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Research article

Transforming growth factor-β (TGF-β) signaling pathway-related genes in predicting the prognosis of colon cancer and guiding immunotherapy☆

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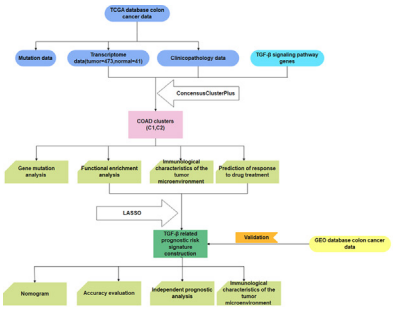
HIGHLIGHTS

- Two transforming growth factor-β (TGF-β) subtypes were identified using consensus clustering.
- The TGF-β high subtype was associated with a poor prognosis and superior immunotherapy response.
- A risk prediction signature was constructed using and validated using publicly available databases.
- Uni- and multivariate Cox regression analyses verified that the model could be an independent prognostic factor.
- Patients in the high-risk subgroup experienced better immunotherapy efficacy.

GRAPHICAL ABSTRACT



We conducted in-depth research on the common functions of TGF-β signaling pathway-related genes to elucidate their potential role in colon cancer prognosis and tumor microenvironment. These genes were incorporated into a risk prediction model to guide individualized precision therapy. Two TGF-β subtypes were determined using consensus clustering based on their expression levels, with the TGF-β high subtype being associated with poor prognosis and immunotherapy advantages. A risk prediction model was constructed using the TCGA colon cancer dataset and validated using the GEO dataset. Univariate and multivariate Cox regression analyses showed that the model was an independent prognostic factor. Based on the median risk score of the TGF-β-related prediction model, colon cancer patients can be divided into high-risk and low-risk subgroups. Patients in the high-risk subgroup had higher levels of immune-suppressive cell infiltration and immune checkpoint expression in their tumor microenvironment, suggesting better immunotherapy efficacy for this subgroup. COAD: Colon adenocarcinoma; GEO: Gene Expression Omnibus; LASSO: Least absolute shrinkage and selector operation; TCGA: The Cancer Genome Atlas; TGF-β: Transforming growth factor-β

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ABSTRACT

Background: Colon cancer is a malignant tumor with high malignancy and a low survival rate whose heterogeneity limits systemic immunotherapy. Transforming growth factor-β (TGF-β) signaling pathway-related genes are associated with multiple tumors, but their role in prognosis prediction and tumor microenvironment (TME) regulation in colon cancer is poorly understood. Using bioinformatics, this study aimed to construct a risk prediction signature for colon cancer, which may provide a means for developing new effective treatment strategies.
Methods: Using consensus clustering, patients in The Cancer Genome Atlas (TCGA) with colon adenocarcinoma were classified into several subtypes based on the expression of TGF-β signaling pathway-related genes, and differences in survival, molecular, and immunological TME characteristics and drug sensitivity were examined in each subtype. Ten genes that make up a TGF-β-related predictive signature were found by least absolute shrinkage and selector operation (LASSO) regression using colon cancer data from the TCGA database and confirmed using a Gene Expression Omnibus (GEO) dataset. A nomogram incorporating risk scores and clinicopathologic factors was developed to stratify the prognosis of patients with colon cancer for accurate clinical diagnosis and therapy.
Results: Two TGF-β subtypes were identified, with the TGF-β-high subtype being associated with a poorer prognosis and superior sensitivity to immunotherapy. Mutation analyses showed a high incidence of gene mutations in

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the TGF-β-high subtype. After completing signature construction, patients with colon cancer were categorized into high- and low-risk subgroups based on the median risk score of the TGF-β-related predictive signature. The risk score exhibited superior predictive performance relative to age, gender, and stage, as evidenced by its AUC of 0.686. Patients in the high-risk subgroup had higher levels of immunosuppressive cell infiltration and immune checkpoints in the TME, suggesting that these patients had better responses to immunotherapy.
Conclusions: Patients with colon cancer were divided into two subtypes with different survival and immune characteristics using consensus clustering analysis based on TGF-β signaling pathway-related genes. The constructed risk prediction signature may show promise as a biomarker for evaluating the prognosis of colon cancer, with potential utility for screening individuals for immunotherapy.

Introduction

Colon cancer is a prevalent and deadly disease that is of public health interest, with an increasing global mortality rate.[1–4] Over 10% of patients have locally advanced illness, and the 5-year survival rate is low.[5–7] Colon adenocarcinoma (COAD) is the most common colon cancer type and the third most common adenocarcinoma globally.[8] Developing effective treatment strategies for colon cancer prevention and treatment is a matter of urgency.

Common forms of colon cancer treatment vary depending on the disease's stage and include surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy.[6,9,10] Immunotherapy is crucial in treating colon cancer, and immune checkpoint inhibitors (ICIs) have shown significant clinical benefits.[11] The current clinical application of programmed death ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade is primarily based on the gene mutation patterns of colon cancer. Patients with colon cancer are classified into defective mismatch repair (dMMR)/microsatellite instability-high (MSI-H) and proficient mismatch repair (pMMR)/microsatellite-stability (MSS) groups.[12] With the Food and Drug Administration's (FDA's) acceptance of the ICI pembrolizumab as the first-line therapy for patients with metastatic MSI-H/dMMR colon cancer in 2020, promising outcomes have been achieved. However, MSI-H/dMMR still shows low prevalence and certain patients with colon cancer with MSI-H/dMMR exhibit intrinsic or acquired resistance to immunotherapy.[13] Thus, continually investigating reliable predictive biomarkers to enable the immunotherapy stratification of patients with colon cancer is critical.

Transforming growth factor-β (TGF-β) is a member of the secretory cytokine family.[14,15] TGF-β comprises three isoforms—TGF-β1, TGF-β2, and TGF-β3—that cooperate to coordinate various physiological and pathological processes, notably concerning the progression of diseases such as inflammation and cancer.[16] TGF-β is a multifunctional cytokine with paradoxical roles in cancer progression. In the early phases of cancer, TGF-β may function as a tumor suppressor; however, as cancer progresses, it transforms into a tumor promoter.[17–19] Furthermore, TGF-β may considerably stimulate epithelial–mesenchymal transition (EMT) in malignant cells, enhancing the capacity of cancer cells to invade, migrate, and evade apoptosis.[20–23] Hypoxic environments activate TGF-β, which can stimulate angiogenesis in various cancer types.[24] Cancer-associated fibroblast (CAF) production and extracellular matrix (ECM) deposition are both induced by TGF-β overexpression, often resulting in cancer.[25] Another essential role of TGF-β in cancer is in immunosuppression and has therefore been long recognized as an immunosuppressive factor in the tumor microenvironment (TME).[26,27] Currently, the study of antitumor drugs that specifically target TGF-β has advanced considerably. These interventions have undergone human clinical trials for validation or have exhibited encouraging outcomes in preclinical animal models.[28]

To date, TGF-β signaling pathway-related risk prediction models have been constructed for various types of cancer, including renal clear cell carcinoma, hepatocellular carcinoma, gastric cancer, and bladder cancer.[29–32] The shared functions of TGF-β signaling pathway-related genes for clustering and constructing prognostic signatures to further advise precise colon cancer therapies are yet to be thoroughly investigated. In this regard, we intended to conduct an investigation focused on colon cancer.

Methods

Patients and data

For the training set, the raw transcriptome, clinicopathology, and mutation data from a COAD patient cohort were retrieved from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). The ribonucleic acid (RNA) expression of each sample, as well as its survival time and status, age, gender, grade, and stage, were then obtained through data compilation. The location and type of the mutant chromosome, as well as the mutant base, were included in the mutation data.

For the validation set, Gene Expression Omnibus (GEO) data for colon cancer (GEO: GSE38832) were searched on the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/), and Identity (ID) probe matrix files and platform annotation files for patients with colon cancer were downloaded. From the GEO database, 122 clinical colon cancer samples were obtained. Using platform annotation data, the probe matrix was changed into a gene matrix to obtain the gene expression microarray dataset.

Liu et al[31] assembled TGF-β signaling pathway genes to identify all TGF-β signaling pathway-related genes from GO:0007179 from AmiGO 2 (http://amigo.geneontology.org/amigo/landing), *TGF-β* from Ensembl Genome Brower (http://grch37.ensembl.org/index.html), BIOCARTA_TGFB_PATHWAY, and KEGG_TGF_BETA_SIGNALING_PATHWAY from gene set enrichment analysis (GSEA) (http://www.gsea-msigdb.org/gsea/index.jsp). Ultimately, 223 genes involved in the TGF-β signaling pathway were acquired [Supplementary Table 1].

Relationship between transforming growth factor-β subtypes and patient prognosis as well as immunological characteristics of the tumor microenvironment

Colon cancer data collation and consensus clustering in The Cancer Genome Atlas database

Based on the TGF-β signaling pathway-related genes, the gene expression of the TGF-β signaling pathway genes in each TCGA colon cancer sample was obtained from the transcriptome expression matrix. Consensus clustering identified COAD molecular subtypes using the "ConcensusClusterPlus" package in R software (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). We examined the optimal number of clusters between $k = 2$ and 10, determined the k-value as the number of clusters with the lowest cross-validation error, and repeated this procedure 1000 times to ensure the stability of the results. The "pheatmap" package in R software generated cluster heatmaps.

