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ITGB5 is a prognostic factor in colorectal cancer and promotes cancer progression and metastasis through the Wnt signaling pathway

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Integrin beta5 (ITGB5) expression levels are dysregulated in a variety of cancers. However, the mechanism and clinical value of ITGB5 in colorectal cancer (CRC) remain unclear. The Gene Expression Omnibus (GEO) database, real-time PCR, Western blotting and immunohistochemistry were utilized to evaluate ITGB5 expression levels in CRC tissue. Clinical data from the GEO database were obtained to further explore the associations of ITGB5 with clinical features and patient survival. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and gene set enrichment analysis (GSEA) were performed to explore the functions and signaling pathways of ITGB5. In addition, ITGB5 expression was inhibited by siRNA, and the roles of ITGB5 in SW480 and RKO cell growth, migration and invasion, as well as in the Wnt/β-catenin signaling pathway, were investigated. Pancancer studies have shown that ITGB5 is highly expressed in a variety of cancers. Moreover, ITGB5 expression is significantly increased in CRC tissues and is correlated with TNM stage, invasion depth, lymph node metastasis and distant metastasis stage. Kaplan–Meier analysis and meta-analysis of the GSE39582 and GSE17538 datasets indicated that a high level of ITGB5 is a high risk factor for overall survival (OS) and disease-free survival (DFS). In addition, receiver operating characteristic (ROC) curve analysis revealed the value of ITGB5 in predicting DFS, and univariate and multivariate analyses showed that ITGB5 may be an independent prognostic factor for DFS. GO and KEGG analyses indicated that many GO terms related to the extracellular matrix (ECM), focal adhesion and ECM–receptor interaction pathways were enriched. GSEA revealed focal adhesion, cancer pathways, ECM–receptor interactions and Wnt signaling pathways in the samples with high ITGB5 expression. Correlation analysis revealed that high ITGB5 expression is significantly correlated with the TGF-β/EMT pathway and WNT targets. Silencing of ITGB5 inhibited SW480 and RKO cell proliferation, invasion and migration. Mechanistically, downregulated ITGB5 expression blocked the Wnt/β-catenin signaling pathway and epithelial–mesenchymal transition (EMT) in CRC cells. Moreover, ITGB5 expression was related to M0 macrophages, M2 macrophages, neutrophils and plasma cell fractions. ITGB5 may be associated with poor prognosis and metastasis in patients with CRC. ITGB5 may hold promise as a prognostic biomarker and a new potential therapeutic target for CRC.

Keywords Integrin beta 5 (ITGB5), Tumor metastasis, Epithelial–mesenchymal transition (EMT), Wnt/β-catenin signaling, Colorectal cancer

The most recent cancer statistics from the American Cancer Society show that colorectal cancer rates are declining for both men and women in the United States, but colorectal cancer remains the third most common cancer in terms of incidence and mortality[1]. In addition, studies have shown that the incidence of CRC tends to

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stabilize or decrease in developed countries and increase in low- and middle-income countries, which is related to many factors, such as economic level and food structure[2–4]. With the advancement of medicinal practices, comprehensive surgical treatments for many colorectal cancer patients have improved survival, but the overall survival (OS) rate of patients with CRC remains unsatisfactory[5–7]. Therefore, it is of great practical and economic significance to study the mechanism underlying the formation, development and metastasis of colorectal cancer and to determine treatment strategies[8–11].

Integrins are a group of transmembrane proteins that form heterodimeric complexes composed of α and β subunits and mediate interactions between cells and the extracellular matrix (ECM). In addition, integrins are considered to be involved in almost all stages of cancer development, from the primary tumor to metastasis[12,13]. The ITGB5 gene encodes the integrin β-subunit, which is highly expressed in cancers such as hepatocellular carcinoma and glioblastoma and functions as an oncogene[14,15]. There are few studies on the role and mechanism of ITGB5 in colorectal cancer, and only a few articles have conducted preliminary research and exploratory analyses, such as biological information analysis[16]. ITGB5 is reportedly associated with multiple signaling pathways. In breast cancer, ITGB5 regulates the MEK-ERK and SRC-FAK signaling pathways to promote TGF-β-induced epithelial–mesenchymal transition (EMT)[17,18]. In hepatocellular carcinoma (HCC), ITGB5 directly interacts with β-catenin and inhibits its degradation, activating the Wnt/β-catenin signaling pathway[14]. In CRC, ITGB5 may modulate the TGF-β signaling pathway[16], but the exact mechanism is unclear.

In this study, we analyzed ITGB5 expression levels in CRC and paracancerous tissues and the relationship between ITGB5 expression and clinical features. Then, we studied the prognostic value of ITGB5 and investigated the molecular functions and related signaling pathways of ITGB5 using Gene Ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis[19,20] and gene set enrichment (GSEA) analysis. In addition, we analyzed the correlation between ITGB5 and tumor-infiltrating immune cells (TIICs). Finally, we found that knockdown of ITGB5 inhibited CRC cell proliferation, migration and invasion by downregulating the Wnt/β-catenin signaling pathway.

Materials and methods

Patient samples

In this study, 15 adjacent normal mucosa and 55 CRC tissue samples were obtained from CRC patients who underwent surgery at the First Affiliated Hospital of Fujian Medical University (Fuzhou, China) from August 2020 to December 2020. The TNM staging system of the United States Joint Commission on Cancer was used to evaluate tumor staging. The study project was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University (Approval No. MRCTA, ECFAH of FMU [2019]021). All patients provided written consent for the use of human tissues in this study. No patients received preoperative radiotherapy or chemotherapy.

Data acquisition

The human microarray dataset was downloaded from the Gene Expression Omnibus (GEO) comprehensive database of the National Biotechnology Information Center. Among all datasets, the GSE39582 and GSE17538 datasets, which included OS, disease-free survival (DFS), or disease-specific survival (DSS) data, were used for analysis of the prognostic value of ITGB5. The GSE39582 microarray dataset contains CRC tissues from 566 patients and normal mucosal tissues from 19 patients, of whom 562 had OS data, 557 had DFS data, and 532 had complete clinical data (including age, sex, depth of invasion, TNM stage, distant metastasis, lymphatic metastasis, and tumor location). In the GSE17538 dataset, 177 patients had OS data, and 145 patients had DFS data. The GSE39582, GSE23878, GSE25070 and GSE44076 datasets were analyzed to investigate the differential expression of ITGB5.

Bioinformatics analysis

R software (version 3.6.3) was used for bioinformatics analysis and data visualization. The GEO dataset was separately normalized using R software, and the differences in the ITGB5 expression levels between normal and CRC tissues in the GSE39582, GSE23878, GSE25070, and GSE44076 datasets were evaluated using the Wilcoxon test. The median of each dataset was selected to distinguish between groups with high and low ITGB5 expression of the patients in this dataset. The Kaplan–Meier method was used to evaluate the prognostic value of ITGB5 in the GSE39582 and GSE17538 datasets. Additionally, a meta-analysis was performed to further evaluate the prognostic value of ITGB5 in the GSE39582 and GSE17538 datasets and its predictive ability using the receiver operating characteristic (ROC) curve method.

Differentially expressed genes (DEGs) ($|\log FC| > 0.5$, $p < 0.05$) were separately extracted from the GSE39582 and GSE17538 datasets. ClusterProfiler: an R package for comparing biological themes among gene clusters.

We also studied the correlations between ITGB5 expression and the enrichment scores of some known biological processes, which can be evaluated according to biological gene signatures[21]. These known biological processes were used in several articles and have proven their reliability[22,23]. Again, we collected these signatures of known biological processes, as shown in Table S1. The known biological processes included angiogenesis; $CD8^+$ T effector; antigen processing machinery; DNA damage repair (gene expression profiling, G); DNA damage repair (mutational profiling, M); cell cycle; DNA replication; epithelial–mesenchymal transition (EMT1-3) signatures; FGFR3-related genes; Fanconi anemia; homologous recombination; immune checkpoint; mismatch repair; KEGG-discovered histones; panfibroblast TGFb response (pan-F-TBRS) signature; nucleotide excision repair; and WNT targets. The specific associations between the enrichment scores of each known biological pathway and ITGB5 in the GSE39582 and GSE17538 datasets were studied in detail.

The TGF-β/EMT pathway plays an important role in tumorigenesis, tumor development and metastasis, especially in cytokine and chemokine secretion. The gene signatures of the growth factor TGF-β/EMT pathway,

including TWIST1, SMAD9, TGRB1, ACTA2, CLDN3, TGFBR2, VIM, ZEB1-AS1 and COL4A1, were collected from the published literature[24,25]. The transcript levels of genes related to the TGF-β/EMT pathway signature were extracted from the CRC patient data in the GSE39582 and GSE17538 datasets (Table S2 and Table S3). The data for the transcripts of the TGF-β/EMT pathway-related genes were extracted, and the relationships among the ITGB5 genes were investigated using the "ggpubr" package.

