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Original Article

GJB5 expression in pancreatic adenocarcinoma: prognostic significance and therapeutic implications

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Abstract: Background: Gap junction protein 5 (GJB5) has been associated with tumorigenesis; however, its exact role in pancreatic adenocarcinoma (PAAD) remains unclear. This study investigates GJB5's expression, its functional roles in tumor progression, and its prognostic significance in PAAD. Methods: This study used multiple bioinformatics tools, including Tissue Infiltrating Microenvironment Estimation Resource 2, University of Alabama at Birmingham Cancer, Tumor Immune System Interaction Database, Gene Expression Profiling Interactive Analysis 2, and cBioPortal. These tools were used to analyze GJB5 expression, its correlation with immune cell infiltration, and its potential as a prognostic biomarker in PAAD. Functional assays, including cell counting kit-8, colony formation, wound healing, and transwell assays, were performed to investigate the impact of GJB5 on PAAD tumor cell behaviors, including proliferation, migration, and invasion. Additionally, pathways associated with GJB5 and its interactions with the tumor microenvironment were explored. Results: GJB5 was significantly overexpressed in PAAD tissues compared to adjacent normal tissues. Promoter hypomethylation, rather than somatic mutation, was identified as the primary mechanism driving GJB5 upregulation. Survival analysis and Cox regression models indicated that upregulated GJB5 expression is an independent prognostic factor for poor survival in patients with PAAD. Furthermore, GJB5 expression was positively correlated with immune cell infiltration in the PAAD microenvironment. Functional assays exhibited that silencing GJB5 reduced cell proliferation, migration, and invasion in PAAD cell lines. Conclusions: GJB5 is a significant prognostic biomarker for PAAD and a potential therapeutic target for reversing tumor progression, providing novel strategies for PAAD treatment.

Keywords: PAAD, GJB5, therapeutic strategy and diagnostic biomarker, prognosis

Introduction

Pancreatic adenocarcinoma (PAAD) is a malignancy associated with poor prognosis, as the five-year relative survival rate remains below 10% [1, 2]. According to 2024 statistics, approximately 508,532 new cases of pancreatic cancer were reported globally, accounting for 2.6% of all cancer cases. Additionally, 467,409 deaths were reported, representing 4.8% of all cancer-related deaths [3]. This highlights the disease's high lethality and diagnostic challenges. Due to the extensive application of genomics and in-depth research on molecular mechanisms in the past decades, multiple genetic mutations, including KRAS, Tumor Protein p53 (TP53), Cyclin Dependent Kinase Inhibitor 2A (CDKN2A), and SMAD Family Member 4 (SMAD4), have been identified as contributing to the initiation and progression of PAAD [4, 5]. Additionally, studies have investigated the roles of the tumor microenvironment (TME), immune cell infiltration, metabolic reprogramming, and extracellular matrix remodeling in PAAD [6-9].

The Connexin family is a group of proteins that form gap junctions between adjacent cells,

facilitating the exchange of small molecules and ions, as well as intercellular signaling [10]. Currently, 21 members of this family, designated Cx32-Cx60, are identified and expressed in various tissues throughout the body in a tissue-specific manner [11]. Connexin proteins have been identified to have essential functions in various physiological processes, including nerve impulse transmission, heart muscle contraction, immune response, wound healing, and embryonic development [12]. Recent studies suggest that connexin is involved not only in inherited diseases but also in the pathogenesis and progression of tumors [12, 13-15]. Aberrant connexin 43 (Cx43) expression is associated with cancer recurrence, metastasis, and unfavorable survival [16]. Therefore, further investigation into the mechanisms underlying the effects of connexin on the pathogenesis and progression of PAAD can provide insights into its diagnosis, prognosis, and therapeutic targeting.

Gap junction protein 5 (GJB5), also known as Cx31.1, is a gap junction protein enriched in the placenta and skin that maintains intercellular communication and differentiation. Its deletion causes 30% embryonic lethality and placental hypoplasia in mice [17]. In tumors, GJB5 acts context-dependently. In primary melanoma, high GJB5 levels are correlated with better prognosis; however, they are also associated with MAPKi resistance. Therefore, it suppresses proliferation and migration in lung cancer [18, 19]. Pan-cancer data from the Cancer Genome Atlas (https://portal.gdc.cancer.gov/projects/TCGA) indicate that GJB5 upregulation in colorectal and esophageal cancers is associated with AKT-mTOR activation, immune infiltration, high TMB/MSI, and shorter overall survival (OS). This suggests that GJB5 plays prognostic and immunoregulatory roles [20]. However, its specific functions in PAAD remain to be investigated. Our preliminary bioinformatic analysis revealed upregulation of this gene in PAAD compared to adjacent normal tissues. Therefore, we conducted additional studies and experiments to explore its properties and prognostic value in PAAD. In summary, this study aims to establish GJB5 as a novel biomarker and therapeutic target, and to provide novel mechanistic insights into PAAD development.

