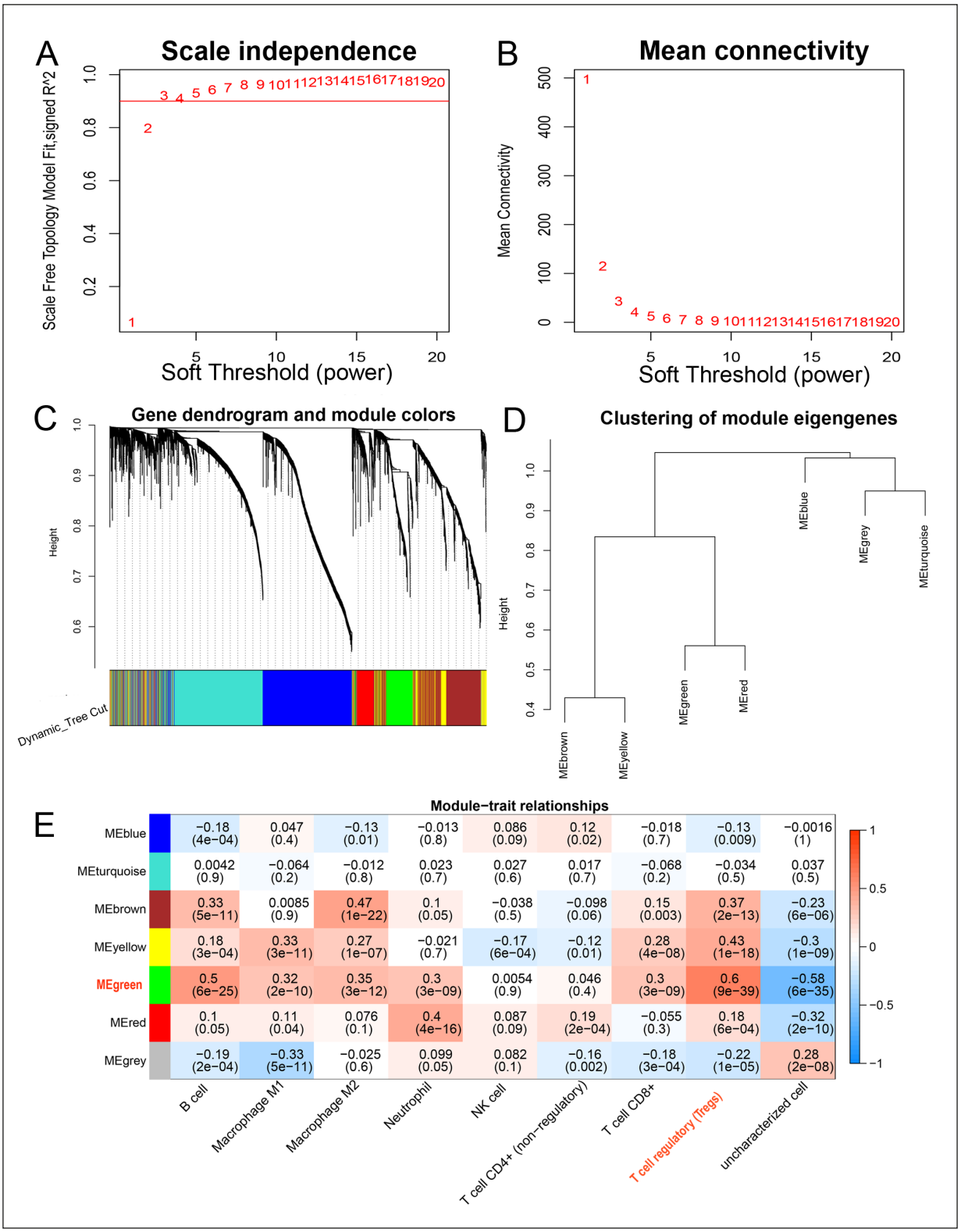

Figure 2. Construction of co-expression network and select out important module using WGCNA. **A-B**, Analysis of network topology and the adjacency matrix is defined using soft-thresholds with β=4; **C**, Clustering dendrogram of DEGs with difference in different immune cell types and 7 gene modules from 398 samples; **D**, Visualization of the gene network using network heatmap plot; **E**, Analysis of module-trait relationships of colon cancer. The green module with a high correlation coefficient is linked to T regulatory cells (Tregs), which includes 393 genes.