Tumor-infiltrating immune cell (TIIC) assessment

The CIBERSORT algorithm and single-sample gene set enrichment analysis (ssGSEA) algorithm were used to quantify the infiltrating fractions of 22 immune cell types, and TIIC scores were calculated for the GSE39582 and GSE17538 samples using the "CIBERSORT" and "ssGSEA" R scripts, respectively. $P < 0.05$ was considered to indicate a statistically significant difference. Differential expression analysis of TIICs between samples with high ITGB5 expression and those with low ITGB5 expression was performed with the Wilcoxon test, and Spearman correlation analysis was used to determine the correlation between the ITGB5 gene and TIICs.

Cell culture and cell transfection

Human CRC SW480 and RKO cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SW480 cells were grown in RPMI 1640 medium (Gibco, Carlsbad, CA, United States), and RKO cells were grown in MEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. ITGB5 small interfering RNAs (siRNAs) and negative controls (NCs) were purchased from Sangon Biotech (Shanghai, China), and the target sequences were as follows: si-ITGB5-1, GCATCCAACCAGATGGACTAT; si-ITGB5-2, GCTCGCAGGTCTCAACATAT T. siRNA transfection was performed with Engreen TM-R4000 (Engreen Biosystem, Beijing, China) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted from CRC tissues, adjacent normal tissues or cell lines transfected with siRNAs for 36–48 h using a TransZol Up Plus RNA kit (TransGen Biotech, Beijing, China). The following sequences of primers were used: ITGB5, F-GTCTGCTAATCCACCCAAAATG and R-TCTCTATCTCACCTCCACAGC; and GAPDH, F-ACAACTTTGGTATCGTGGAAGG and R-GCCATCACGCCACAGTTTC. ITGB5 mRNA expression was analyzed using RT-qPCR. All assays were performed in triplicate.

Western blot (WB) analysis

Radioimmunoprecipitation assay (RIPA; Beyotime, Haimen, China) buffer mixed with phenylmethanesulfonyl fluoride (PMSF; Beyotime) (RIPA: PMSF = 100:1) was added to adjacent normal mucosa or CRC tissues or to each group of cells (transfected with siRNAs for 48–72 h) for protein extraction. Approximately 60 μg of total protein was loaded onto a 10% SDS-PAGE gel (Beyotime) and transferred onto a PVDF membrane. After blocking with 5% skim milk for 2 h, the cells were incubated with anti-GAPDH (1:1000; Sigma, St. Louis, MO, USA), anti-ITGB5 (1:1000; Boster, China), β-catenin, E-cadherin, N-cadherin, MMP-9, c-MYC (1:1000; ABclonal, China) and vimentin (1:1000; Proteintech, China) antibodies overnight at 4 °C. Then, the membranes were incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies for 1.5 h. The membranes were visualized using enhanced chemiluminescence (ECL) reagent (Beyotime).

Tissue immunohistochemical staining

Immunohistochemistry was performed on 4-μm thick paraffin-embedded sections of adjacent normal mucosal tissues and CRC tissue. The slides were incubated overnight at 4 °C with an anti-ITGB5 antibody (1:100 dilution, Boster, China). The slides were washed with PBS and then mounted with antifade reagent (Invitrogen, Carlsbad, CA, United States). Finally, the stained slides were observed using an Olympus CX41 fluorescence microscope (Olympus, Tokyo, Japan). ITGB5 expression levels were independently evaluated by three observers. Protein expression was assessed using the proportion of positive cells to total cells (on a scale of 0–3:0, no positive tumor cells; 1, < 10% positive tumor cells; 2, 10-50% positive tumor cells; and 3, > 50% positive tumor cells) and staining intensity (on a scale of 0–3:0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining). Specimens with a total of > 3 points were recorded as having high ITGB5 expression; otherwise, specimens were recorded as having low ITGB5 expression.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) was used to determine the proliferative capacity of SW480 and RKO cells. A total of 1×10^3 cells/well were seeded into 96-well plates and incubated for 0, 24, 48, or 72 h. Before measurement on a microplate reader at 450 nm, the medium was replaced with fresh medium containing 10% FBS and 10 μl of CCK-8 reagent, and the cells were then cultured for 2 h. The black wells that were measured as background signals contained only culture medium and CCK-8 reagent.

Transwell migration and invasion assays

The migration and invasion abilities of SW480 and RKO cells were measured using the Transwell method. For the invasion assay, SW480 cells (1.2×10^5 cells/well) and RKO cells (1.5×10^5 cells/well) were suspended in 250 μl of serum-free culture medium and seeded into the upper chamber coated with Matrigel (100 μl) (Becton Dickinson, Franklin Lake, NJ, United States), and 750 μl of medium containing FBS (10% FBS in SW480 cells and 20% FBS in RKO cells) was added to the lower chamber. For the migration assay, SW480 cells (0.8×10^5 cells/well) and RKO cells (1×10^5 cells/well) were placed into the upper chamber without Matrigel, and the medium in the lower chamber was the same as that used for the invasion assay. Following incubation for 48 h at 37 °C and

5% CO_2, the cells were fixed with methanol for 15 min and stained with crystal violet for 30 min. Images were obtained by using an Olympus CX41 microscope (Nikon, Tokyo, Japan), and the number of stained cells was assessed by manual counting.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. The data are expressed as the means ± SDs, and the quantitative data were analyzed with Student's *t* test. ITGB5 expression in the GSE39582 cohort and the immunohistochemistry results were tested using the χ^2 test of independent samples. Univariate and multivariate Cox analyses were used to evaluate the independent prognostic value of ITGB5. $P<0.05$ was considered significant.

Results

ITGB5 expression levels across cancers

ITGB5 expression levels were tested in more than 30 tumor types using the TIMER2.0 database (http://timer.cistrome.org/). ITGB5 expression levels in 14 types of tumor tissues were significantly different from those in the corresponding adjacent normal tissues (Fig. 1A). ITGB5 gene expression was greater in thyroid cancer, gastric adenocarcinoma, small cell carcinoma, lung adenocarcinoma, hepatocellular carcinoma, head and neck squamous cell carcinoma, esophageal carcinoma, colon adenocarcinoma, glioblastoma, cholangiocarcinoma and