Material and methods

Data collection

The distinct expression patterns of GJB5 in tumors and adjacent normal tissues across multiple cancers were investigated using the Tumor Immune Estimation Resource 2.0 (TIMER2) (http://timer.cistrome.org/) [21], and the SangerBox online tools (http://sangerbox.com/home.html) [22]. The raw RNA-seq data and clinical information of GSE28735 (45 paired samples), GSE62452 (69 tumor samples and 61 adjacent normal samples), and GSE71729 (223 tumor samples and 134 adjacent normal samples) were downloaded from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov,/). Data for PAAD cohorts were collected from the TCGA database. All raw data were normalized and $\log_2$ (x + 0.001) transformed before further analysis. The Gene Expression Profiling Interactive Analysis 2.0 (GEPIA2) was used to obtain the top 50 genes significantly associated with GJB5 in PAAD (http://gepia2.cancer-pku.cn/) [23]. We used the STRING (https://cn.string-db.org/) online bioinformatics tool to construct a protein-protein interaction (PPI) network using experimental and consensus evidence.

Diagnostic and prognostic value of GJB5

We used the pROC package in R version 3.6.3 to generate a receiver operating characteristic (ROC) curve and evaluated the diagnostic value of our findings. Additionally, we performed Kaplan-Meier survival analysis and Cox regression modeling using the “survminer” and “survival” (version 3.2-7) packages in R, respectively. We calculated the *P*-value, hazard ratio (HR), and 95% confidence intervals (CIs) for our results.

Genetic alteration analysis

The genetic alteration landscape of GJB5 across various cancer types was evaluated using the TCGA PanCancer Atlas Studies data in the cBioPortal database (https://www.cbioportal.org/) [24]. Detailed information on alterations in the gene’s coding regions was obtained using the “Mutations” module.

Information on somatically acquired mutations in human cancers for input genes can also be

obtained from the publicly accessible Catalogue of Somatic Mutations in Cancer database [25], available at http://www.sanger.ac.uk/cosmic/.

Analysis of DNA promoter methylation

The GJB5 promoter methylation levels across various clinical characteristics were determined by analyzing box plots generated from the TCGA database using the University of Alabama at Birmingham Cancer (UALCAN) (http://ualcan.path.uab.edu/analysisprot.html) [26].

Analysis of the relationship between drug treatment and GJB5 expression

The CellMiner online database [27] (https://discover.nci.nih.gov/cellminer/home.do) was used to determine the correlation between GJB5 expression level and pharmacological treatment efficacy.

Analysis of infiltration of immunocytes

Tumor Immune System Interaction Database (TISIDB) is a public, online database that provides information on the expression profiles, precise regulatory mechanisms, and clinical relevance of a wide range of immune genes in tumor and normal tissues [28]. Using the TISIDB (http://cis.hku.hk/TISIDB/) database, we analyzed the correlation between GJB5 and the tumor immune microenvironment (TIME), including tumor-infiltrating lymphocytes, immunocyte co-inhibitors, and co-stimulators. Heat maps and scatter graphs were generated to illustrate these correlations in this platform.

Gene set enrichment analysis (GSEA)

To investigate the potential mechanisms underlying the initiation and progression of PAAD associated with GJB5 expression, we used GSEA [29]. We used the clusterProfiler package (version 4.4.4) [30] and obtained gene collections from the Molecular Signatures Database. We identified differentially expressed genes by stratifying samples into high and low groups based on the median GJB5 expression level and comparing gene expression between the groups. These genes were then enriched based on hallmark gene sets in the database.

Analysis of the associated gene's function

Gene ontology (GO) analysis is an extensively used approach for genes and gene annotation, enabling the identification of molecular function, biological process, and cellular components. The Kyoto encyclopedia of genes and genomes (KEGG) database provided a systematic framework for analyzing gene functions by associating gene sets with corresponding pathways.

GO and KEGG analyses of the gene set were performed using R to investigate their functional implications. Furthermore, the Metascape (http://metascape.org/) tool [31] was used to analyze the functions of GJB5-related genes in PAAD. We investigated the association between signaling pathways and GJB5-related genes using Gene Set Cancer Analysis Lite (GSCALite) (https://www.editorialmanager.com/jtrm/default1.aspx) [32].