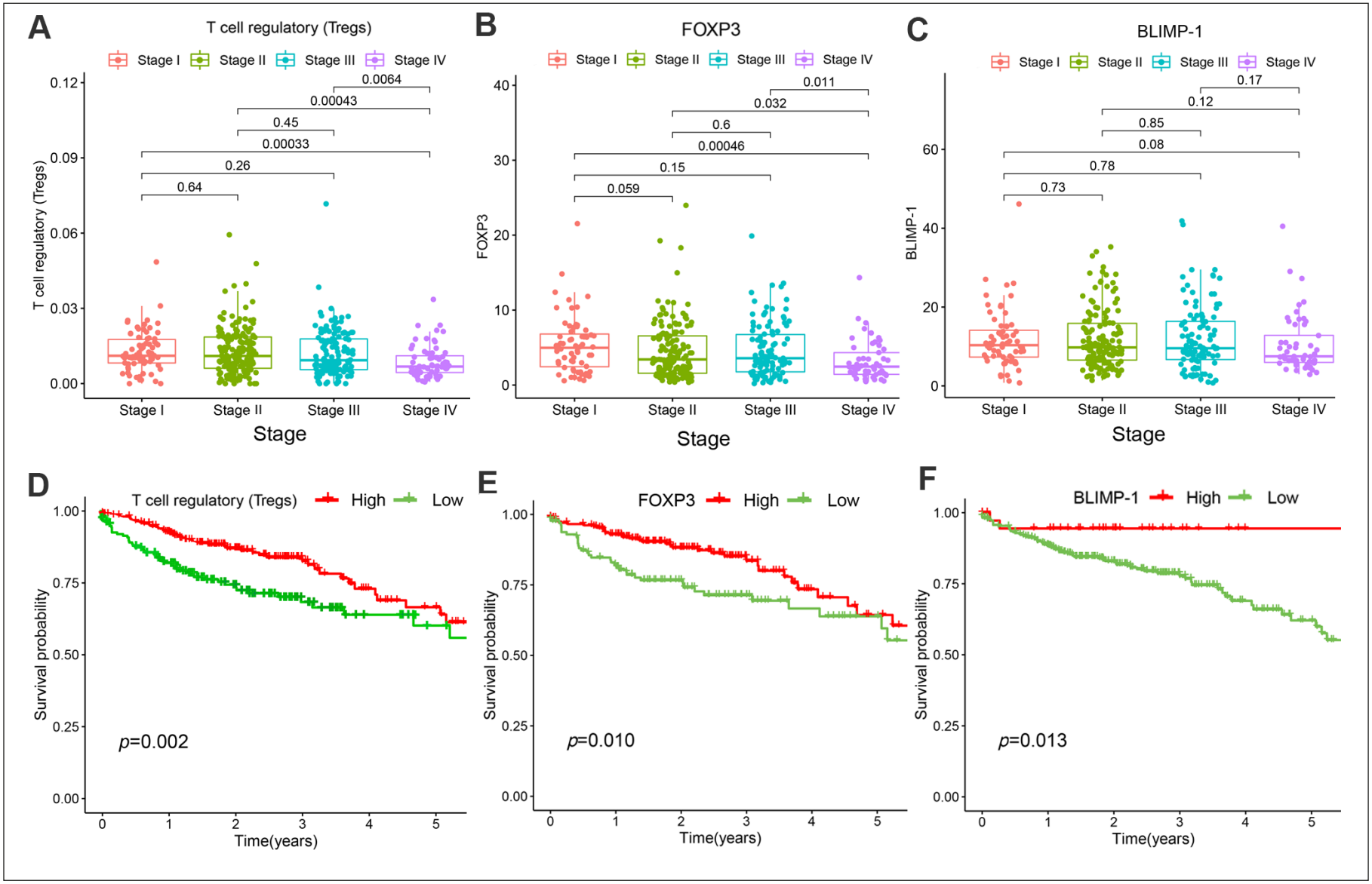

Figure 3. Associations of Tregs infiltration with clinicopathologic features in colon cancer. **A**, Expression of Tregs in different pathological stage; **B**, Expression of FOXP3 in different pathological stage; **C**, Expression of BLIMP-1 in different pathological stage; **D**, Survival analysis between patients with high and low Tregs; **E**, Survival analysis between patients with high and low FOXP3; **F**, Survival analysis between patients with high and low BLIMP-1. High and low subgroups are determined by the median cutoff of expression levels.