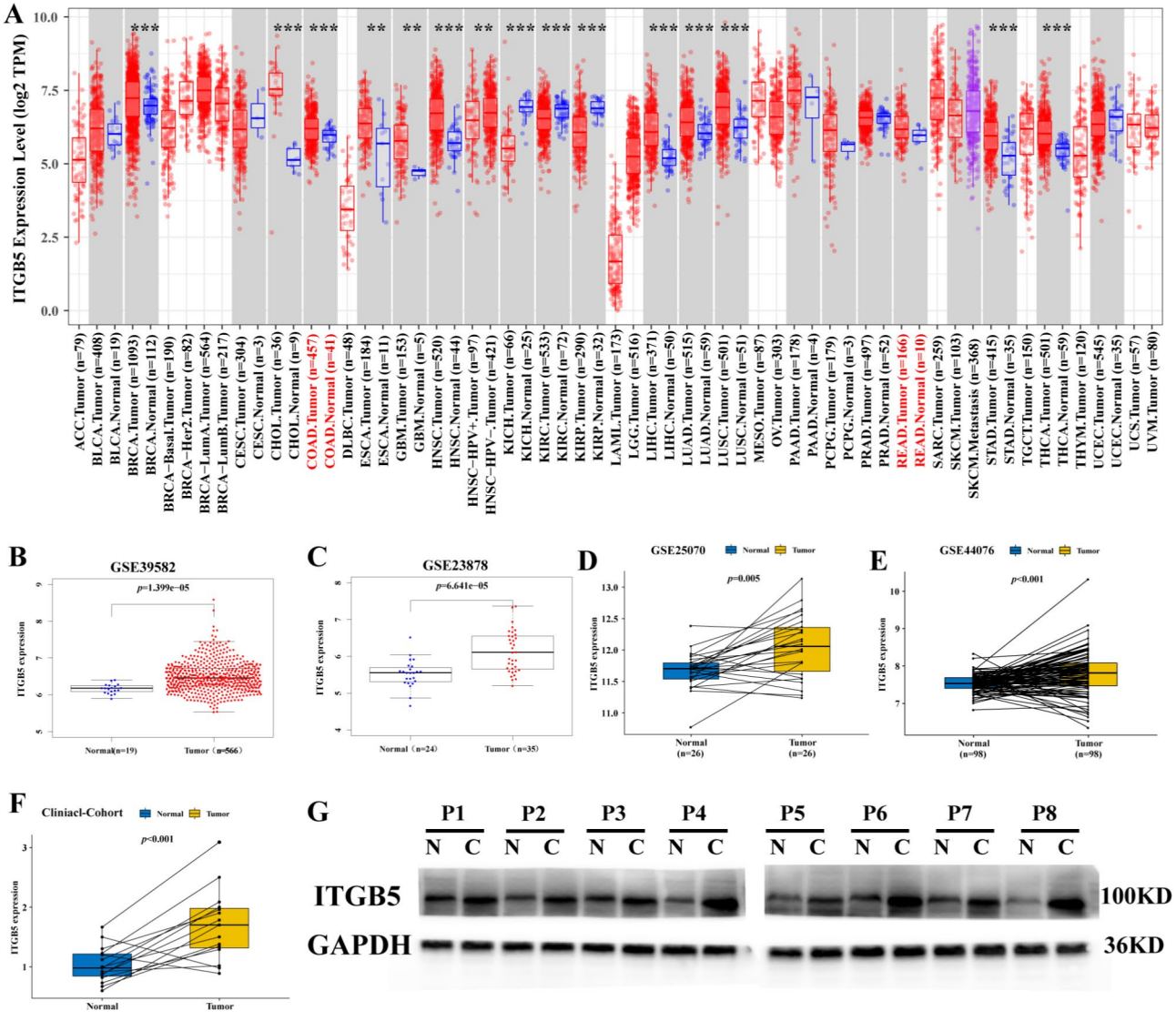


Fig. 1. ITGB5 expression in cancers. (**A**) Analysis of ITGB5 expression across cancers. (**B**–**E**) Two paired datasets (GSE39582 and GSE23878) and two unpaired datasets (GSE25070 and GSE44076) from the NCBI GEO database showed that ITGB5 was highly expressed in CRC. (**F**) PCR analysis of 15 pairs of CRC and normal tissues in the clinical cohort. (**G**) Western blot analysis of 15 pairs of CRC tissues and normal tissues also indicated that ITGB5 mRNA and protein levels were high in CRC tissues (*$P<0.05$; **$P<0.01$).

invasive breast carcinoma and lower in three different subtypes of kidney cancer (Fig. 1A). This result suggested that ITGB5 may play an important role in cancer development and progression.

ITGB5 expression is increased in CRC

According to two paired datasets (GSE39582 and GSE23878) and two unpaired datasets (GSE25070 and GSE44076) downloaded from the GEO database, ITGB5 expression was significantly greater in CRC tissues than in normal colorectal mucosal tissues (Fig. 1B–E). We assessed ITGB5 mRNA expression in 15 fresh pairs of CRC and normal adjacent tissues using qRT-PCR and obtained similar results (Fig. 1F). Next, we found that ITGB5 protein levels were elevated in CRC tissues, as assessed in 15 pairs of CRC and normal adjacent tissues by Western blotting (Fig. 1G), and the ITGB5 protein levels in the cancer tissues were markedly greater than those in the normal adjacent tissues. Additionally, immunohistochemistry was used to analyze 15 normal mucosal tissues and 55 CRC tissues, among which CRC tissues presented increased ITGB5 protein expression (Fig. 2A, B).

ITGB5 expression is associated with clinicopathological characteristics

High ITGB5 expression was associated with advanced TNM stage ($p = 0.045$), advanced invasion ($p = 0.027$) and distant metastasis ($p = 0.01$) according to the chi-square test of the GSE39582 dataset (Table S4). Similar results were obtained in the GSE17538 dataset, and increased ITGB5 expression correlated with TNM stage ($p = 0.006$) (Table S5). Moreover, the Wilcoxon rank-sum test revealed that increased ITGB5 expression was correlated with stage III/IV ($p = 0.025$), T3-4 ($p = 0.022$), the presence of lymph node metastasis ($p = 0.036$) and the presence of distant metastasis ($p = 0.003$) (compared to stage I/II, T1-2, the absence of lymph node metastasis and distant metastasis) in the GSE39582 (Fig. 2C, E–G) and GSE17538 (stages III/IV compared with stages I/II, $p = 0.038$) datasets (Fig. 2D). Furthermore, immunohistochemistry revealed that ITGB5 was highly expressed in patients with advanced TNM stage ($p = 0.049$), advanced invasion depth ($p = 0.024$), lymph node metastasis ($p = 0.049$) and distant metastasis ($p = 0.043$) (Table S6) in the clinical CRC cohort.

High ITGB5 expression is correlated with poor prognosis

Kaplan-Meier survival analysis revealed that high ITGB5 expression was correlated with poor OS in the GSE39582 ($p = 0.014$) and GSE17538 ($p = 0.024$) datasets (Fig. 3A, C), poor DFS in the GSE39582 ($p < 0.001$) and GSE17538 ($p = 0.002$) datasets (Fig. 3B, D), and poor DSS in the GSE17538 ($p = 0.007$) dataset (Fig. 3E). Meta-analysis of the GSE39582 and GSE17538 datasets revealed that high ITGB5 expression was a high-risk factor for OS ($p = 0.0002$) (Fig. 3F) and DFS ($p < 0.0001$) (Fig. 3G).

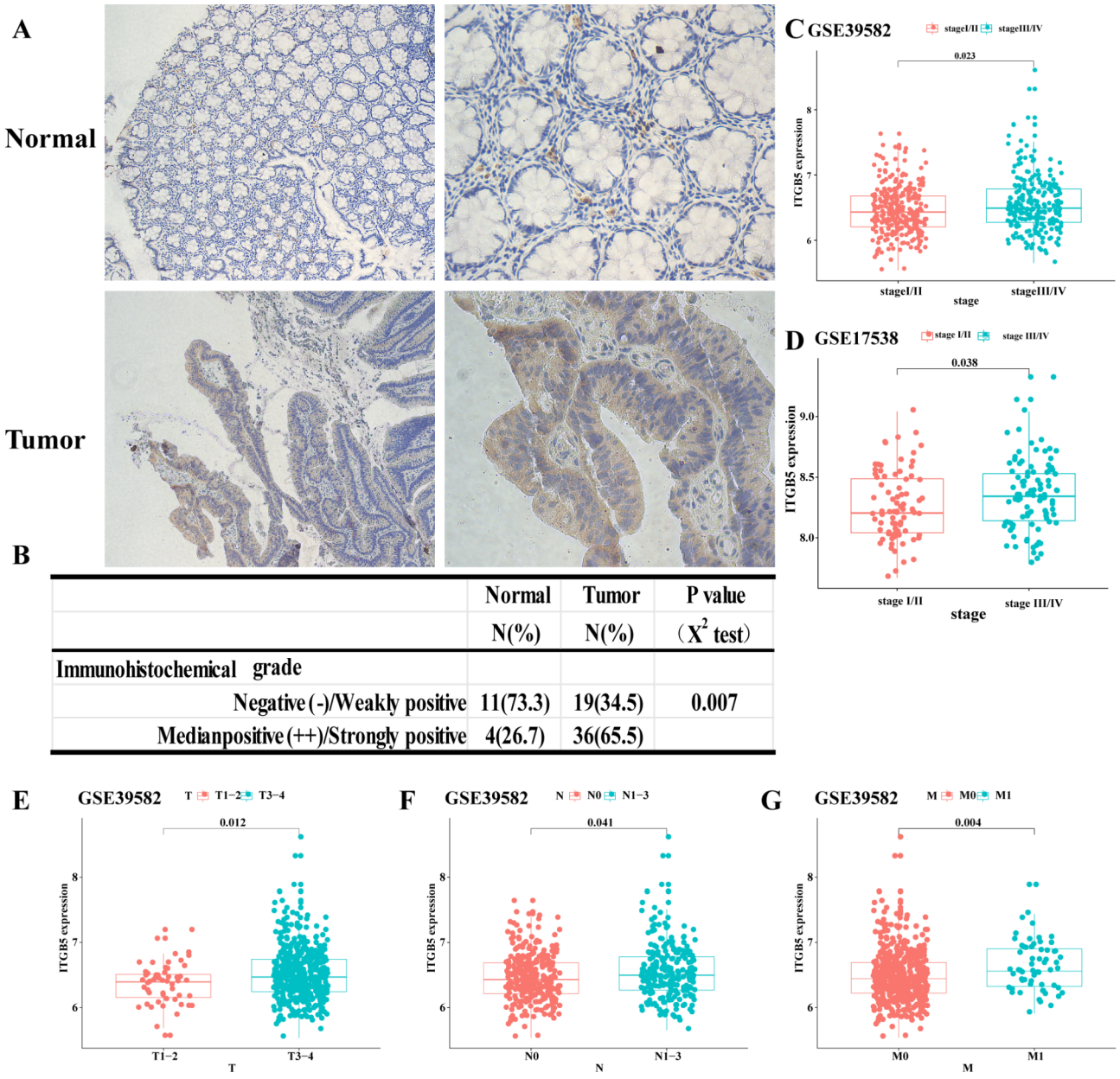
Univariate Cox analysis of the GSE39582 dataset revealed that stage III/IV, lymph node metastasis, distant metastasis and ITGB5 expression correlated with poor OS (Table 1) and DFS (Table 2). Moreover, T3-4 was associated with poor DFS. In addition, the presence of distant metastasis and ITGB5 expression served as independent predictors of OS and DFS (Tables 1 and 2), as revealed by multivariate Cox analysis of the GSE39582 dataset. Interestingly, we also found that ITGB5 expression and TNM stage served as independent predictors of DFS according to the independent univariate analysis and multivariate analysis of the GSE17538 dataset (Table 3).