Cell culture

The PDAC cell lines PANC-1 and SW1990 were obtained from the Chinese Academy of Sciences Cell Bank. These cell lines were cultured in DMEM supplemented with 10% fetal calf serum (Gibco Thermo Fisher Scientific, Inc., USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco Thermo Fisher Scientific, Inc., USA). The cells were maintained under standard cell culture conditions at 37°C in a humidified atmosphere containing 5% CO_2.

GJB5 knockdown

To suppress GJB5 expression in PANC-1 and SW1990 cells, we designed and synthesized three small interfering RNAs (siRNAs) targeting GJB5 (siGJB5-1, siGJB5-2, and siGJB5-3), and a negative control (NC) RNA (Sangon Biotech Co., Ltd.). The cells were seeded in 6-well plates (1×10^5 cells/well) and allowed to reach 70% confluency at 37°C. Then, the cells were transfected with either the siRNAs or NC using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocols. The antisense sequences of the three siRNAs were as follows: siGJB5-1: 5'-CUGGAGUAUCUUUGAGGGAUU-3'; siGJB5-2: 5'-GGACUUCGACUGCAAUACUUU-3'; siGJB5-3: 5'-AUAUAUCCUCCCUCCUGUGUU-3'.

Efficiency evaluation of siRNA

Western blotting was used to determine the relative expression of GJB5 in PNAC-1 and SW1990 cells.

Colony formation assay

To evaluate the effects of GJB5 knockdown on PAAD cell proliferation, we performed a colony formation assay. After 48 h of transfection with siGJB5 or NC, PANC-1 and SW1990 cells were seeded into 6-well plates at a density of 800 cells/well and incubated at 37°C with 5% CO_2 for two weeks. The colonies were subsequently fixed with methanol for 30 min and stained with crystal violet for 30 min. The number of colonies containing more than 50 cells was manually counted under a microscope. Each group was tested in triplicate wells.

Wound healing assay

The siRNA-transfected cells were grown in a 6-well plate until approximately full confluency, after which scratches were created using a 200 μL pipette tip. Subsequently, the serum-free medium was added to the wells to prevent any potential effects on cell growth. At 0, 24, and 48 h after scratching, images of the cells were captured under a microscope.

Transwell assay

A stratum of adhesive Matrigel matrix (Corning Company, USA) was applied to the upper chamber of a Transwell according to a serum-free medium: glue ratio of 1:8, while the lower chamber was covered with 700 μL complete medium. The upper chamber was then seeded with 5×10^4 cells in 200 μL serum-free medium from each group, followed by incubation at 37°C with 5% CO_2 for 24 h. After fixing the cells with 4% paraformaldehyde for 20 min, they were stained with crystal violet for 30 min. Any remaining cells in the upper chamber were carefully removed with sterile cotton. The transwells were allowed to dry at room temperature and photographed under a microscope. Furthermore, the presence or absence of a Matrigel matrix adhesive layer distinguished the transwell invasive assay from the migration assay.

Statistical analysis

A biostatistician reviewed all analyses. Graphs were generated with GraphPad Prism 9.0 and R 4.3.0. Spearman's rank test was used to determine correlation. Normally distributed continuous variables were compared using unpaired two-tailed Student's t-tests, while non-normally distributed data were analyzed with the Mann-Whitney U test. For three or more groups, data were analyzed using a one-way analysis of variance followed by Tukey's post-hoc test. Survival curves were constructed using the Kaplan-Meier method and compared by the two-sided log-rank test. A $P < 0.05$ was considered statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

Results

Aberrant expression of GJB5 in PAAD

The abnormal expression of specific genes found in tumor samples could indicate their potential role in the initiation and progression of the tumor. In the pan-cancer TIMER2 dataset, we observed a significant upregulation in GJB5 expression across 11 tumor types compared to their respective normal tissues. GJB5 expression was significantly upregulated in 11 tumor types, including CESC, CHOL, COAD, ESCA, HNSC, HNSC-HPV (-), LUAD, LUSC, READ, SKCM, and UCEC. However, GJB5 was significantly downregulated in BRCA, GBM, and PRAD ($P < 0.05$ for all) (**Figure 1A**). Furthermore, the combined analysis of the TCGA and GTEx databases revealed that PAAD tumor samples exhibited significantly higher GJB5 expression than adjacent normal tissues (**Figure 1B**). Subsequently, TCGA PAAD cohort, GSE28735, GSE62452, and GSE71729 exhibited a significant upregulation of GJB5 in PAAD tumor tissues compared to normal tissues (**Figure 1C-F**).