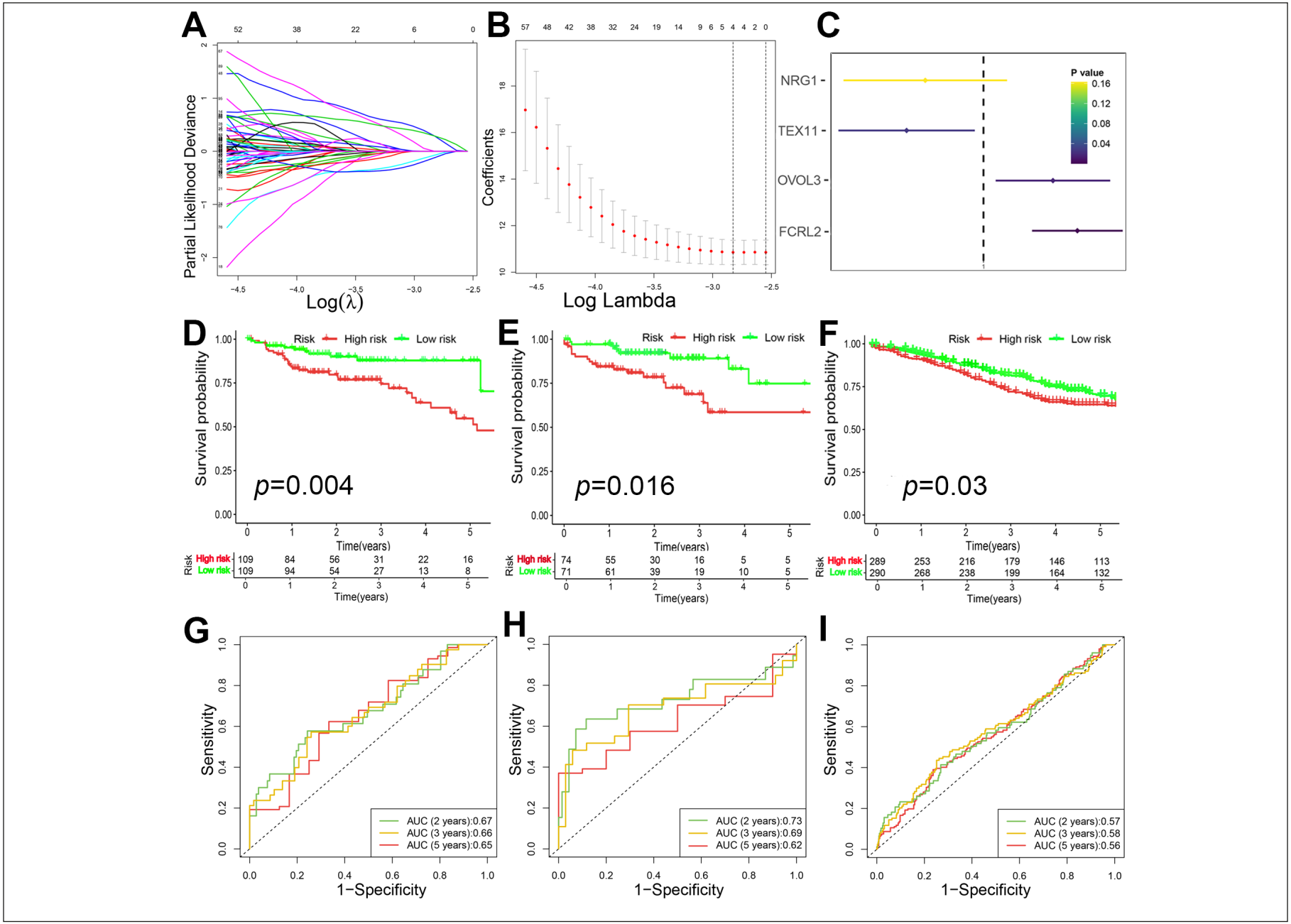

Figure 4. Construction and Validation of Tregs-related risk signature (TRRS). **A-B**, LASSO regression analysis; **C**, Multivariate stepwise Cox regression analysis; **D**, Survival curves in TCGA training set; **E**, Survival curves in TCGA testing set; **F**, Survival curves in GEO set; **G**, Time-ROC curves of overall survival for model validation in TCGA training set; **H**, Time-ROC curves of overall survival for model validation in TCGA testing set; **I**, Time-ROC curves of overall survival for model validation in GEO set.