According to the ROC curve analysis of the ability of ITGB5 expression to predict DFS, the area under the curve (AUC) of the GSE39582 dataset was 0.627 at 3 years, 0.613 at 5 years and 0.612 at 8 years (Fig. 3H). In addition, the AUC was 0.605 at 3 years, 0.673 at 5 years and 0.777 at 8 years in the GSE17538 dataset (Fig. 3I). ROC curve analysis also indicated that the ITGB5 gene had a greater ability to predict DFS than did the TNM stage, T stage, or N stage (Fig. 3J, K). Furthermore, according to the multivariate models in the GSE39582 dataset, nomograms were generated to predict 3-, 5-, and 8-year DFS in CRC patients (Fig. 4).

Analysis of the biological functions and signaling pathways of the ITGB5 gene

To investigate genes that interact with ITGB5, a protein-protein interaction (PPI) network was generated using the STRING database (https://string-db.org/). The network contained several genes that encode α subunits of integrins, such as ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA8, ITGA10, ITGA11 and ITGAV (Fig. 5A). We extracted two groups of DEGs ($|\log \text{fold change}| > 0.5$, $p < 0.05$) from the comparison between the ITGB5 high- and low-expression groups. In GSE39582, the sets contained 307 DEGs; 255 were upregulated, and 52 were downregulated (Table S7). The GSE17538 dataset contained 206 DEGs; 178 were upregulated, and 28 were downregulated (Fig. 5B) (Table S7). Then, Venn diagrams were generated with 165 common DEGs between the two sets; 146 genes were commonly upregulated, and 19 genes were commonly downregulated (Fig. 5B). GO analysis (p value < 0.05 and q value < 0.05) of the common DEGs revealed that the top 3 enriched biological process (BP) terms were ECM organization, extracellular structure organization, and collagen fibril organization. The top 3 terms enriched in the cellular component (CC) were collagen-containing ECM, collagen trimer, and extracellular matrix component. The top 3 enriched molecular function (MF) terms were ECM structural constituent, glycosaminoglycan binding, and ECM structural constituent conferring tensile strength (Fig. 5C). Additionally, KEGG pathway enrichment analysis (p value < 0.05) revealed that the top 5 enriched pathways were protein digestion and absorption, focal adhesion, the PI3K-Akt signaling pathway, human papillomavirus infection, and ECM-receptor interaction (Fig. 5D). Furthermore, GSEA revealed that focal adhesion, cancer pathways, ECM-receptor interactions and Wnt signaling pathways were enriched in in the samples with high ITGB5 expression in the GSE39582 and GSE17538 datasets (Fig. 5E, F).

We examined the relationships between different levels of ITGB5 expression and known biological processes using Spearman correlation analysis. A heatmap of the correlation matrix (Fig. 6A, B) demonstrated that ITGB5 expression was strongly positively correlated with the WNT target, angiogenesis, EMT1-3 and Pan-F-TBRS signatures but strongly negatively correlated with the cell cycle, DNA damage repair, DNA replication,



B

	Normal	Tumor	P value
	N(%)	N(%)	(X^2 test)
Immunohistochemical grade			
Negative (-)/Weakly positive	11(73.3)	19(34.5)	0.007
Medianpositive (++)/Strongly positive	4(26.7)	36(65.5)	

Fig. 2. Association between ITGB5 expression and clinicopathologic features. (**A**,**B**) Immunohistochemical analysis of ITGB5 protein expression indicated that the ITGB5 gene was highly expressed in CRC. (**C**,**D**) Analysis of ITGB5 expression in the GSE39582 dataset and GSE17538 dataset using the Wilcoxon test revealed that ITGB5 was more highly expressed in patients with stage III/IV disease than in patients with stage I/II disease. (**E**–**G**) ITGB5 levels in the T3-4, N1-3 and M1 samples were greater than that in the T1-2, N0 and M0 samples, as determined using the Wilcoxon test.

homologous recombination, nucleotide excision repair and mismatch repair pathways in both the GSE39582 and GSE17538 datasets. ITGB5 may play a role in promoting the occurrence and development of colorectal cancer through WNT targets, angiogenesis, EMT1-3 and other mechanisms. More importantly, correlations between ITGB5 expression and the TGF-β/EMT pathway-related gene signature were detected in the GSE39582 and GSE17538 datasets (Fig. 6C, D). As expected, the expression of most TGF-β/EMT pathway-related genes was markedly greater in the high-ITGB5 expression group than in the low-ITGB5 expression group. All of these results strongly indicate that high ITGB5 expression is significantly correlated with the TGF-β/EMT pathway and WNT targets.

Verification of the role of ITGB5 overexpression in CRC

To further analyze the function of ITGB5, we detected ITGB5 expression levels in 8 colorectal cancer cell lines by qPCR and WB respectively. We found that SW480 had the highest ITGB5 expression level, while RKO had

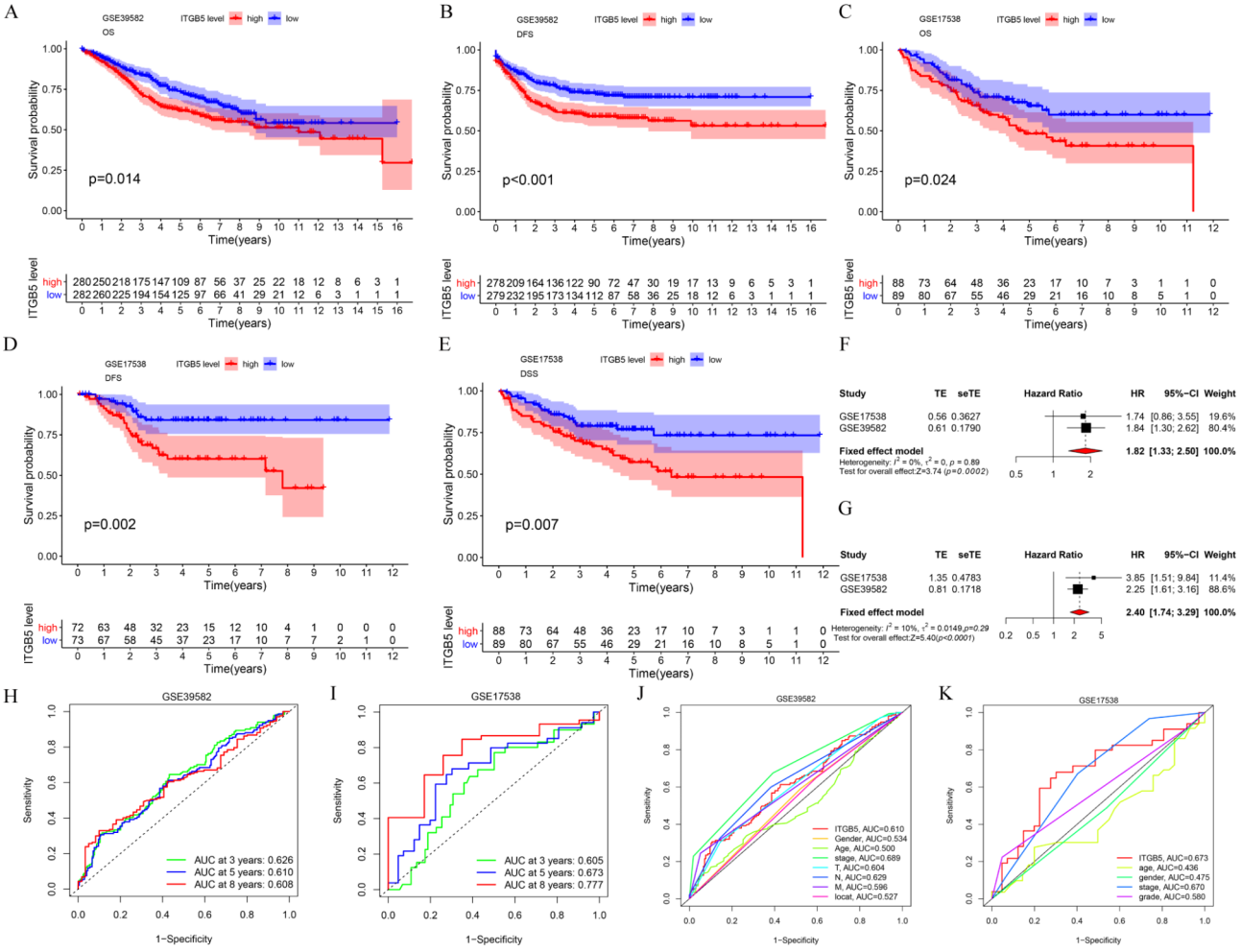


Fig. 3. High ITGB5 expression is correlated with poor prognosis. High ITGB5 expression was associated with poor OS (**A**,**C**), poor DFS (**B**,**D**) and poor DSS (**E**). High ITGB5 expression is a high risk factor for OS (**F**) and DFS (**G**), as revealed by meta-analysis of the GSE39582 and GSE17538 datasets. (**H**,**I**) ITGB5 expression can predict DFS. (**J**,**K**) The ITGB5 gene has greater predictive ability for DFS than the TNM stage, T stage, or N stage.