Analysis of differentially expressed genes in colon cancer tumor and normal tissues

Differential gene expression in tumor and normal tissues was assessed using the empirical Bayesian method of the "limma" package in R software. Screening criteria for differential messenger RNA (mRNA) expression included an adjusted P-value <0.001 and $|\log_2$ Fold change (FC)$| \geq 1$. The protein–protein interaction (PPI) plots from the Search Tool for the

Retrieval of Interacting Genes/Proteins (STRING) database illustrated the relationships among the genes exhibiting differential expression.

Survival and differentially expressed genes analysis of different transforming growth factor-β subtypes

Kaplan–Meier (K–M) analysis was used to compare the overall survival (OS) between distinct TGF-β subtype cohorts of patients with colon cancer using the "survminer" and "survival" packages in R software. The intersection of TGF-β signaling pathway gene expression and cluster data were merged to obtain the TGF-β signaling pathway gene expression levels of different subtypes, as well as to evaluate their differential expression among the different subtypes. mRNA differential expression was assessed using a significance level of *P*-value <0.001 and $|\log_2 FC| \geq 1$. For visualization, volcano and heat maps were constructed using the "pheatmap" package in R software.

Gene ontology, Kyoto Gene and Genome Encyclopedia, and gene set enrichment analysis of different TGF-β subtypes

To examine the distinct signaling pathways and biological impacts between different TGF-β subtypes, the "clusterProfiler" package in R software was used for gene ontology (GO) and Kyoto Gene and Genome Encyclopedia (KEGG) annotation. In GO and KEGG enrichment studies, a *q*-value (*P*-value after correction) < 0.05 determined statistically significant elements. GSEA analysis was performed using GSEA software (http://www.broadinstitute.org/gsea/index.jsp) to evaluate molecule phenotype in the MSigDB collection (c2.cp.kegg.v7.4.symbols.gmt).

Gene mutation analysis of different transforming growth factor-β subtypes

Tumor mutational burden (TMB) is the total amount of somatic coding mutations, base substitutions, and insertion-deletion errors per million bases in human malignancies with various numbers of somatic mutations. COAD mutation data from the TCGA database were processed using the "Maftools" package in R software. Waterfall plots were constructed to display the mutated genes, examine the mutation type and frequency in the samples, and determine whether TMB differentiated among different TGF-β subtypes.

Immunological characteristics of the tumor microenvironment of different transforming growth factor-β subtypes

The stromal, immune, and tumor purity scores of the TME were calculated using the "ESTIMATE" package in R software and displayed in violin plots to emphasize the differential expression of stromal, immune, and tumor cells between the various TGF-β subtypes. The immunological features of the TME were then investigated. Immune cell infiltration analysis was used to visualize the proportionate number of immune cells in each sample. Cell type identification by estimating relative subsets of RNA Transcripts (CIBERSORT) (http://cibersort.stanford.edu/) analysis of COAD expression data was used to compute the relative percentages of 22 immune cell types and the results were presented using a landscape map. The differences in immune cells, human leukocyte antigen (HLA), and immune checkpoints between TGF-β subtypes were next investigated. The results were shown in violin plots.

Prediction of response to drug treatment of different transforming growth factor-β subtypes

Drugs were cycled and visualized using "pRRophetic," "limma," "ggpubr," and "ggplot2" packages in R software to predict drug sensitivity in each sample and compare differences in drug sensitivity among different TGF-β subtypes.

Transforming growth factor-β-related prognostic risk signature construction, validation, and immunological microenvironmental analysis in colon cancer

Merging The Cancer Genome Atlas, Gene Expression Omnibus database expression, and survival data

The expression data of the TGF-β signaling pathway-related genes in patients with colon cancer from the GEO database was first extracted. Thereafter, TCGA, GEO database expression, and survival data were merged to obtain sample survival time and status and TGF-β signaling pathway-related gene expression. Univariate Cox regression analysis was performed to identify prognosis-related genes ($P < 0.05$).

Construction of transforming growth factor-β-related prognostic risk signature

TCGA samples served as the training set for the prognostic signature, while GEO data served as the test set for signature validation. Least absolute shrinkage and selector operation (LASSO) regression analysis was utilized to calculate correlation coefficient values of the TGF-β prognostic risk signature after identifying statistically relevant TGF-β related prognostic genes using univariate Cox regression analysis. The risk score was calculated using the following formula: risk score = $\sum$βixRNAi, where βi is the coefficient of the ith gene in the LASSO regression analysis. Each sample's risk category was established based on the median value of the risk score. High-risk subgroup samples have risk ratings greater than the median.

Validation of transforming growth factor-β-related prognostic risk signature

Risk signature predictive potential was evaluated using K–M survival analysis and the log-rank test ($P < 0.05$). Survival differences between high- and low-risk subgroups in TCGA and GEO colon cancer populations were investigated, and risk curves and heatmaps were generated. Uni- and multivariate Cox regression analyses were performed to determine whether a predictive risk signature could be used as an independent prognostic variable for individuals with colon cancer. To evaluate the accuracy of risk scores to predict 1-, 3-, and 5-year survival in patients with colon cancer in TCGA and GEO, we used the "survivalROC" package in R software to produce receiver operating characteristic (ROC) curves and calculate the area under the ROC curves (AUCs). Furthermore, we constructed a nomogram that incorporates risk scores and clinicopathologic factors to stratify the prognosis of patients with colon cancer for accurate clinical diagnosis and therapy. Calibration curves were used to test the nomogram's capacity and accuracy in predicting clinical outcome events, representing the difference between predicted and actual values.

Immune microenvironment analysis of transforming growth factor-β prognostic risk signature

To examine the tumor immune microenvironment of patients with different risks, the "ESTIMATE" package was used to determine the stromal, immune, and integrated scores of the TME, and boxplots were generated. Correlation analysis of immune cell infiltration level and risk scores was conducted to understand the regulatory relationship. The "ggplot" package in R software was further used to plot the correlation scatter plot of immune cells with $P < 0.05$. The Wilcox test was used to calculate the difference in immune checkpoints between different TGF-β risk subgroups, and the findings were shown in boxplots.

Statistical analysis

R software (version 4.1.2) was used for statistical analysis and data visualization. Pearson or Spearman correlation analysis was used to determine correlations between continuous data; *t*-tests were employed for continuous variables with a normal distribution across binary groups; the Mann–Whitney *U* test was used for non-normally distributed data. Risk score and prognosis were compared using uni- and multivariate Cox regression models. The two-sided *P*-value was regarded as significant when it was less than 0.05.

Results

Identification of transforming growth factor-β-related subtypes

The list of TGF-β signaling pathway-related genes was compiled from a large body of research, and Liu et al[31] previously published 223 TGF-β signaling pathway genes [Supplementary Table 1]. By setting $k = 2$ to match the optimal number of COAD clusters, the cohort was partitioned