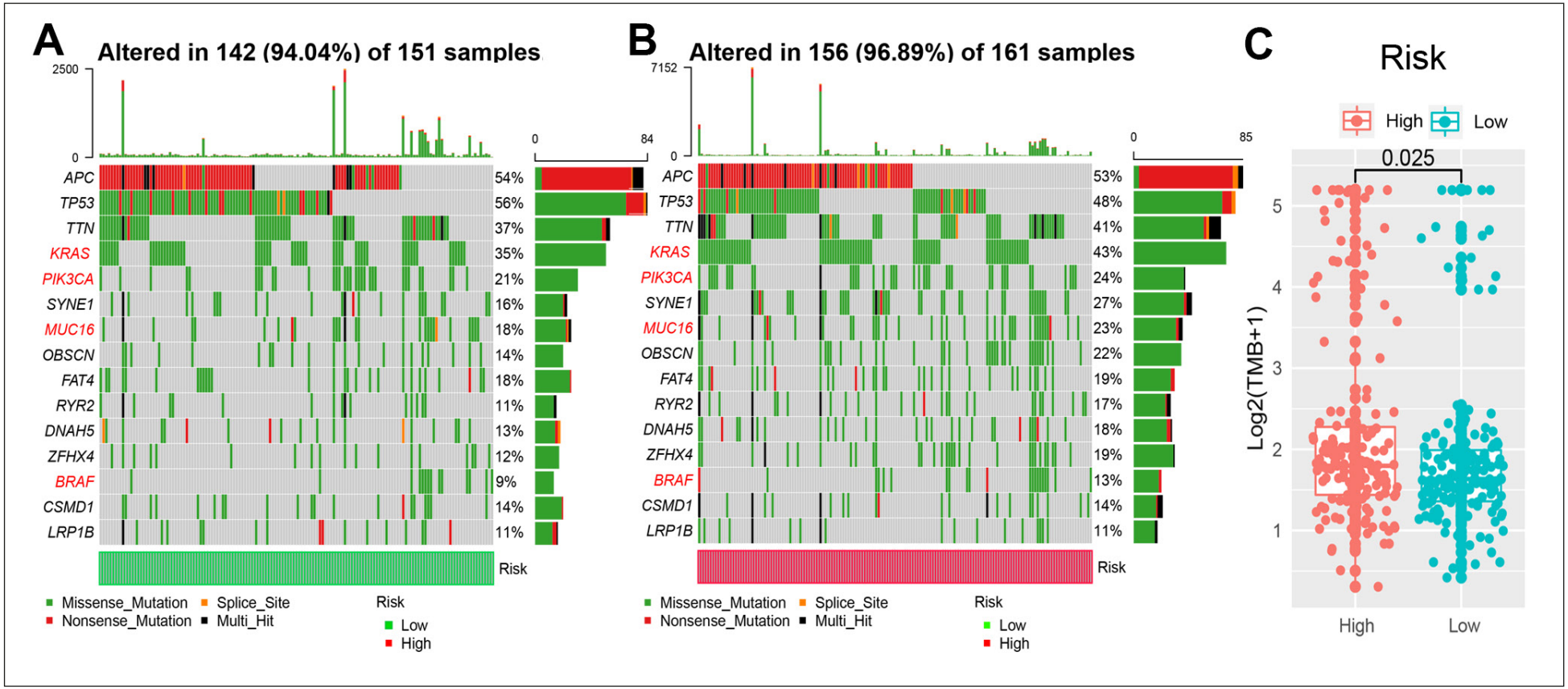

Figure 5. The mutation patterns of colon cancer in different risk status. **A**, A waterfall plot presents the mutation distribution of the key genes in low-risk cohort; **B**, A waterfall plot presents the mutation distribution of the key genes in high-risk cohort; **C**, TMB with difference in high- and low-risk cohorts. High and low are determined by the median cutoff of TRRS riskscore.