Clinical characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex (male vs. female)	1.276 (0.941–1.729)	0.116	–	–
Age (≥60 vs. <60)	1.387 (0.967–1.989)	0.076	–	–
Stage (III/IV vs. I/II)	1.861 (1.372–2.522)	< 0.001	1.455 (0.624–3.395)	0.385
T (T3–4 vs. T1–2)	1.906 (0.974–3.729)	0.06	–	–
N (N1–3 vs. N0)	1.607 (1.189–2.172)	0.002	0.874 (0.402–1.901)	0.734
M (M1 vs. M0)	5.212 (3.617–7.511)	< 0.001	4.156 (2.683–6.439)	< 0.001
Location (proximal vs. distal)	0.915 (0.673–1.245)	0.573	–	–
ITGB5 expression (continuous)	1.842 (1.27–2.672)	0.001	1.538 (1.046–2.262)	0.029

Table 1. Univariate and multivariate analyses of overall survival (OS) in the GSE39582 dataset.

the lowest ITGB5 expression level (Fig. S1A, B). Therefore, SW480 and RKO were selected for follow-up study. We downregulated ITGB5 in SW480 and RKO cells using ITGB5-1 (si-ITGB5-1) and ITGB5-2 (si-ITGB5-2) siRNAs, and the knockdown effect was tested by qRT-PCR (Fig. 7A) and WB (Fig. 7B). All the results indicated that si-ITGB5-1 and si-ITGB5-2 significantly reduced the expression of ITGB5 compared with that in the NC group ($p < 0.001$). Moreover, there was no significant difference in the downregulation effect of the two siRNAs, so si-ITGB5-2 was selected for subsequent WB validation.

Clinical characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex (male vs. female)	1.217 (0.88–1.682)	0.235	–	–
Age (≥60 vs. <60)	0.851 (0.602–1.204)	0.363	–	–
Stage (III/IV vs. I/II)	2.978 (2.116–4.191)	<0.001	10.453 (4.557–23.975)	<0.001
T (T3–4 vs. T1–2)	4.34 (1.607–11.717)	0.004	3.748 (1.377–10.203)	0.01
N (N1–3 vs. N0)	2.216 (1.598–3.072)	<0.001	0.198 (0.094–0.416)	<0.001
M (M1 vs. M0)	6.263 (4.255–9.219)	<0.001	3.087 (1.938–4.915)	<0.001
Location (proximal vs. distal)	1.247 (0.891–1.745)	0.197	–	–
ITGB5 expression (continuous)	2.239 (1.553–3.228)	<0.001	1.721 (1.176–2.52)	0.005

Table 2. Univariate and multivariate analyses of disease-free survival (DFS) in the GSE39582 dataset.

Clinical characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex (male vs. female)	1.276 (0.941–1.729)	0.116	–	–
Age (≥60 vs. <60)	1 (0.52–1.925)	0.999	–	–
Stage (III/IV vs. I/II)	2.828 (1.414–5.657)	0.003	2.473 (1.223–5.003)	0.012
Grade (well vs. moderate/poor)	1.384 (0.423–4.526)	0.591	–	–
ITGB5 expression (continuous)	3.852 (1.508–9.838)	0.005	3.029 (1.168–5.003)	0.023

Table 3. Univariate and multivariate analyses of disease-free survival (DFS) in the GSE17538 dataset.

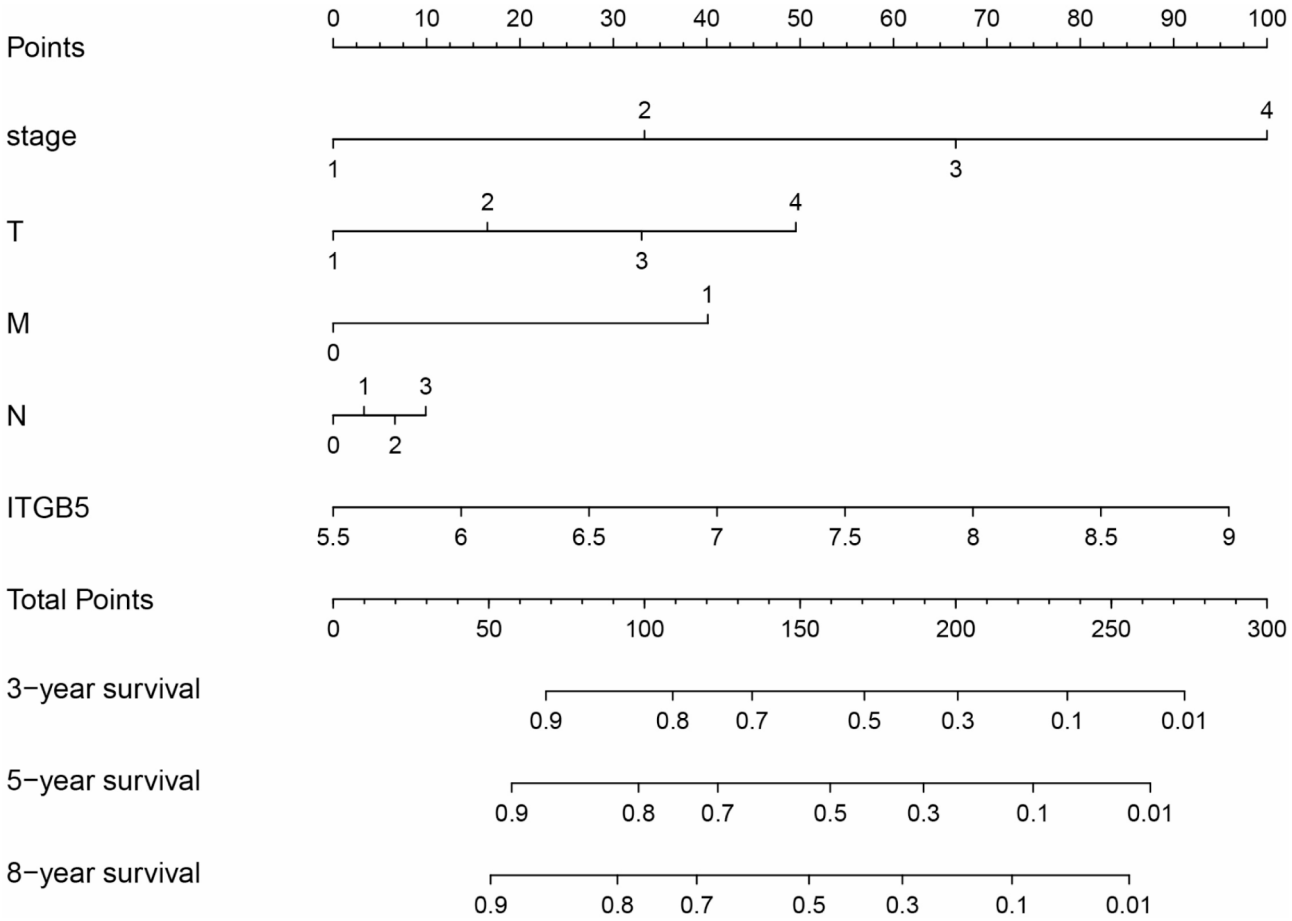


Fig. 4. Nomograms for predicting 3-year, 5-year and 8-year DFS.