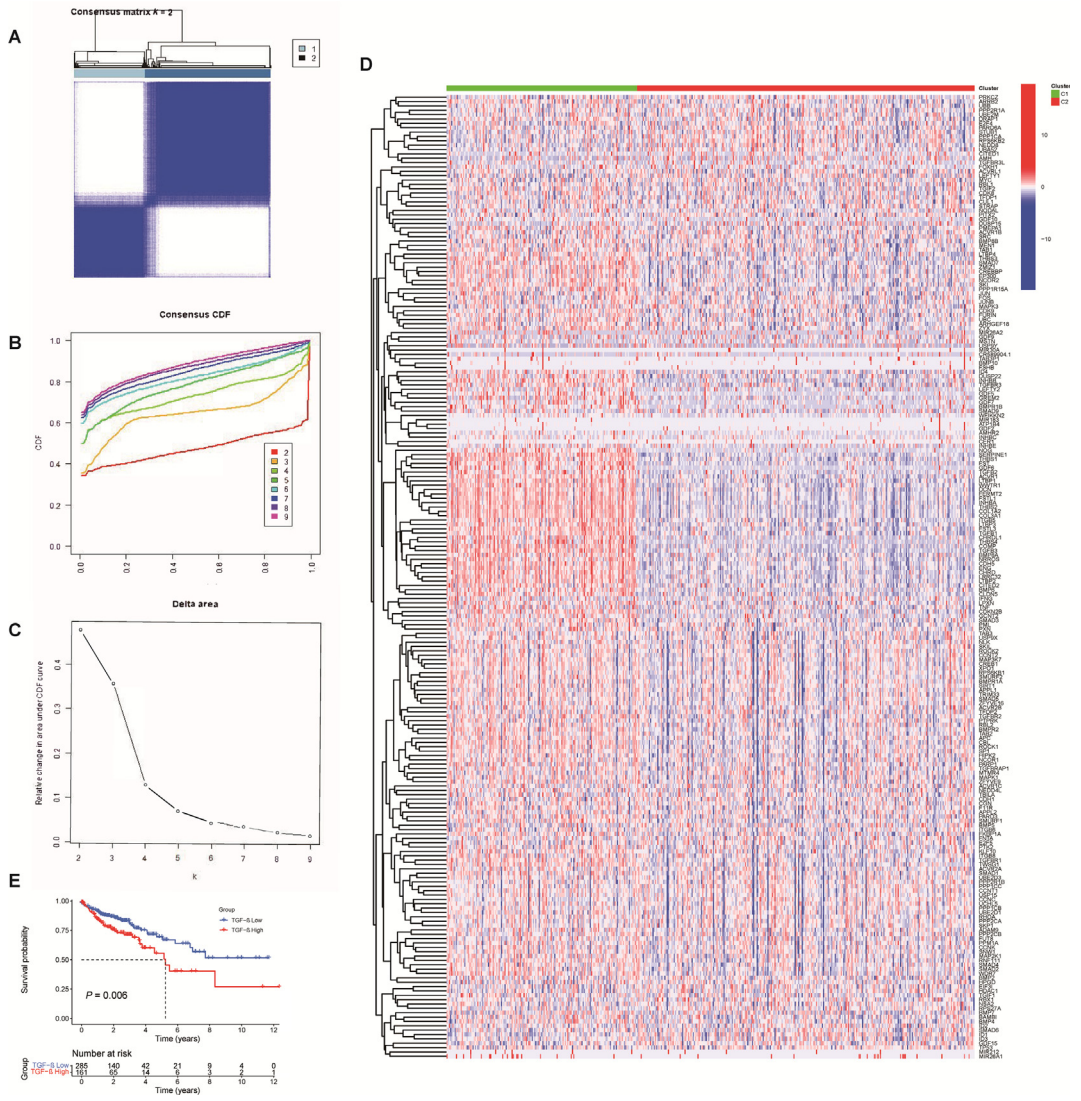

Figure 1. Consensus clustering for identifying TGF-β-related subtypes. (A–C) The area under the CDF curve for $k = 2$–9 is shown by the delta area curve, and the heatmap shows the consensus clustering solution for the 223 genes in the COAD samples ($k = 2$). (D) Expression patterns of the 223 TGF-β-related genes in different subtypes are depicted in the heatmap. Red indicates higher gene expression; blue indicates lower gene expression. (E) Kaplan–Meier curves of OS in TGF-β-high and -low subtypes. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. CDF: Cumulative distribution function; COAD: Colon adenocarcinoma; OS: Overall survival; TGF-β: Transforming growth factor-β.

into two distinct clusters, each exhibiting unique expression patterns of genes associated with the TGF-β signaling pathway [Figure 1A–C]. Additionally, cluster C1 could be classified as a TGF-β-high subtype since TGF-β signaling pathway-related genes were expressed at elevated levels, while cluster C2 is the opposite [Figure 1D]. The survival analysis indicated that clinical outcomes varied between these TGF-β subtypes. Specifically, cluster C1 was associated with an unfavorable prognosis, whereas cluster C2 was linked to favorable clinical outcomes [Figure 1E].

Relationship between transforming growth factor-β subtypes and patient prognosis as well as immunological characteristics of the tumor microenvironment

Identification of differentially expressed genes and signaling pathways of different transforming growth factor-β subtypes

The DEG analysis of the TCGA colon cancer cohort (473 cancer and 41 paracancer samples) revealed differential expression of genes associated with the TGF-β signaling pathway between colon cancer and normal samples, as shown in the heatmap [Supplementary Figure 1]. The correlation among these DEGs was shown through PPI network analysis using the STRING database [Supplementary Figure 2].

We investigated differential gene expression in several subtype samples based on the above results. Different TGF-β subtypes contained 139 DEGs, of which 88 and 51 were up- and downregulated in colon cancer tissues, respectively. Volcanic and heatmaps provided a clearer illustration of DEG distribution between these subtypes [Figure 2A and B].

To better comprehend the underlying molecular processes regulating the prognosis of patients with colon cancer, the relevant signaling pathways of these important DEGs in various subtypes were examined. Different subtypes of TGF-β differential genes were mainly related to signaling receptor activator activity, receptor–ligand activity, actin binding, ECM structural constituents, glycosaminoglycan binding, heparin-binding, integrin binding, growth factor binding, collagen binding, and proteoglycan binding. KEGG analysis showed that DEGs were mainly related to the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt/PKB) signaling pathway, neuroactive ligand–receptor interaction, human papillomavirus infection, axon guidance, cytokine–cytokine receptor interaction, and phagosomes

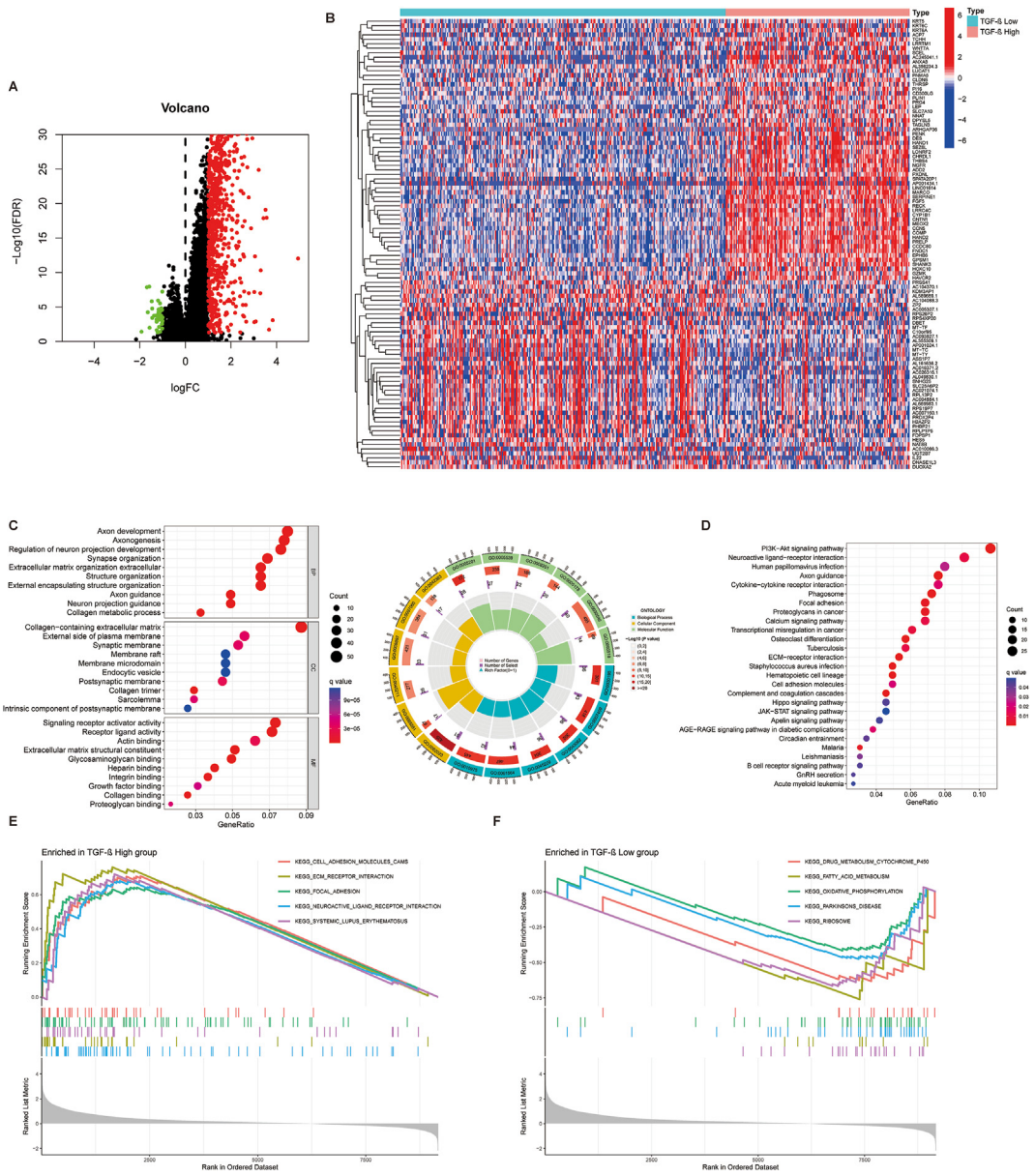

Figure 2. Identification of potential signaling pathways and DEGs in different TGF-β subtypes. (A) Volcano plot of the quantified DEG distribution between TGF-β-high and -low subtypes with thresholds of $|\log_2 FC| > 1$ and $P < 0.001$. (B) Heatmap of the DEG expression in different subtypes. (C) GO enrichment analysis. The dot size in the point plot represents gene counts (the left side of the figure). In the circle plot, the first circle represents the GOid; the second circle represents genes in GO, and the color represents the degree of differential gene enrichment; the third circle represents differential gene enrichment (the right side of the figure). (D) KEGG pathway enrichment analysis is shown as a dot map displaying enriched pathways. (E and F) GSEA analysis identified potential signaling pathways between TGF-β-high (E) and TGF-β-low (F) subtypes. DEG: Differentially expressed gene; FC: Fold change; FDR: False discovery rate; GO: Gene ontology; GSEA: Gene set enrichment analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; TGF-β: Transforming growth factor-β.

[Figure 2C and D]. Further GSEA analysis was conducted to identify the signaling pathways and molecular processes of the various TGF-β-high and -low subtypes (false discovery rate [FDR] < 0.05). TGF-β-high subtype genes were mainly involved with cell adhesion molecules, ECM receptor interaction, focal adhesion, and neuroactive ligand–receptor interaction. The TGF-β-low subtype gene set was mainly associated with drug metabolism cytochrome p450, fatty acid metabolism, oxidative phosphorylation, Parkinson's disease, and ribosomes [Figure 2E and F].