ed to the expression of Tregs, with the exception of *OVOL3* (Figure S2A-S2D). The multivariate Cox regression analyses further observed that age (p=0.003), TRRS (p<0.001) and tumor stage were significantly associated with patient survival in the TCGA cohort (Figure 6E), showing an independent survival correlation. In GEO group, age (p<0.001) and TRRS (p=0.009) were also consistent with these findings (Figure S2E). By combining TRRS with TNM stage and age, we constructed a novel nomogram to predict the survival probability of 1-, 3- and 5-year OS for patients with colon cancer (Figure 7A). Calibration plots (bootstrapping) 1-, 3-, and 5-year OS were close to diagonal line in TCGA group (Figure 7B) and GEO group (Figure 7E), respectively. In the TCGA set (Figure 7C), the 1-, 3- and 5-year AUC values of the nomogram were 0.82, 0.83 and 0.77, respectively, while those of the GEO set (Figure 7F) were 0.77, 0.74 and 0.71, respectively. Obviously, the AUCs of nomogram in the TCGA database were higher than those of single clinical parameters (3-year AUC of nomogram: 0.83; 3-year AUC of TNM stage: 0.51; 3-year AUC of age: 0.57), in agreement with results of GEO database (Figure 7D-7G). All results indicated the better capacity to forecast survival.

Discussion

Colon cancer is an aggressive malignancy with high risk of recurrence metastasis[22], and it is necessary to develop an accurate prognostic model for predicting the risk score of relapse and metastasis. First, Tregs-related module was regarded as the most significant fraction through WGCNA algorithm in our study. We identified four genes (*NRG1*, *TEX11*, *OVOL3*, *FCRL2*) from Tregs-related module and developed the TRRS of colon cancer *via* LASSO and multivariate Cox regression analyses. Using the TRRS and clinical parameters, we developed a more robust nomogram with higher AUC values (over 0.70) based on receiver operating characteristic (ROC) curves and calibration curves compared with a single TRRS, and this nomogram had reliable predictive capabilities for 1-, 3-, and 5-year OS in colon cancer.

Herein, Tregs were mainly enriched in stage I and II, and indicated a favorable prognosis. Meanwhile, *FOXP3* and *BLIMP1* expression was higher in early-stage colon cancer with better survival compared with advanced colon cancer. Tregs played a nonnegligible immunoregulation role in colon cancer[23]. Infiltrating Tregs tend to function as a protumor role in various solid neoplasms, such as ovarian[24] and hepatocellular carcinoma[25,26]. However, the role of Tregs is controversial in colon cancer due to the subpopulations of Tregs. Circulating Treg cells recruited towards intratumor stroma and release multiple suppressive molecules and led to poor prognosis[27]. Indeed, previous studies[23,28,29] had found that in predicting survival of colon cancer, infiltrating *FOXP3*$^+$ T cells may represent better prognosis. For instance, a meta-analysis by Shang et al[30] suggested that patients with colorectal cancer had improved survival with increased *FOXP3*$^+$ Tregs by analyzing eight datasets. Salama et al[31] discovered that the extent of *FOXP3*$^+$ Tregs infiltration was more pronounced in early colorectal cancer and correlated with a positive prognosis. To date, with the development of the ability to identify Tregs subpopulations like *LAG3*$^+$ *TIM3*$^+$ subset and *FOXP3*$^+$ *BLIMP-1*$^+$ subset, the role of Tregs in colon cancer becomes clearer[27,32]. Generally speaking, the density of *FOXP3*$^+$ *BLIMP-1*$^+$ subset may improve prognosis of patients with early colon cancer[32]. Tregs-related module annotated by the WGCNA algorithm is important and reasonable for colon cancer. The construction of prognostic models by extracting Tregs infiltration-related genes has practical implications.