Exploring the associations between GJB5 expression and clinical pathological features

We analyzed the correlations between GJB5 expression levels and various clinical characteristics using the UALCAN database. Our analysis revealed that higher GJB5 expression was significantly associated with advanced age, TP53 mutation status, more frequent alcohol

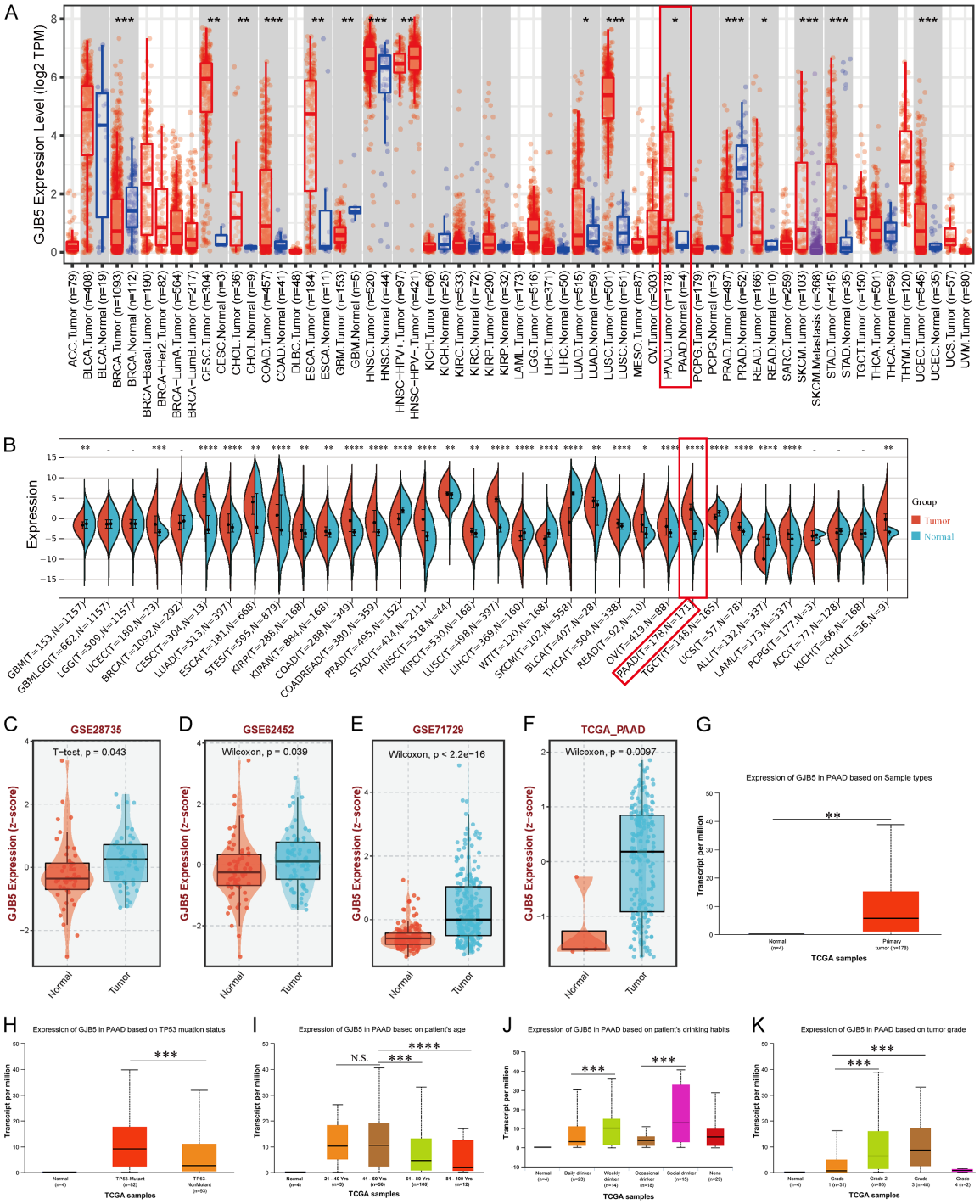


Figure 1. GJB5 expression landscape. The expression profile of GJB5 among pan-cancers with TIMER2 (A). Distribution of GJB5 expression across pan-cancer based on TCGA cohorts combined with GTEx databases (B). The expression level of the GJB5 gene was upregulated in GSE28735 (45 paired samples) (C), GSE62452 (D), GSE71729 (E), and TCGA-PAAD cohorts (F). Upregulated GJB5 expression was observed in tumor subgroups with TP53 mutation status, older age, more frequent drinking habits, and higher tumor grade (G-K). (C, D) Statistics performed by Paired Samples t-Test, (G) statistics performed by unpaired t-test with Welch's correction, (H-K) statistics performed by One-way analysis of variance (n.s. $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). GJB5: Gap junction protein 5; PAAD: Pancreatic adenocarcinoma.

intake, and higher tumor grade (**Figure 1G-K**). These findings provide valuable insight into potential clinical variables associated with elevated GJB5 expression.