Gene mutation and the tumor microenvironment in different transforming growth factor-β subtypes

Generally, primary initiators and determinants of colon carcinogenesis include mutations and aberrant methylation patterns. Accordingly, we examined the subtype differences and mutations from a genomic standpoint. We noted different somatic mutation types and frequencies in TGF-β-high and -low subtypes. Adenomatous polyposis coli (*APC*), *TP53*, *TTN*, *KRAS*, *PIK3CA*, and *SYNE1* were the most common mutations, with approximately similar relative frequencies across subtypes. The TGF-β-high subtype had a greater incidence of mutations (98.71%) than that of the TGF-β-low subtype (94.21%) [Figure 3A and B].

Emerging evidence suggests that TGF-β significantly influences the consequences of immune responses in numerous types of malignancies.[33] The TGF-β-high subtype showed higher stromal, immune, integrated, and lower tumor purity scores than those of the TGF-β-low subtype [Figure 4A]. Using CIBERSORT, we next examined the differences in infiltration of 22 immune cells between the two subtypes in patients with COAD, and the correlation between immune cells in the samples was also

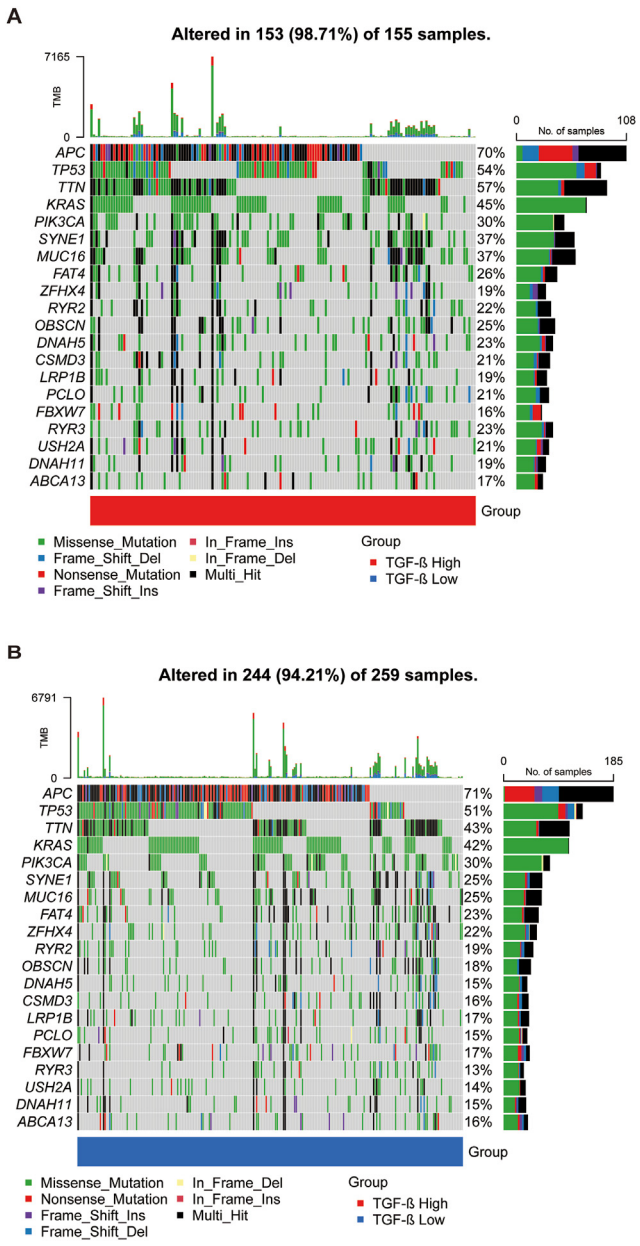

Figure 3. Waterfall plot of somatic mutation comparisons between different TGF-β subtypes. (A and B) Visualization of the top 10 most frequently mutated genes in the TGF-β high (A) and TGF-β low subtypes (B). No.: Number; TGF-β: Transforming growth factor-β; TMB: Tumor mutational burden.

studied [Figure 4B and C]. The differential study of immune cells revealed that patients with TGF-β-high subtypes had considerably larger percentages of plasma cells, naïve B cells, resting CD4 T cell memory, and M0 and M1 macrophages and lower percentages of activated dendritic cells (DCs) [Figure 4D]. The differential analysis of immune checkpoints and *HLA* genes revealed upregulation in most TGF-β-high subtypes, whereas the TGF-β-low subtype showed the reverse [Figure 4E and F], suggesting that immunosuppressive cells could be critical in creating an immunosuppressive TME in patients with the TGF-β-high subtype. Simultaneously, immune checkpoints and *HLA* genes showed higher expression levels, suggesting that patients with the TGF-β-high subtype could benefit more from immunotherapy, unlike those with the TGF-β-low subtype.

Drug sensitivity analysis of different transforming growth factor-β subtypes

To assess the sensitivity of the different subtypes to conventional targeted drugs, we calculated the half-maximal drug inhibitory concentration (IC_{50}) values of nine common drugs in both subtypes [Figure 5]. Statistical analysis revealed significant differences between the two clusters ($P < 0.05$). Vascular endothelial growth factor receptor (VEGFR) inhibitor AMG.706, Akt/PKB inhibitor A.443654, proto-oncogene tyrosine-protein kinase Src (Src) family lymphocyte-specific protein tyrosine kinase (LCK) inhibitor A.770041, Src/Abl tyrosine kinase (Abl) kinase inhibitor AZD.0530 (Saracatinib), rapidly accelerated fibrosarcoma (RAF) inhibitor AZ628, B-cell receptor (BCR)-Abl AP.24534 (ponatinib), PI3K inhibitor AZD6482, heat shock protein 90 (HSP90) inhibitor AUY922 (luminespib) and c-jun N-terminal kinase (JNK) inhibitor AS601245 could be more appropriate for patients with TGF-β-high subtype colon cancer.

Transforming growth factor-β-related prognostic risk signature construction, validation, and immunological microenvironmental analysis in colon cancer

Construction and validation of transforming growth factor-β risk signature

We developed a prognostic risk signature comprising TGF-β signaling pathway-related genes applicable to all patients with colon cancer. Through univariate Cox analysis, we identified 12 TGF-β signaling pathway-related genes significantly associated with the OS of patients with colon cancer [Figure 6A]. Subsequently, using LASSO regression analysis, we evaluated and selected ten TGF-β signaling pathway-related genes (*ADAM9*, *CDK9*, *CER1*, *FOXH1*, *FSTL3*, *INHBB*, *NOG*, *PPP2CB*, *SERPINE1*, and *TGFB3*) to construct a predictive risk signature. These ten genes significantly impacted the prognosis of patients with colon cancer [Figure 6B]. The risk signature was developed based on the following algorithm: risk score = (−0.128)*$ADAM9$ + 0.198*$CDK9$ + (0.107)*$CER1$ + 0.155*$FOXH1$ + 0.043*$FSTL3$ + 0.068*$INHBB$ + 0.281*NOG + (−0.214)*$PPP2CB$ + 00.018*$SERPINE1$ + 0.054*$TGFB3$. Based on the median risk score, risk scores were determined for each patient. Patients with a risk score above the median were classified as high-risk.

Furthermore, an investigation was conducted to examine the correlation between risk score and survival status. The results revealed that the low-risk subgroup exhibited a considerably greater survival outcome compared with that of the high-risk subgroup. According to the risk heatmap analysis, the expression levels of high-risk genes, including *CDK9*, *CER1*, *FOXH1*, *FSTL3*, *INHBB*, *NOG*, *SERPINE1*, and *TGFB3*, increased with increasing risk score [Figure 6C]. Additionally, K–M analysis was employed to further assess the survival status of patients with colon cancer. In the TCGA group, the survival rates varied significantly between the high- and low-risk subgroups, with higher risk scores being associated with lower OS. Results from the GEO group's validation were consistent [Figure 6D].

Application of the transforming growth factor-β risk signature in association with the tumor microenvironment

Univariate analysis of age, gender, stage, and risk score revealed that a high TGF-β risk score was significantly associated with a worse OS [Figure 7A]. According to the multivariate analysis, the TGF-β risk score for patients with colon cancer may serve as an independent prognostic predictor [Figure 7B]. In Figure 7C, we compared the accuracy of the prognostic signature with clinicopathological characteristics such as age, sex, and stage to predict patient survival. The risk score exhibited superior predictive performance relative to age (AUC = 0.628), gender (AUC = 0.497), and stage (AUC = 0.678), as evidenced by its AUC of 0.686. Figure 7D subsequently displays the accuracy of the prognostic risk signature as determined by the predicted ROC curves over the subsequent 1, 3, and 5 years in the TCGA database; the accuracy of the risk prediction signature was also confirmed in the GEO database for the same periods, with model accuracies of 0.778, 0.677, and 0.616, respectively [Figure 7E]. To provide clinicians with a quantitative method for predicting the probable risk of cancer progression, we constructed a nomogram incorporating clinicopathologic variables and risk scores to predict the 1-, 3-, and 5-year prognosis of patients with colon cancer. The calibration curves showed good agreement between actual and predicted 1-, 3-, and 5-year survival rates [Figure 8A and B].