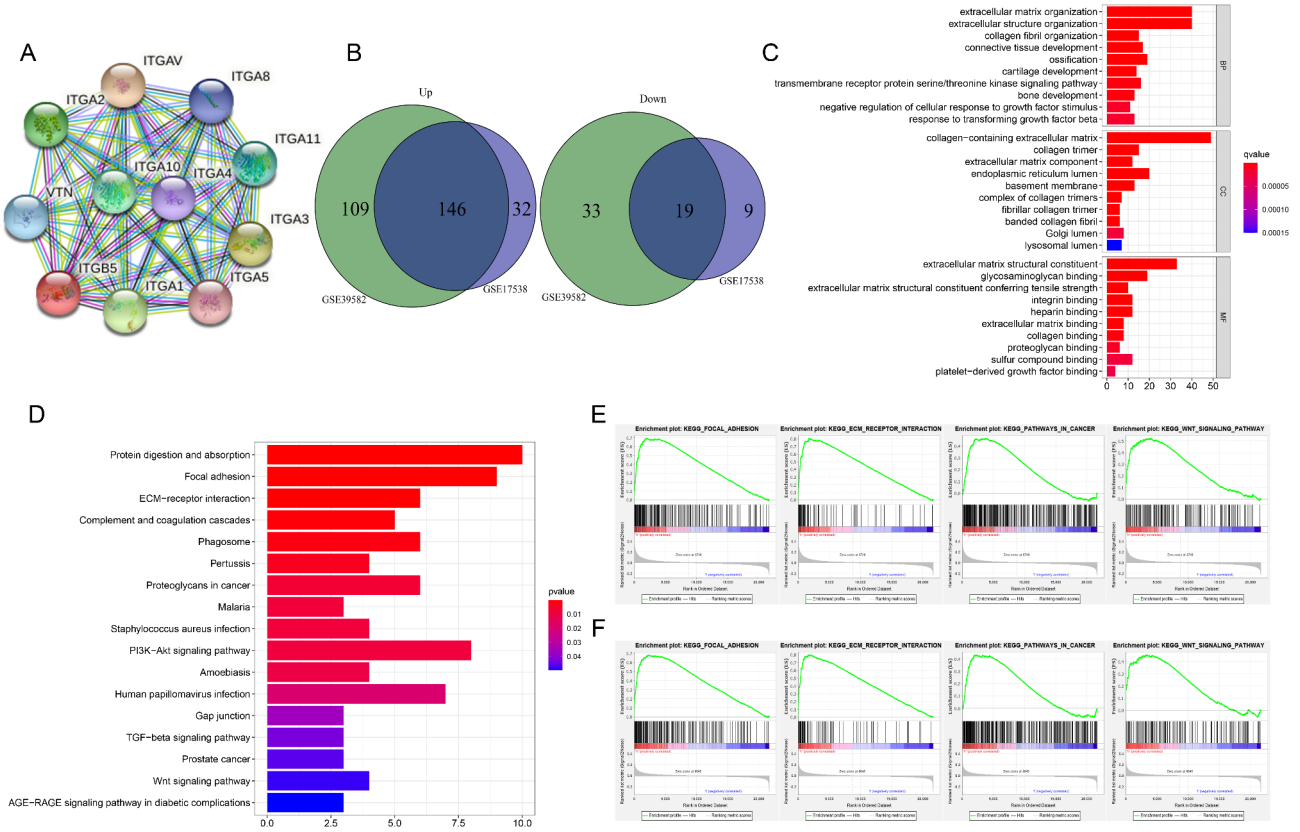


Fig. 5. Molecular functions of the ITGB5 gene. (**A**) PPI network of the ITGB5-related genes. (**B**) Common differentially expressed genes (DEGs) extracted from the comparison of the high vs. low ITGB5 expression groups in the GSE39582 and GSE17538 datasets. (**C**) GO analysis of the common DEGs. (**D**) KEGG pathway analysis of the common DEGs. (**E**,**F**) GSEA was performed on the ITGB5 gene in the GSE39582 and GSE17538 datasets.

The CCK-8 cell proliferation assay showed that compared with that in the NC group, the percentage of proliferating SW480 cells in the si-ITGB5-1 and si-ITGB5-2 groups was significantly greater at 48 h and 72 h, but there was no significant difference between the si-ITGB5-1 and si-ITGB5-2 groups. The same trend was also confirmed in the proliferation experiment with RKO cells (Fig. 7C). In brief, downregulating ITGB5 expression slowed the growth of SW480 and RKO cells.

Transwell cell invasion assays revealed that compared with that in the NC group, the number of SW480 cells in the si-ITGB5-1 and si-ITGB5-2 groups was significantly lower, but no significant difference was noted between the si-ITGB5-1 and si-ITGB5-2 groups. The same trend was confirmed in the RKO cell invasion experiment (Fig. 7D). Transwell cell migration assays revealed that compared with that in the NC group, the number of migrated SW480 cells in the si-ITGB5-1 and si-ITGB5-2 groups was also significantly lower, but no significant difference was noted between the si-ITGB5-1 and si-ITGB5-2 groups. The same trend was also confirmed in the RKO cell migration experiment (Fig. 7E). Overall, these results indicated that the downregulation of ITGB5 decreased the invasion and migration capacities of SW480 and RKO cells.

Exploration and verification of the signaling pathways associated with ITGB5 overexpression in CRC

As mentioned above, GSEA of the GSE39582 and GSE17538 datasets revealed that the Wnt/β-catenin signaling pathway was enriched in samples with high ITGB5 expression, and the results of the analysis of the relationship between ITGB5 and known biological processes indicated that high ITGB5 expression is significantly correlated with the TGF-β/EMT pathway and WNT targets. WB experiments indicated that the downregulation of ITGB5 with si-ITGB5 blocked the Wnt/β-catenin signaling pathway and downregulated β-catenin, and the expression of Cyclin D1 and c-Myc, which are downstream target genes of the Wnt/β-catenin signaling pathway, was downregulated in both SW480 and RKO cells (Fig. 7F, G). Downregulation of ITGB5 also decreased the occurrence of EMT in tumor cells, as indicated by decreased expression of MMP-9, Vimentin and N-cadherin but increased expression of E-cadherin in both SW480 and RKO cells (Fig. 7F, G).

Analysis of the relationship between ITGB5 and the abundance of infiltrating immune cells

GO analysis also revealed enrichment in relevant immune terms, such as negative regulation of immune system process, humoral immune response, and regulation of inflammatory response (Table S8). According

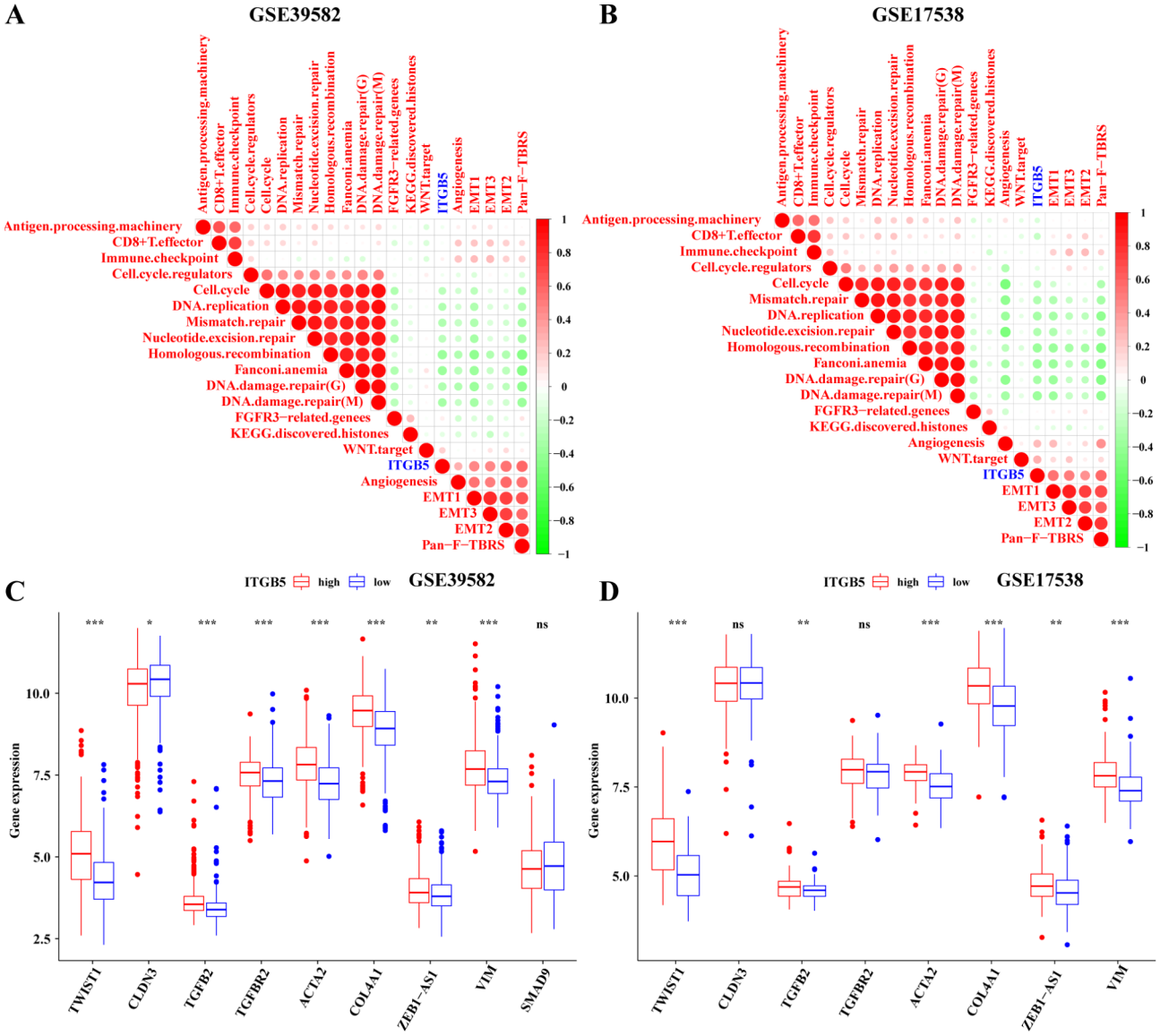


Fig. 6. Relationship between ITGB5 and known biological processes. (**A**) Correlation analysis between the expression of ITGB5 and known biological processes in the GSE39582 and (**B**) GSE17538 datasets. (**C**) Correlation analysis between the expression of ITGB5 and the expression of genes related to the TGF-β/EMT pathway in the GSE39582 and (D) GSE17538 datasets (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