The predictive risk label (also known as TRRS) consisting of four genes can be considered as a prognostic factor independent from other clinicopathologic parameters. Traditionally, clinical factors have long been considered as the most important factor in screening high-risk patients with short survival and guiding intervention. However, with the extraction and annotation of genomic data, several studies[33,34] have observed that genetic features are strongly linked to cancer prognosis, and have constructed models accordingly, such as ferroptosis-related genes and m6A-related genes. In our study, the riskscore of TRRS is calculated from genes related to Tregs infiltration. Furthermore, our model used a smaller number of genes compared to other signatures[35,36], showing practical and convenient advantages while retaining the predictive ability of a qualified AUC. The higher the score calculated from the TRRS, the worse the prognosis of the patient. Further analysis found that the high-risk group was associated with high TMB, which is consistent with a previous report by Chen et al[37].

In addition, to obtain a more robust predictive model, we further constructed a nomogram to pre-

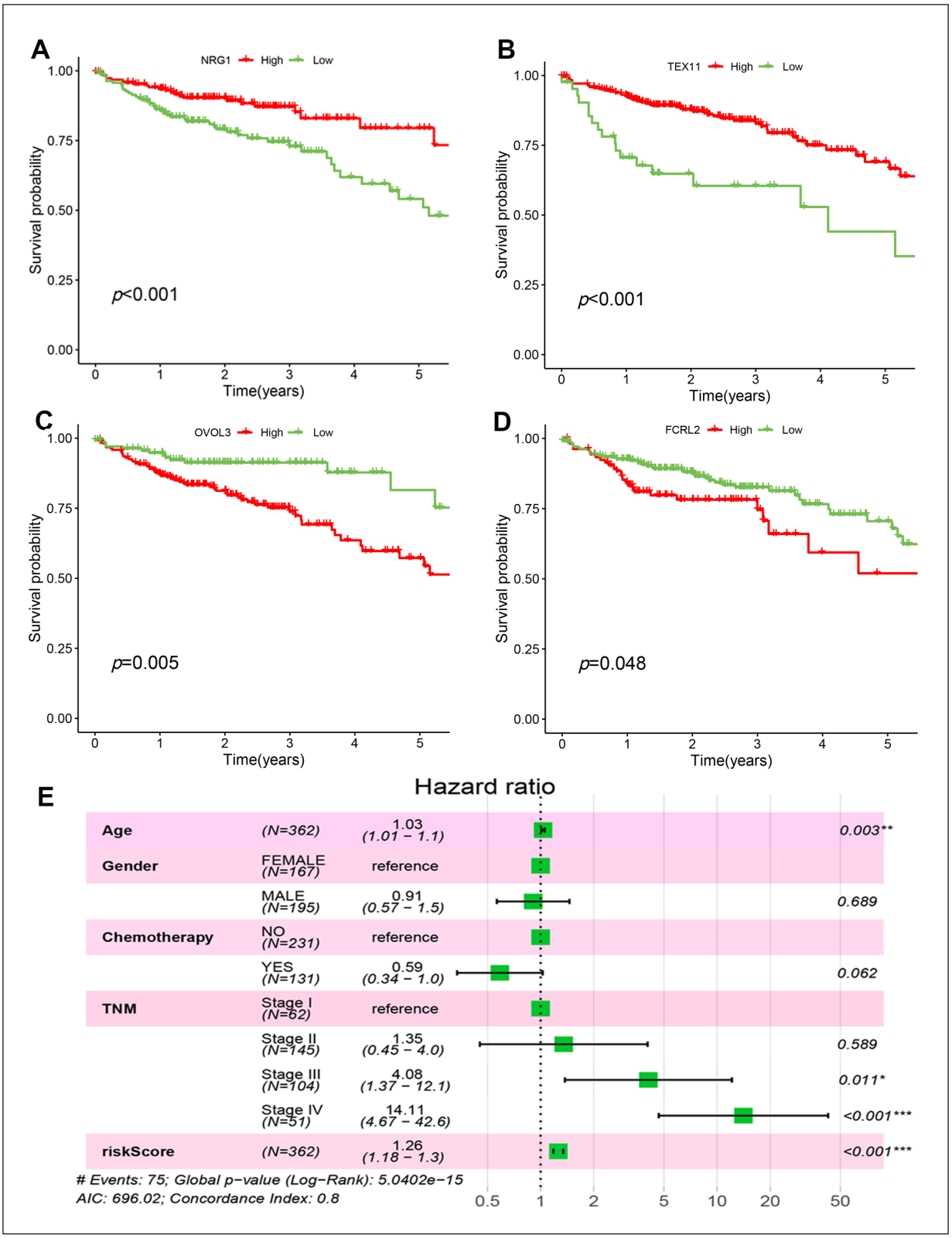

Figure 6. Kaplan-Meier curves and independence validation of TRRS using multivariate analysis. **A**, Kaplan-Meier curves of NRG1; **B**, Kaplan-Meier curves of TEX11; **C**, Kaplan-Meier curves of OVOL3; **D**, Kaplan-Meier curves of FCRL2; **E**, The survival-related independent factors by multivariate analysis taking into account clinical variables and TRRS in the TCGA cohort.