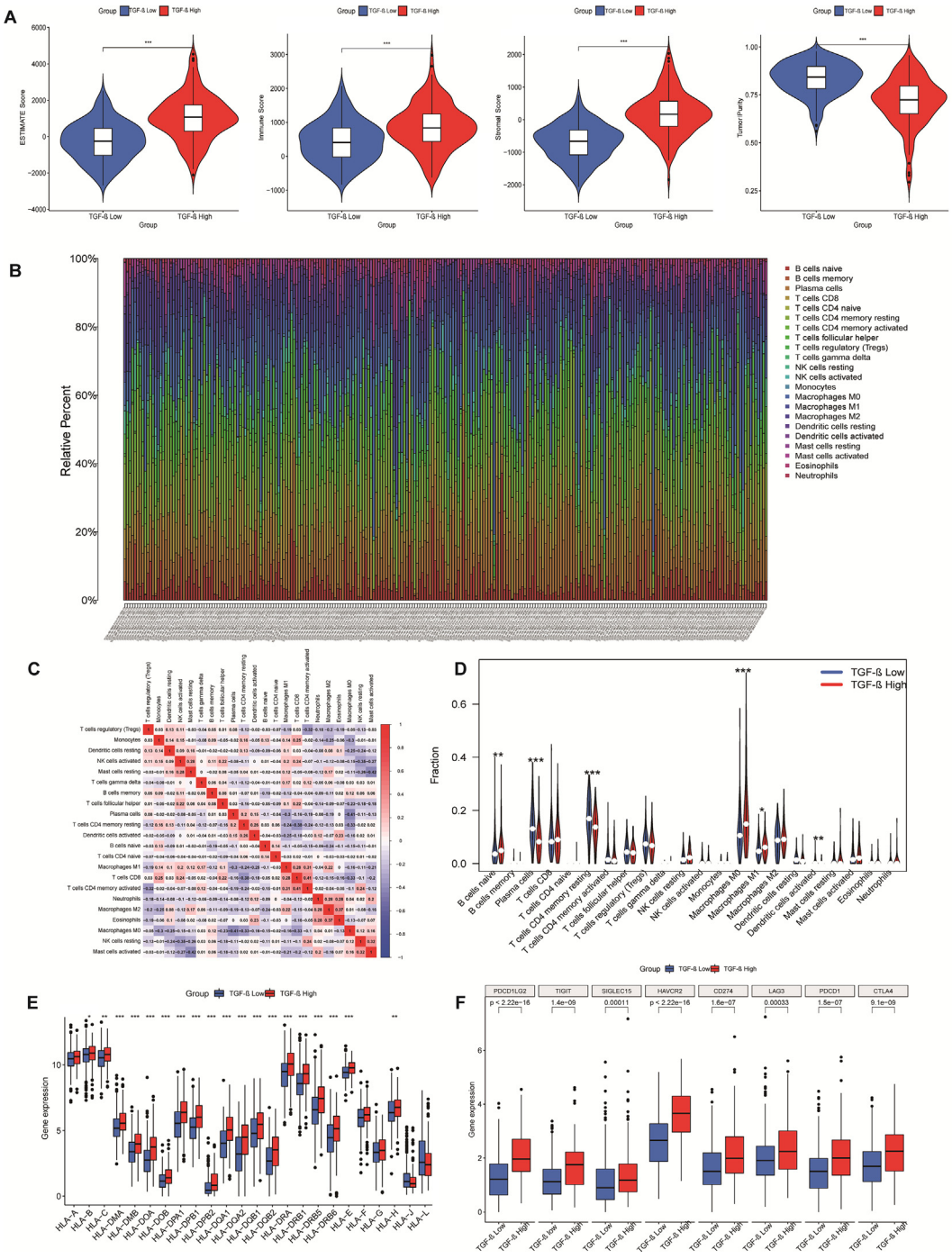

Figure 4. TGF-β-high and -low subtype immune profiles. (A) Violin plot of the median and interquartile estimates of immune and tumor purity scores of the tumor microenvironment. (B) Landscape map of the proportions of TGF-β-high and -low subtype immune infiltration; the horizontal coordinate represents each colon cancer patient. (C) Correlation of various immune cells in colon cancer. (D) Violin plot of the significantly different immune cells between different TGF-β subtypes. (E and F) Boxplot showing differential expression of multiple immune checkpoints (E) and *HLA* genes (F) in TGF-β-high and -low subtypes. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. *HLA*: Human leukocyte antigen; NK: Natural killer cell; TGF-β: Transforming growth factor-β; Tregs: Regulatory T cells.

Evaluation of the stromal, tumor purity, and immune scores indicated that the TGF-β high-risk subgroup exhibited higher stromal and immune scores and lower tumor purity scores compared with those of the TGF-β low-risk subgroup [Figure 9A]. Given the crucial biological function of TGF-β in the antitumor immune response, the relationship between the TGF-β risk score and the TME was investigated in depth. The results showed that high-risk scores negatively correlated with DCs, resting CD4 memory cells, and activated CD4 memory cells and positively correlated with regulatory T cells (Tregs) and M0 macrophages [Figure 9B]. Upon examining immune checkpoint variations, overexpression was observed in most patients in the TGF-β high-risk subgroup [Figure 9C]. These results showed that immunotherapy is more beneficial for patients in the TGF-β high-risk subgroup.

Discussion

Colon cancer is among the most prevalent malignancies, and its low survival rate is still a pressing issue.[4] Studying the TGF-β signaling

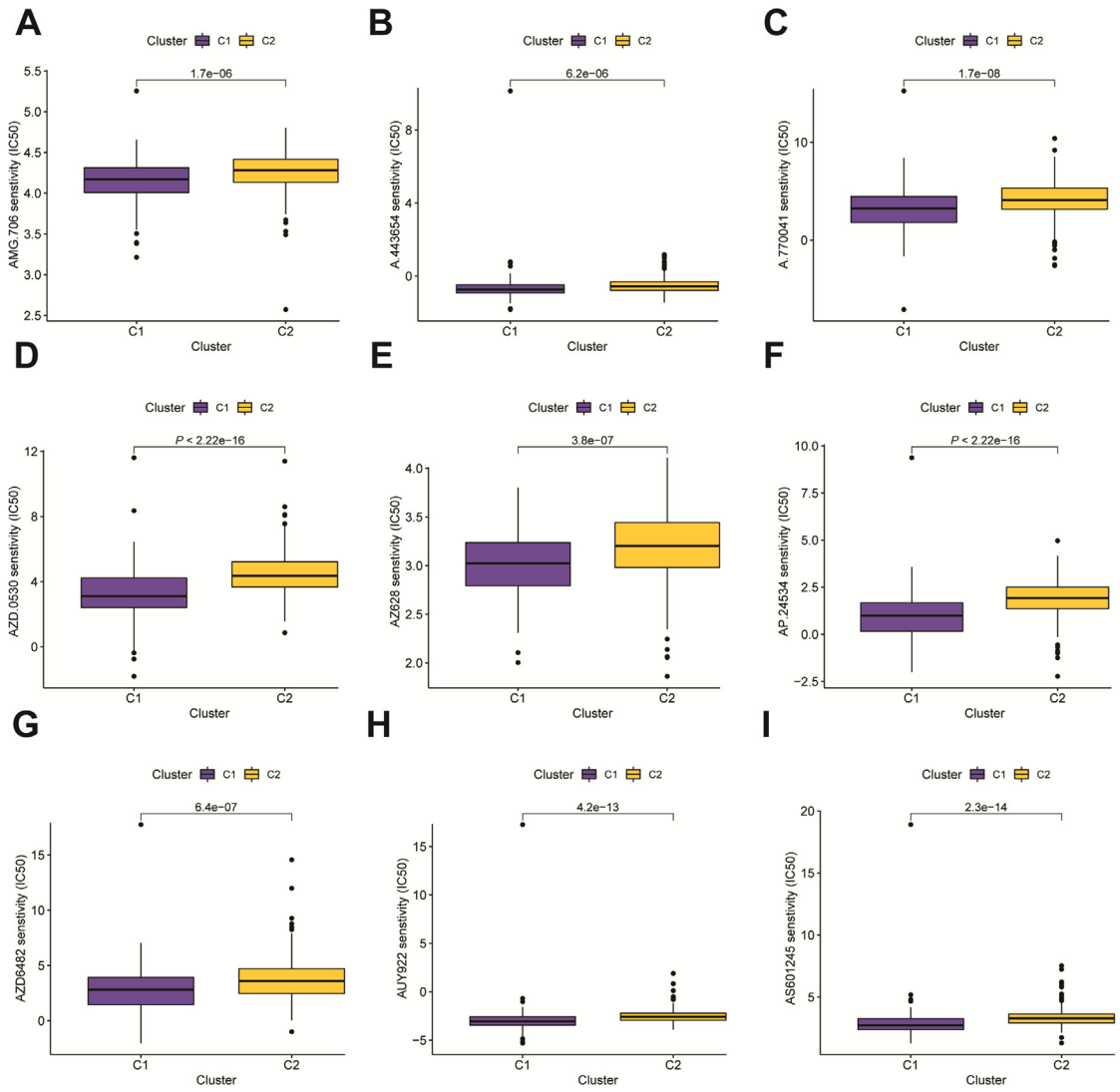

Figure 5. IC_{50} values of nine common drugs in different TGF-β subtypes. (A) VEGFR inhibitor AMG.706; (B) Akt/PKB inhibitor A.443654; (C) Src family LCK inhibitor A.770041; (D) Src/Abl kinase inhibitor AZD.0530 (Saracatinib); (E) RAF inhibitor AZ628; (F) BCR-ABL AP.24534 (ponatinib); (G) PI3K inhibitor AZD6482; (H) HSP90 inhibitor AUY922 (luminespib); (I) JNK inhibitor AS601245. Abl: Abl tyrosine kinase; Akt/PKB: Protein kinase B; BCR: B-cell receptor; HSP90: Heat shock protein 90; IC_{50}: Half-maximal inhibitory concentration; JNK: c-Jun N-terminal kinase; LCK: Lymphocyte-specific protein tyrosine kinase; PI3K: Phosphatidylinositol 3-kinase; RAF: Rapidly accelerated fibrosarcoma; Src: Proto-oncogene tyrosine-protein kinase Src; TGF-β: Transforming growth factor-β; VEGFR: Vascular endothelial growth factor receptor.

pathway is critical to tumor research owing to its elaborate functional role in contributing to cancer progression, metastasis, treatment resistance, and other aspects.[27] The shared functions of TGF-β signaling pathway-related genes for clustering and constructing prognostic signatures to further advise precise therapy are poorly understood. Therefore, we sought to address this in the present study.