to the TIMER database, the expression level of ITGB5 was positively correlated with the infiltration of M0 macrophages, M2 macrophages and neutrophils and negatively correlated with the plasma cell fraction in CRC (colon adenocarcinoma and rectal adenocarcinoma) (Fig. S1C). Differential analysis using the CIBERSORT algorithm revealed that high ITGB5 expression in the GSE39582 and GSE17538 datasets was associated with high proportions of infiltrating M0 macrophages, M2 macrophages and neutrophils and a lower fraction of infiltrating plasma cells (Fig. 8A, B). Differential analysis using the ssGSEA algorithm revealed that the high expression of ITGB5 in the GSE39582 and GSE17538 datasets was associated with high proportions of infiltrating macrophages, MDSCs, immature dendritic cells and natural killer T cells and a lower percentage of activated B cells and activated CD4 T cells (Fig. 8C, D). In addition, Spearman's correlation analysis revealed that ITGB5 expression was positively correlated with M0 macrophages (Fig. 8E, I), M2 macrophages (Fig. 8F, J) and neutrophils (Fig. 8G, K) but negatively correlated with the fraction of plasma cells in the two CRC datasets (Fig. 8H, L). These results suggest that ITGB5 is associated with immunosuppressive factors.

Discussion

The ITGB5 gene encodes the integrin β-subunit, which is highly expressed in many cancers, and ITGB5 has carcinogenic effects on breast cancer[17,18], hepatocellular carcinoma[14], glioblastoma[15], gastric cancer[26] and pancreatic cancer[27]. ITGB5 overexpression can be used as a prognostic biomarker for glioblastoma[14], and high ITGB5 expression has been found to predict adverse OS in CRC patients[9,16]. Our multidimensional

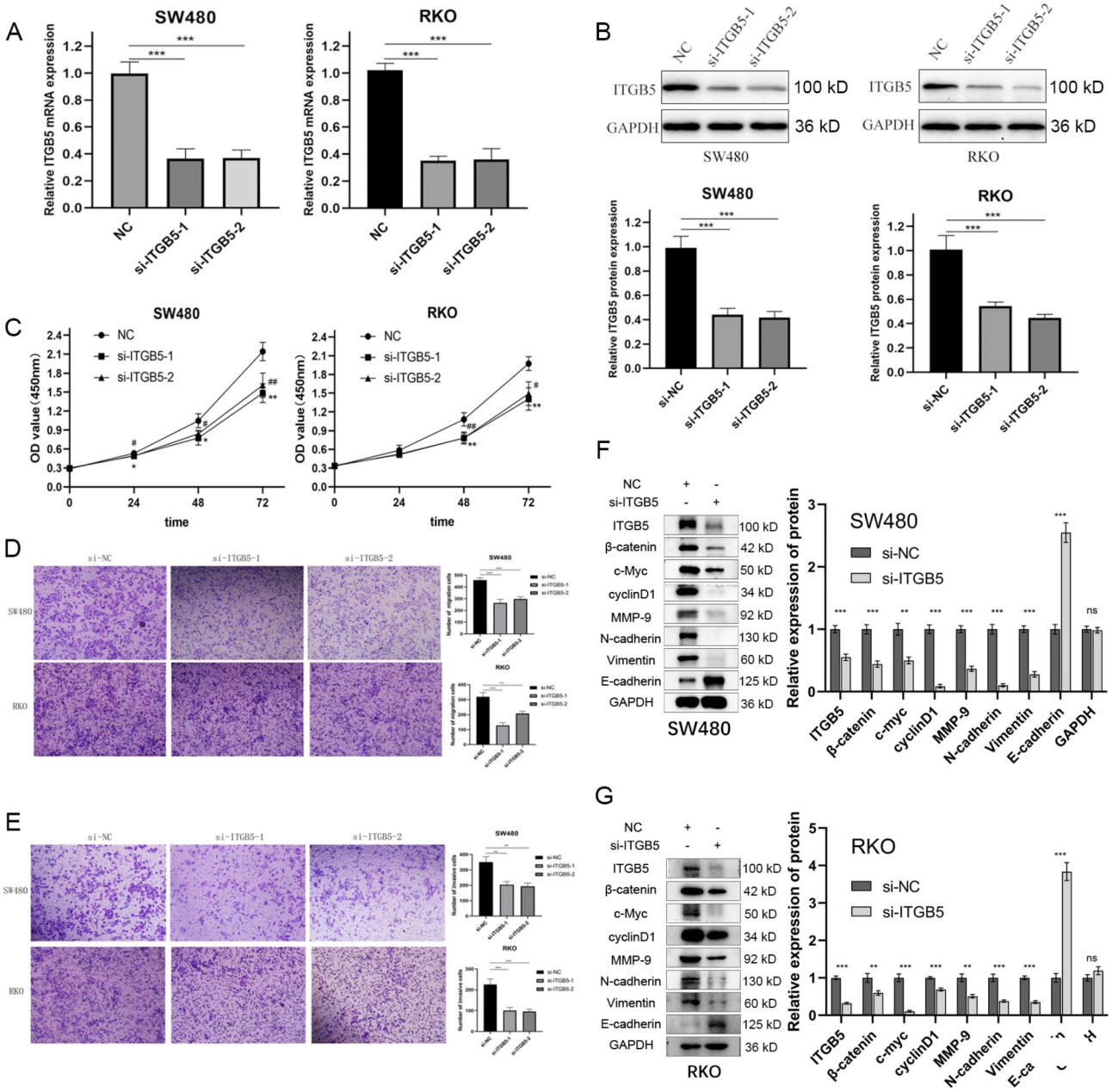


Fig. 7. ITGB5 plays an oncogenic role in CRC. (**A**) qPCR and WB (**B**) were performed to measure the efficiency of ITGB5 silencing using siRNA-1/-2 in SW480 and RKO cells. (**C**) Knockdown of the ITGB5 gene reduced the proliferation of SW480 and RKO cells, as determined by CCK-8 assays. (**D**,**E**) ITGB5 gene silencing attenuated the migration and invasion abilities of SW480 and RKO cells, as measured by Transwell assays. (**F**,**G**) ITGB5 knockdown blocked the Wnt/β -catenin signaling pathway and EMT. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; $^{\#}P<0.05$, $^{\#\#}P<0.01$.

in-depth study confirmed the prognostic value of ITGB5 in colorectal cancer but suggested that it may have more important value in DFS given that high ITGB5 expression is associated not only with poorer OS but also with poorer DSS and DFS. In addition, meta-analysis of two GEO datasets suggested that high ITGB5 levels are a high risk factor for OS and DFS. In addition, ROC curve analysis revealed that ITGB5 expression had a certain predictive value for DFS. Finally, univariate and multivariate analyses revealed that ITGB5 may be an independent prognostic factor for OS and DFS. This may be because ITGB5 overexpression in colorectal cancer tissues is associated with late TNM staging[16,28,29]. In this study, multiple datasets and clinical samples confirmed that the ITGB5 expression level was related to the TNM stage, the depth of late invasion, and the occurrence of lymph node metastasis and distant metastasis, especially distant metastasis, which are the most critical factors affecting the prognosis of CRC patients. In addition, we demonstrated that downregulation of ITGB5 in SW480 and RKO cells attenuated cell proliferation and invasion. ITGB5 can be used as a marker of