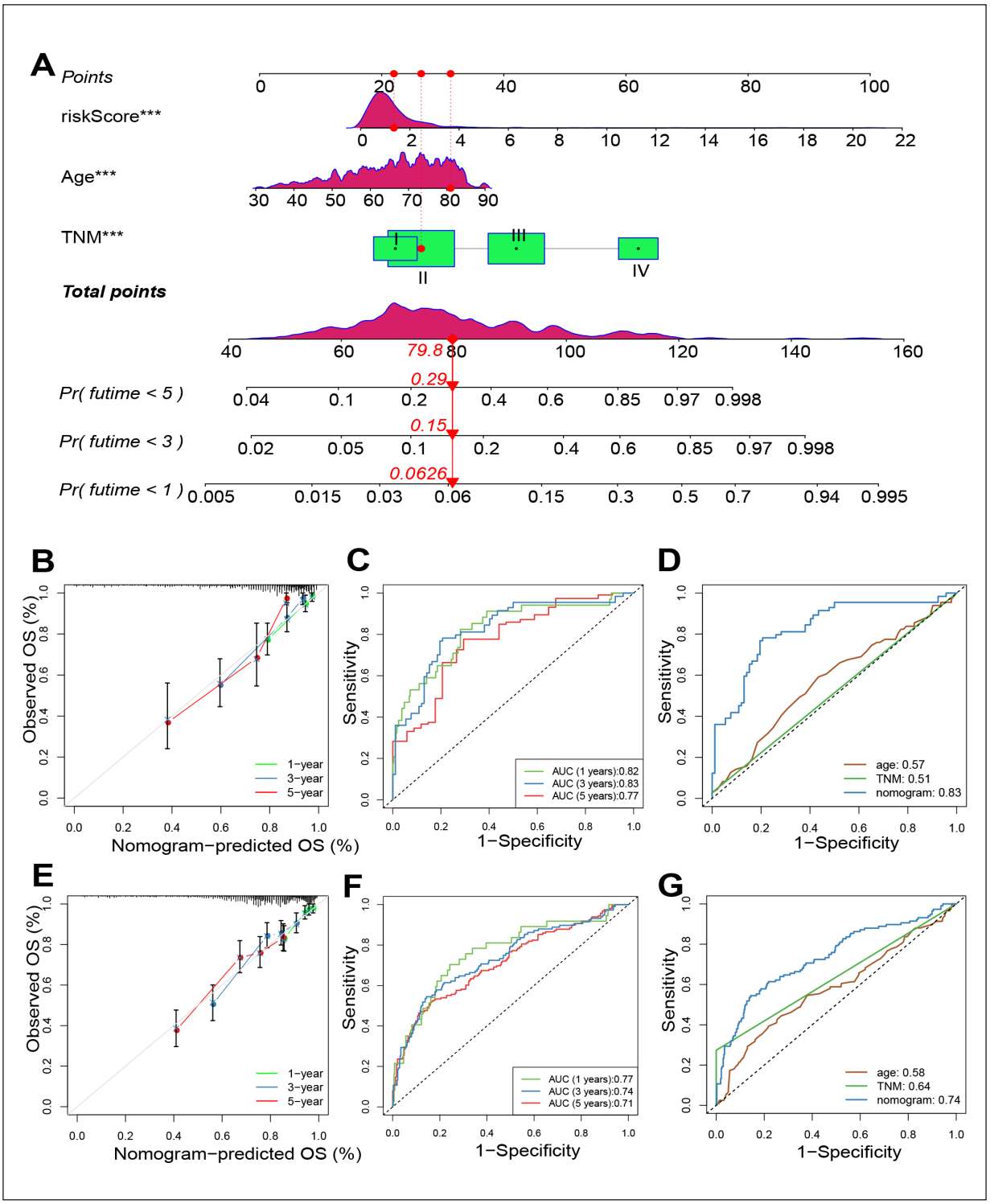

Figure 7. Construction and validation of the novel nomogram; **A**, A comprehensive nomogram in combination of TRRS and important clinical variables for predicting the survival rates of colon cancer patients in 1-, 3-, and 5-years in the TCGA cohort; **B**, Calibration curves of the nomogram for the prediction of 1-, 3- or 5-year overall survival (OS) in the TCGA cohort; **C**, Time-ROC curves with AUC values based on the TCGA cohort; **D**, Evaluation of performance of nomogram relative to a single clinical parameters and TRRS as a predictor based on the TCGA cohort; **E**, Calibration curves of the nomogram for the prediction of 1-, 3- or 5-year OS in the GEO cohort; **F**, Time-ROC curves with AUC values based on GEO cohort; **G**, Evaluation of performance of nomogram relative to a single clinical parameters and TRRS as a predictor based on GEO cohort.

dict 1-, 3-, and 5-year OS of patients with colon cancer by combining clinical factors and TRRS. Based on the nomogram, higher scores mean lower overall survival, providing important indication for the treatment of patients. In the future, the combination of clinicopathologic features and tumor biology in prognostic models will greatly enhance diagnosis, treatment and prognosis risk stratification[38,39].

In this study, *NRG1* and *TEX11* were favorable prognostic factors for colon cancer, while *OVOL3* and *FCRL2* were unfavorable prognostic factors. *FCRL2* is mainly connected with chronic lymphocytic leukemia[40,41]. *OVOL3* is highly expressed in breast cancer, correlated with poor overall survival[42]. *NRG1* is one of members of the epidermal growth factor (EGF)-like family and is involved in encoding a membrane glycoprotein. It functions as a tumor-diver gene to promote