Our study focused on TGF-β signaling pathway-related genes and identified two TGF-β subtypes through consensus clustering, with the TGF-β-high subtype associated with a poor prognosis and a superior response to immunotherapy. GSEA analysis revealed that the TGF-β-high subtype gene set was mainly associated with cell adhesion molecules, ECM receptor interaction, and focal adhesion. Most of these gene enrichment pathways influence the TME, affecting the onset, progression, and dissemination of cancer.[34–37]

The TGF-β-high subtype showed a higher frequency of gene mutations. Previous research has linked colon cancer to *APC* and *TP53* mutations, which had the greatest mutation frequencies in our study. Mutations in the *APC* gene, which encodes a protein involved in β-linked protein degradation, can lead to colorectal polyps and malignant tumors. The mutation cluster region regulates the Wnt signaling pathway for colon cancer cell adhesion, invasion, progression, differentiation, and stemness.[38,39] *TP53*, a frequent genetic mutation associated with colon cancer, has oncogenic features controlling cancer cell growth and metastasis.[40,41] TMB can indirectly reflect the tumor's potential and extent for producing neoantigens and impact immune response intensity, predicting immunotherapy outcomes for an extensive range of malignancies. Our results imply that patients with the TGF-β-high subtype are more capable of generating neoantigens and influencing the immune response. Moreover, administering ICIs as part of the therapeutic approach yields more favorable clinical outcomes.[42,43]

The relationship between TGF-β-high/-low subtypes and the tumor immune microenvironment of colon cancer was also highlighted. We performed an immune microenvironment analysis and found that TGF-β expression levels correlated with tumor-infiltrating immune cells, *HLA* genes, and immune checkpoints. Tumor-infiltrating immune cells affect the prognosis and survival of individuals diagnosed with colon cancer as they are implicated in each stage of tumor development, invasion, immune evasion, and metastasis.[44–47] *HLA*, also referred to as the major histocompatibility complex in humans, comprises a group of genes intricately related to cell recognition and exerts a substantial impact on immune function

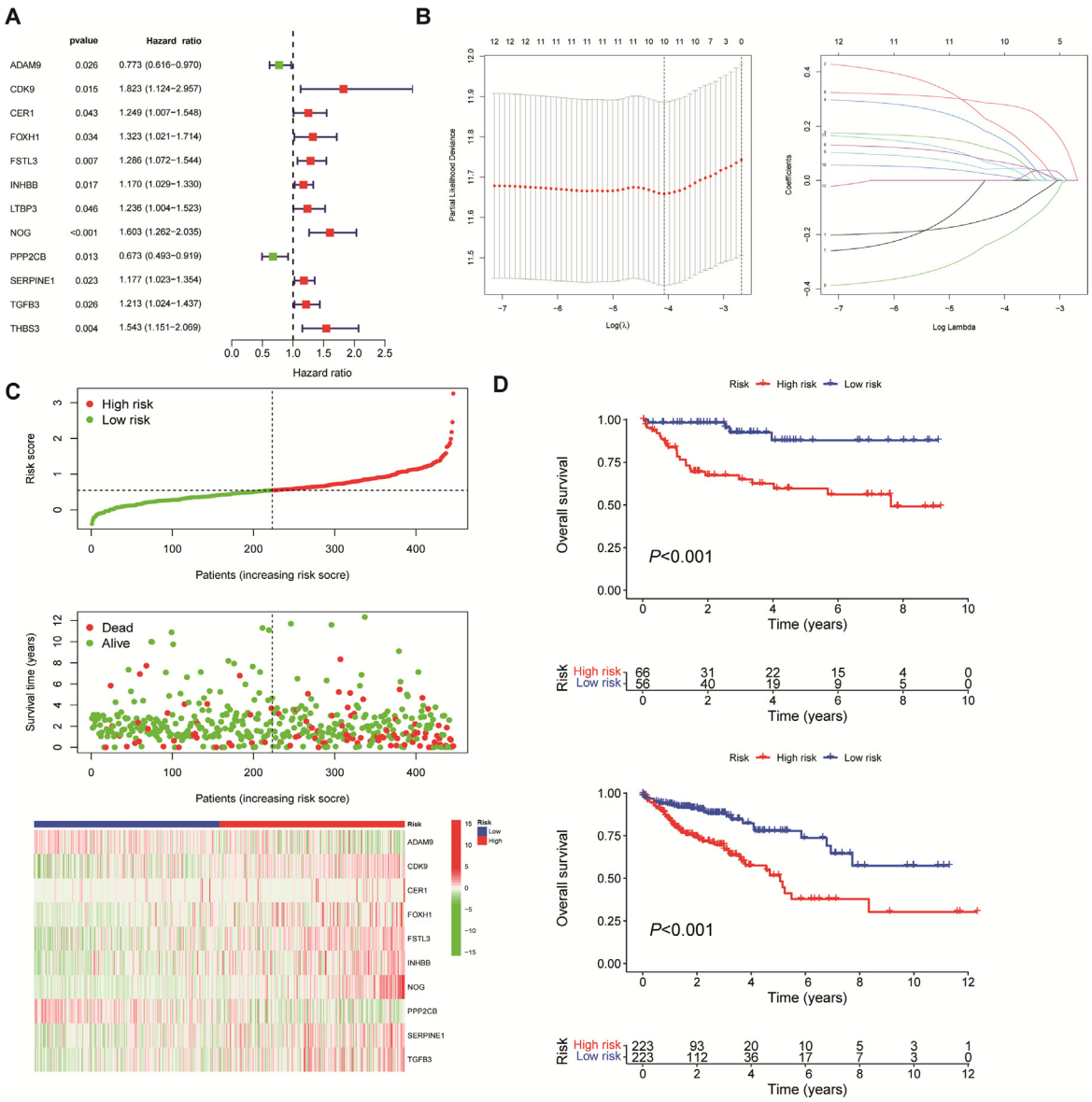

Figure 6. Construction and validation of the TGF-β risk signature. (A) Univariate Cox analysis of the prognostic value of TGF-β signaling pathway-related genes in relation to OS in TCGA database. (B) LASSO regression analysis identifies the 10 genes most associated with OS in the TCGA dataset. (C) Scatter plot of each patient's risk scores and survival status distribution and heatmap of TGF-β risk signature genes in TCGA database. (D) Kaplan–Meier analysis of the prognostic significance of the TGF-β risk signature in TCGA (upper) and GEO (lower) cohorts. AUC: Area under the receiver operating characteristic curve; GEO: Gene Expression Omnibus; LASSO: Least absolute shrinkage and selector operation; OS: Overall survival; ROC: Receiver operating characteristic curve; TCGA: The Cancer Genome Atlas; TGF- β: Transforming growth factor-β.

regulation. Immune checkpoints are important regulators of the immune system that modulate autoimmune tolerance. The TGF-β-high subtype was associated with the immunosuppressive phenotype and high immune checkpoint and *HLA* gene expression levels, indicating that individuals possessing this subtype might respond better to immunotherapy. Various therapeutic agents that may be beneficial for the TGF-β-high subtype were identified, thus advancing the goal of treating colon cancer in an approach that can be customized to each patient.