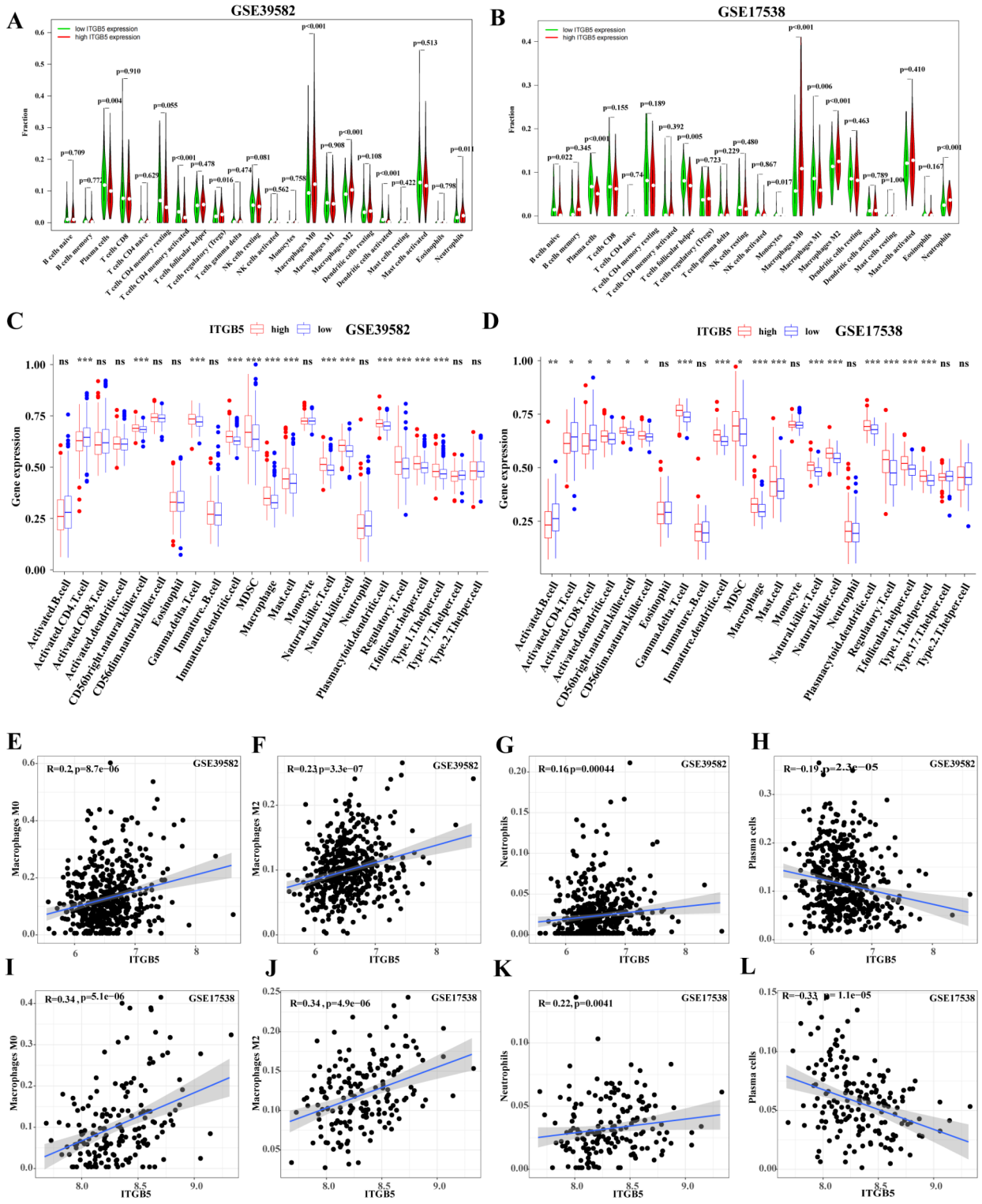


Fig. 8. Immune cell infiltration analysis. Differential analysis of the CIBERSORT algorithm (**A**,**B**) and the ssGSEA algorithm (**C**,**D**) of the fractions of 22 types of infiltrating immune cells in the high and low ITGB5 expression groups in the GSE39582 and GSE17538 datasets. (**E**–**H**) Correlation analyses of ITGB5 expression with the fractions of M0 macrophages, M2 macrophages, neutrophils and plasma cells in the GSE39582 dataset. (**I**–**L**) Correlation analyses of ITGB5 expression with the fractions of M0 macrophages, M2 macrophages, neutrophils and plasma cells in the GSE17538 dataset.

malignant progression and poor prognosis in CRC patients. Therefore, how ITGB5 plays a role in promoting the malignant progression of CRC is unclear.

Integrins are a large class of cell adhesion receptors that connect cells and the ECM and between cells[9–11]. ITGB5 encodes the β subunit of the integrin family and can interact with the α subunit to assemble heterodimeric integrin proteins[8,13,16]. We obtained DEGs by comparing the differential expression of ITGB5 in CRC datasets. GO analysis of the common DEGs in the two GEO datasets revealed enrichment in many GO terms related to the ECM, and KEGG pathway analysis revealed enrichment of focal adhesion and ECM receptor interaction pathways. In addition, GSEA of CRC datasets indicated that the focal adhesion and ECM receptor interaction pathways were enriched in samples with high ITGB5 expression, consistent with the molecular functions of integrins reported in the literature[8–11].

The primary function of integrins expressed on the cell surface is to adhere to ECMs, junctions that provide traction critical for tumor cell survival and invasion. Cell migration and invasion are also controlled by integrins by influencing the activity and localization of matrix-degrading proteases (MMPs)[30,31]. Moreover, the Wnt signaling pathway is closely related to cancer cell proliferation, dryness, apoptosis, autophagy, metabolism, inflammation and immunity, the microenvironment, drug resistance, ion channels, heterogeneity, and EMT/migration/invasion/metastasis[32,33]. The Wnt signaling pathway affects occurrence and development of CRC and has potential value as a cancer treatment target[34,35]. The Wnt signaling pathway is activated by ITGB5 and β-catenin in liver cancer[14]. We also preliminarily confirmed that ITGB5 may be closely related to the WNT signaling pathway and that Wnt/β-catenin signaling is one of the major signaling pathways that promotes the EMT process in tumor cells[36–38]. CRC is induced to develop a motional, nonpolarized mesenchymal cell phenotype with invasive ability, antiapoptotic effects and alterations in ECM component biosynthesis[39]. Finally, the biological characteristics of CRC are altered by changes in the expression level of MMPs to promote invasion and metastasis[40]. We confirmed that knocking down the ITGB5 gene can block the Wnt pathway in SW480 and RKO cells. Moreover, the expression of key EMT proteins decreased, the expression of EMT phenotype proteins decreased, and the level of the MMP-9 protein decreased, ultimately inhibiting the proliferation, invasion and migration of CRC cells. Therefore, ITGB5 may promote tumor growth and metastasis by upregulating EMT processes and Wnt signaling pathways.

The mechanism of action of ITGB5 in CRC may be multifaceted, and many studies have confirmed that integrins on cancer cells and other cell types in the tumor microenvironment play important roles in controlling intracellular activities and intercellular communication[41,42]. Moreover, cancer cells regulate the tumor microenvironment through interactions between integrins and the ECM and regulate the infiltration of different types of cells, including immune cells, into the microenvironment. Different types of immune infiltration and their interactions are thought to play important roles in cancer development and metastasis[43,44]. It has been reported that M2-TAMs and TAMs promote invasion and angiogenesis in CRC and may be potential therapeutic targets[45–47]. Our results also showed enrichment in the humoral immune response, regulation of the inflammatory response, negative regulation of the immune system and other immune-related GO terms. According to the TIMER database, ITGB5 expression levels were positively correlated with the fractions of infiltrating M0 macrophages, M2 macrophages and neutrophils and negatively correlated with the fraction of infiltrating plasma cells in CRC[48]. Similar results were also obtained using the CRC datasets. Additionally, the high-ITGB5 expression group had greater proportions of immune-infiltrating M0 macrophages, M2 macrophages and neutrophils but a lower percentage of immune-infiltrating plasma cells in the two CRC datasets. ITGB5 may promote CRC progression and metastasis by affecting tumor-associated macrophages and neutrophils. However, the mechanism by which ITGB5 affects the infiltration of immune cells in the tumor microenvironment remains to be further studied and explored.

However, there are still some limitations to our study. First, the mechanism by which ITGB5 promotes CRC invasion, migration and metastasis by activating the Wnt/β-catenin signaling pathway needs more in-depth research and exploration. Second, the specific mechanism by which ITGB5 is involved in immunosuppressive regulation needs to be further studied. Third, whether ITGB5 can be used to assess tumor immune cell infiltration and predict effective immunotherapy strategies needs to be validated in real-world data from CRC patients treated with immune checkpoint inhibitors.

In conclusion, we studied the role of ITGB5 in CRC by performing bioinformatics analysis, clinical data and basic experiments. ITGB5, a risk factor for OS and DSS and an independent high-risk factor for DFS, was found to be highly expressed in CRC. In the CRC microenvironment, the ITGB5 gene is associated with tumor-associated macrophages and neutrophils. Through the Wnt signaling pathway, ITGB5 may have a significant effect on promoting cancer. We believe that ITGB5 is a prognostic biomarker and a potential therapeutic target in CRC and is worthy of further exploration.

Data availability

The datasets generated and analyzed during the current study are available in the [the [GEO] repository [www.ncbi.nlm.nih.gov/geo/]. The other data used to support the findings of this study are included within the supplementary information files.

Received: 15 April 2024; Accepted: 4 March 2025
Published online: 18 March 2025

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Acknowledgements

We thank the Cancer Research Center and the Central Laboratory of the First Affiliated Hospital of Fujian Medical University for providing the research and experimental platform.

Author contributions

SC is the designers of this study. CZ analyzed and wrote the manuscript. SW, YF and CZ completed the real-time PCR, Western blotting and immunohistochemistry analyses of CRC tumors and cells. XY and LS downloaded the data and performed the statistical analysis. All the authors have read and approved the final manuscript.

Funding

This work was supported by the Joint Funds for the Innovation of Science and Technology, Fujian Province (No.2023Y9071) and the Fujian Provincial Health Technology Project (No. 2021CXB006).

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

The study project was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University (Approval Nos. MRCTA and ECFAH of FMU [2019]021). The Ethics Committee of the First Affiliated Hospital of Fujian Medical University approved the study protocol. All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent

Written informed consent was obtained from all individual participants involved in this study.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-93081-7.

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