the oncogenesis and progression of glioma[43]. Shu et al[44] reported that *NRG1* regulates ERK1/2-Fbxw7-c-Myc pathway to accelerate metastasis in triple-negative breast cancer. Currently, these four genes have not been previously studied to demonstrate their association with colon cancer, but they may serve as relevant targets for our further studies of the mechanism of colon cancer progression.

Limitations

There are limitations to our study. The prediction model was constructed based on a public dataset only and was not confirmed in a clinical cohort. In addition, the incomplete nature of the clinical variables in the dataset may have reduced the statistical validity. Nevertheless, our findings still have noteworthy implications for upcoming mechanistic studies and prognostic assessment of colon cancer patients.

Conclusions

In conclusion, we identified the crucial Tregs-related module using comprehensive bioinformatics analysis. The constructed prognostic TRRS and novel nomogram showed good predictive accuracy for survival of colon cancer, and these four genes can be used for further studies of therapeutic targets.

Ethics Approval and Consent to Participate

Not applicable.

Conflicts of Interest

The authors declare no competing interests.

Funding

None.

Authors' Contributions

J.W and H.J.L designed the project and drafted the manuscript. H.J.L performed the bioinformatics analysis and wrote the paper. J.K.P and Y.Q.W performed literature research and provided introduction/discussion data. H.W.J provided specialized collaborations in data analysis. All authors read and approved the final manuscript.

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None.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

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