We also constructed and validated a TGF-β-related prognostic signature comprising ten genes, including *ADAM9*, *CDK9*, *CER1*, *FOXH1*, *FSTL3*, *INHBB*, *NOG*, *PPP2CB*, *SERPINE1*, and *TGFB3*. Previous studies determined that *ADAM9*, *CDK9*, *FOXH1*, *FSTL3*, *INHBB*, *PPP2CB*, *SERPINE1* and *TGFB3* play roles in cancer. Disintegrin and metalloprotease (*ADAM*) proteins are involved in various physiological and pathological processes, including cancer.[48,49] *ADAM9* overexpression in various cancer types, is associated with reduced survival, poor tumor classification, and metastasis.[50,51] In colon cancer, *ADAM9* impacts 5-fluorouracil resistance and growth factor-mediated E-calmodulin recirculation.[52,53] Cell cycle protein-dependent kinases (*CDKs*) are essential for normal cell cycle progression.[54] *CDK9* regulates gene expression, genomic stability, DNA damage response, and epigenetic changes.[55–60] *CDK9* chaperones are members of the T and K families of cell cycle proteins; they phosphorylate RNA polymerase II and are linked to numerous malignancies.[61–63] Numerous studies have revealed that specific *CDK9* inhibition may be a potential therapy as a promising target for overcoming drug resistance and extending survival in patients with cancer.[64–67] The human forkhead-box (*FOXH*) family is influenced by cancer development.[68] *FOXH1* mRNA is expressed in human embryonic stem cells and mediates the TGF-β signaling pathway through interaction with *Smad2* and *Smad4* complexes.[69–73] Follistatin-like 3 (*FSTL3*) is a key

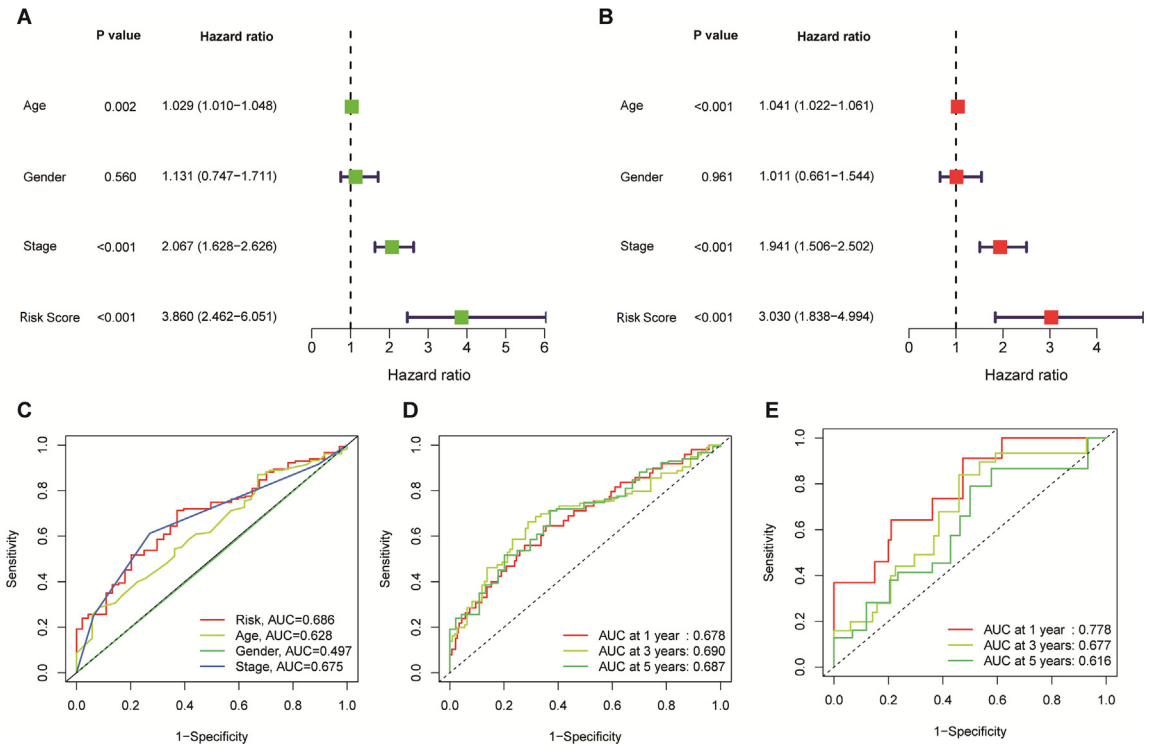

Figure 7. Association of TGF-β risk signature with prognosis. (A and B) Uni- and multivariate Cox analyses assessing the independent prognostic value of TGF-β risk signature in patients with colon cancer. (C) ROC curves of the accuracy of various indicators predicting cancer progression over 5 years in the TCGA database. (D) 1-, 3-, and 5-year ROC curves for the risk signature in the TCGA database. (E) 1-, 3-, and 5-year ROC curves for the risk signature in the GEO database. AUC: Area under the receiver operating characteristic curve; GEO: Gene Expression Omnibus; ROC: Receiver operating characteristic; TCGA: The Cancer Genome Atlas; TGF- β: Transforming growth factor-β.

biomarker in cancer development. Elevated *FSTL3* levels in individuals with gastric cancer result in a worse prognosis.[74] Furthermore, a nomogram containing *FSTL3* predicted laryngeal squamous cell carcinoma patient survival.[75] *FSTL3* overexpression is associated with poor prognosis in colorectal cancer and could create a suppressive immunological microenvironment to speed up lymph node metastasis.[76] Inhibin (*INH*), a dimeric glycoprotein with one alpha and two beta subunits, plays a significant role in human reproduction, endocrine-responsive tumors, and breast cancer.[77–81] Nasopharyngeal carcinoma tissues exhibit lower *INHBB* expression, correlated with lymph node metastases, disease stage, and clinical progression.[82] However, *INHBB* expression is increased in B-Raf proto-oncogene, serine/threonine kinase (BRAF) V600E mutant thyroid and rectal cancer tissues.[83–86] Additionally, *INHBB* is strongly associated with colon cancer metastasis through highly dysregulated methylation.[84,87] *PPP2CB*, associated with the Wnt signaling system, is an independent prognostic factor for bladder cancer due to its involvement in immune cell infiltration and tumor cell EMT.[88] Plasminogen activator inhibitor-1 (*PAI-1*), a member of the serine protease inhibitor (*SERPIN*) superfamily, is a crucial inhibitor of fibrinogen/fibrinolytic enzyme systems and a physiological antagonist of fibrinogen activator.[89,90] It reduces fibrinolysis through tissue remodeling, cell migration, and pericellular protein hydrolysis.[91–96] *LINC00491* positively regulates *SERPINE1* expression, playing an oncogenic role in COAD pathogenesis.[97–100] Functional data suggest elevated *TGFB3* expression in advanced tumors links it to tumorigenesis.[101] *TGFB3* stimulates ECM production but inhibits protease production, leading to excessive connective tissue deposition.[102,103] Moreover, *TGFB3* inhibition reduces collagen fibril structure in COAD.[104]

In K–M analysis, the survival rates varied significantly between the high- and low-risk subgroups, with higher risk scores being associated with lower OS. Next, we examined how TGF-β scores affect patient survival and the TME. In previous studies, TGF-β affected both innate and adaptive immune cells in the TME.[105] In innate immunity, TGF-β reduces bone marrow cell proliferation and differentiation in early cancer stages. In advanced cancers, myeloid cells produce TGF-β, further inhibiting the antitumor immune response and contributing to tumor metastasis. In macrophages, TGF-β protects tumors from antitumor activity by inducing IRAK-M and antagonizing the toll-like receptor signaling pathway. TGF-β also inhibits the activation of natural killer cells and their cytotoxic potential. TGF-β is crucial for adaptive immunity, inhibiting T cell activation, proliferation, differentiation, and migration. Furthermore, TGF-β can differentiate CD4+ helper T cells into effector subtypes and transform naïve T cells into Tregs. TGF-β also blocks cytotoxic CD8+ T cell activation and maturation by inhibiting tumor antigen processing and DC expression. Moreover, TGF-β promotes the antigen-induced expression of Programmed Death Receptor 1(PD-1), potentially leading to T cell depletion. However, its function in regulating B cell-mediated antitumor immunity remains unstudied.[106,107]

Our analysis of the TME revealed higher stromal and immune scores and upregulated immune checkpoint expression in the TGF-β high-risk subgroup, implying that treatment of patients in this subgroup with ICIs could result in a relatively better prognosis. High-risk scores positively correlated with M0 macrophages and Tregs and negatively correlated with DCs, resting CD4 memory cells, and activated CD4 memory cells. Tregs play an important role in maintaining self-tolerance and have a negative role in evoking an effective antitumor immune response.[108] Evidence suggests that Treg depletion or suppression of their function could enhance antitumor effects. Consistent with our results, the large number of Tregs suppress effective antitumor immune responses in high-risk patients, leading to tumor progression and worse clinical

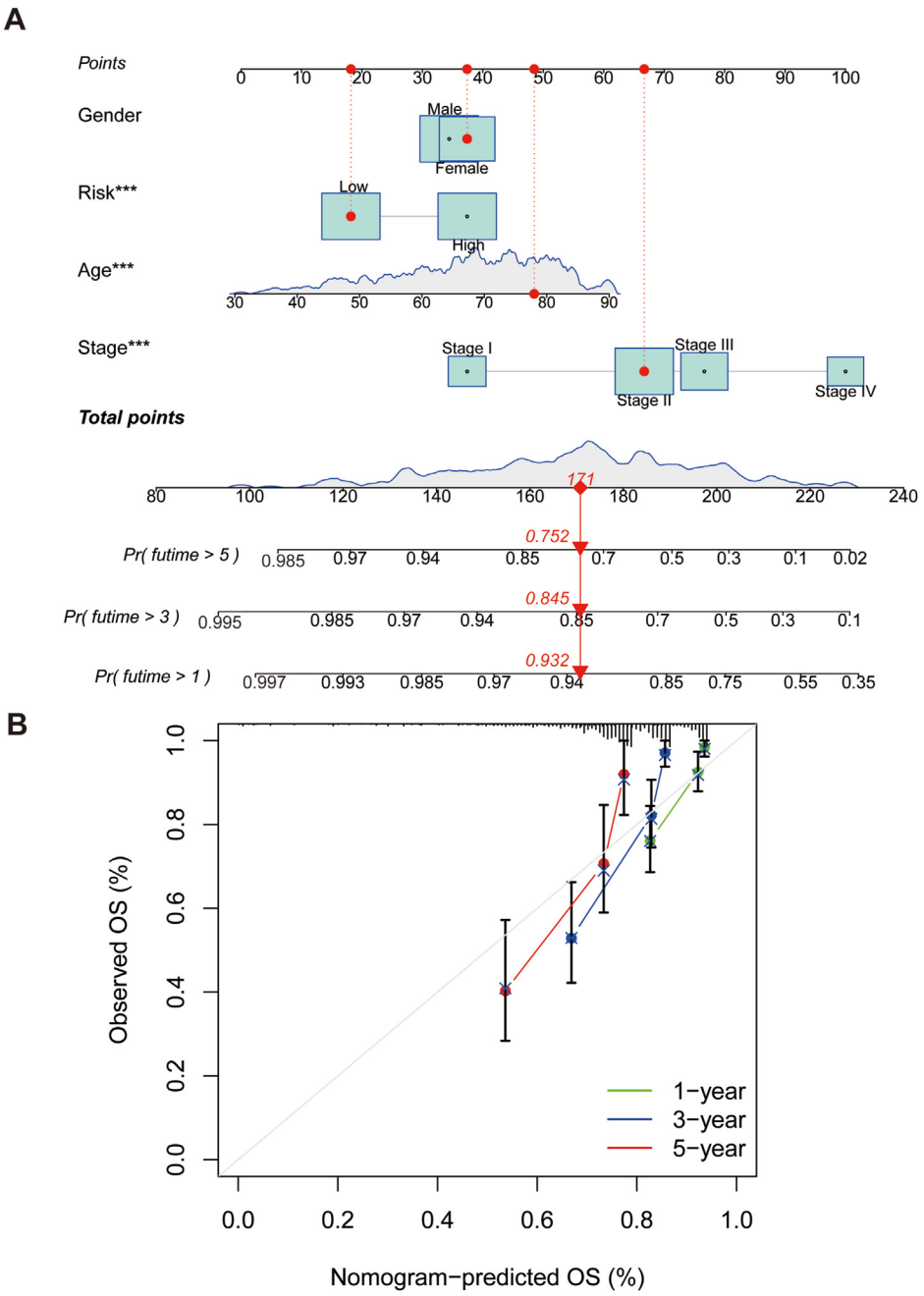

Figure 8. TGF-β risk signature combined with clinicopathologic factors to construct a nomogram. (A) Nomogram. (B) Calibration curve. OS: Overall survival; Pr: Probability; TGF-β: Transforming growth factor-β.

outcomes.[109] DCs are the most efficient antigen-presenting cells and are required for adaptive immune responses.[110] Increased DC numbers and their improved function enhance antitumor immunity.[111] Our results confirm that the antigen-presenting ability of DCs reduces with their reduction in number, further leading to disease progression and decreased patient survival. Thus, patients with a high-risk score had greater pre-existing inhibitory immunity activity levels and higher levels of immunosuppressive cells, such as Tregs, which suppress the anticancer activity of the tumor immune microenvironment and promote tumor progression. The risk score and the expression of typical immune checkpoints such as CD27, CTLA-4, and CD70, which are thought to limit anticancer immunity in the TME, were strongly positively correlated. Collectively, these findings demonstrated that the TME with a higher risk score was more sensitive to immunotherapy and responded better to immunotargeted treatment.

We comprehensively investigated the common functions of TGF-β signaling pathway-related genes, clarified their possible roles in colon cancer prognosis and the TME, and incorporated genes into a risk prediction signature to inform patient risk stratification and treatment choice. However, several issues with our study were unavoidable. First, all our results are based on public data, which need to be validated in clinical cohorts. *In vitro* or *in vivo* experimental investigation is also necessary for mechanistic investigations. Second, our clinical data are primarily retrospective; therefore,

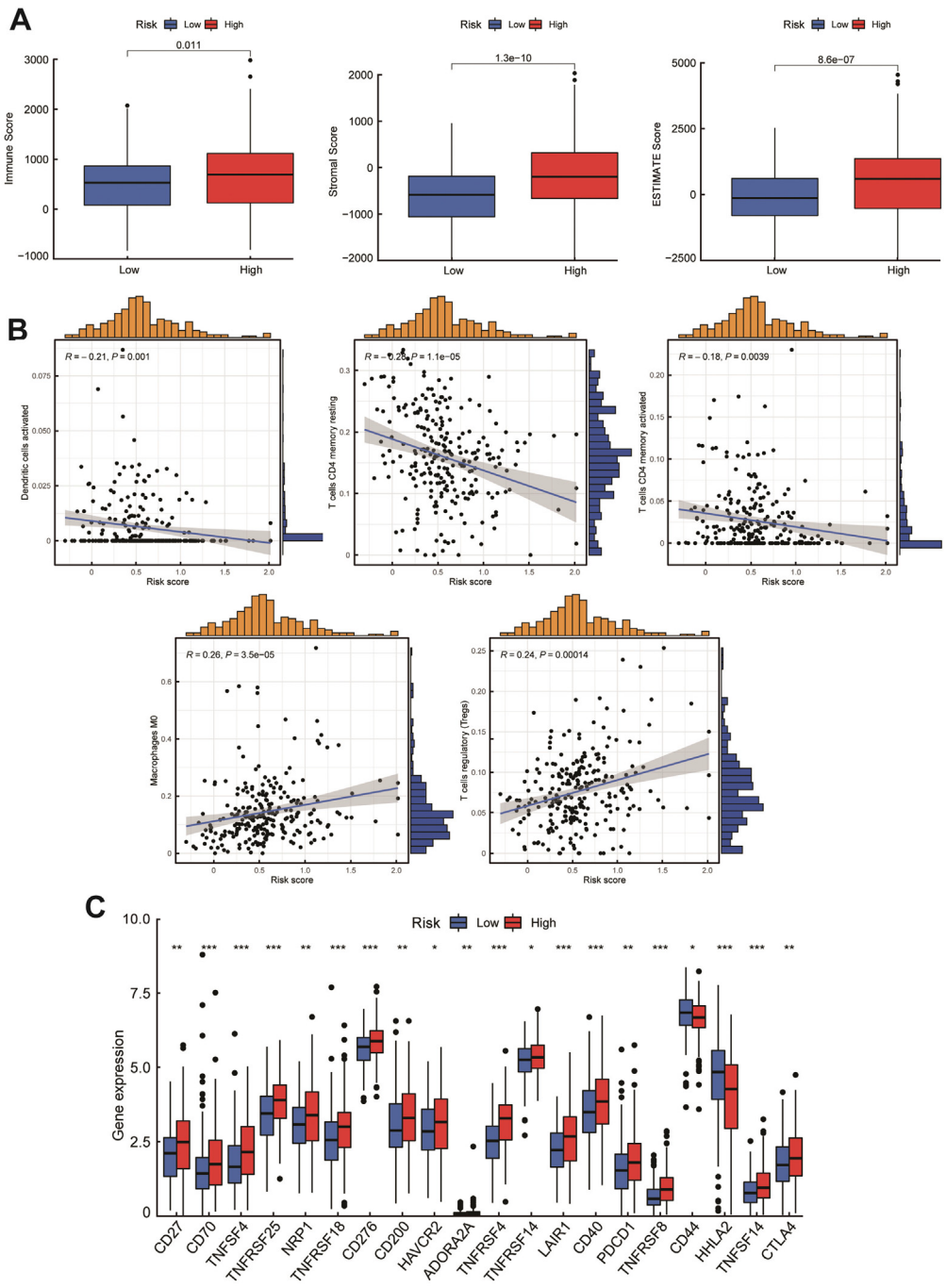

Figure 9. TGF-β risk signature and tumor immune microenvironment. (A) Bar graph of median and interquartile estimates of tumor microenvironment immune and tumor purity scores. (B) Scatter plot of the correlation between risk scores and immune cell infiltration. (C) Box plot of the differential expression of multiple immune checkpoints between TGF-β high- and -low-risk subgroups. TGF-β: Transforming growth factor-β.

prospective data is urgently needed to strengthen the validity of our findings. Third, the clinical application of TGF-β features needs further exploration. Fourth, more research is necessary to confirm our hypothesis that the median TGF-β risk score serves as the cut-off value for all studies.

In conclusion, colon cancer is heterogeneous; thus, in the present study, patients with colon cancer were classified into two subtypes with different survival and immune characteristics using consensus clustering analysis based on the TGF-β signaling pathway-related genes. The constructed risk prediction signature is a promising biomarker for evaluating colon cancer prognosis and immunotherapy effectiveness and precisely screening individuals for immunotherapy. Nevertheless, further validation through more extensive research is required.

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Authors contributions

Jie Chen conceived and designed the study; Chao Ji, Silin Liu, Che Wang, Jin Wang, Jue Pan, and Mengjiao Cai collected the data; Jie Chen analyzed the data; Chao Ji, Jin Wang, Jue Pan, Jinyu Qiao, and Yu Liang contributed to drawing/analysis tools; Jie Chen wrote the manuscript. All authors reviewed and approved the submission of the final version of the manuscript.

Ethics statement

In this study, we used publicly available datasets for bioinformatics analysis. We affirm the importance of data privacy and confidentiality in protecting the rights of participants. We aimed to maintain the privacy rights of participants, uphold scientific integrity, and promote the responsible development of bioinformatics research while using publicly available databases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cpt.2023.12.002.

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