Genetic modifications and promoter methylation profiling of the GJB5 gene in PAAD

Investigating genetic alterations and GJB5 promoter methylation can provide valuable insights into the cause of its abnormal expression and potential correlations with clinical-pathologic characteristics. Investigating the mechanisms underlying GJB5 deregulation can enhance our understanding of its role in cancer development and progression.

cBioPortal is a database that integrates retrieval, downloads, analysis, and visualization of cancer genomics data. Its primary function is to perform various analyses of mutations and to visualize the results [24]. The cBioPortal database was used to analyze GJB5 genetic mutations and alterations in the landscape. The findings identified only a few mutations and alterations in PAAD (**Figure 2A**). Furthermore, we explored the mutational landscape and site of GJB5 in pan-cancers using the "Mutation" module on cBioPortal. There were 53 variants of uncertain significance, which were consistent with previous results (**Figure 2B**). Using the COMSIC online tool, the types of mutation were observed. The primary mutation type was missense substitution (33.33%), and the primary substitution mutation type was C > A (100%) (**Figure 2C**). In general, the GJB5 mutation in PAAD was not significant; however, it did not exclude the potential role for GJB5 in pancreatic cancer.

Using UALCAN, we observed that GJB5 promoter methylation was significantly higher in TP53 non-mutant tumor tissues than in TP53 mutant tumor tissues (**Figure 2D**). Additionally, the promoter methylation level of GJB5 was significantly lower in PAAD tissues than in normal tissues (**Figure 2E**). GJB5 promoter methylation was higher in more advanced-aged patients (**Figure 2F**). Notably, no significant differences in GJB5 promoter methylation were observed across various clinical characteristics, including race, grade, and stage (**Figure 2G-I**). These observations suggest that abnormal GJB5 expression in specific cancer types can be driven by promoter hypomethylation. These insights contribute to a more comprehensive understanding of PAAD tumorigenesis and can guide the development of potential treatment strategies.

Assessing the clinical relevance of GJB5 expression analysis as a prognostic and diagnostic biomarker

Using R software, we performed statistical analyses on OS, disease-specific survival (DSS), and progression-free interval (PFI) in PAAD cohorts. Our results demonstrated a significant correlation between high expression of GJB5 and shorter OS (HR = 1.73, 95% CI: 1.14-2.63, P = 0.010), shorter DSS (HR = 1.69, 95% CI: 1.06-2.70, P = 0.027), and shorter PFI (HR = 1.52, 95% CI: 1.03-2.24, P = 0.036) (**Figure 3A-C**). These findings suggest that high GJB5 expression is associated with poorer clinical outcomes in patients with PAAD. The relationship between GJB5 expression level and clinicopathological variables in patients with PAAD is presented in **Table 1**.

Furthermore, Cox proportional hazard regression analysis revealed that GJB5 was a significant risk factor in multiple types of cancer, including PAAD (HR = 1.18, 95% CI: 1.09-1.29, P < 0.001), SKCM (HR = 1.08, 95% CI: 1.03-1.12, P < 0.001), LUAD (HR = 1.07, 95% CI: 1.02-1.12, P < 0.001), KIPA (HR = 1.06, 95% CI: 1.00-1.12, P = 0.04), and LAML (HR = 1.06, 95% CI: 1.00-1.12, P = 0.04) (**Figure 3D**). The results suggest that a relatively high level of GJB5 is frequently associated with a poorer prognosis for tumor patients. Then, univariate and multivariate Cox regression analyses were performed to determine the effect of variables on prognosis. The results suggested that age could be considered as an independent prognostic factor for patients with PAAD (**Figure 3E**, **3F**). Furthermore, the area under the diagnostic ROC (AUC) was calculated to be 0.855 (95% CI: 0.693-1.000) (**Figure 3G**). Furthermore, time-dependent ROC analysis was performed to determine the predictive capability of GJB5 in PAAD. The AUCs at 1, 3, and 5 years were 0.655, 0.773, and 0.820, respectively, indicating the significant performance of GJB5 in predicting the prognosis of patients with PAAD (**Figure 3H**). Subsequently, we developed a comprehensive nomogram to provide quantitative predictions of 1-, 3-, and 5-year OS probabilities in patients with PAAD. The calibration curves of the nomogram exhibited significant agreement between the actual and predicted OS rates (**Figure 3I**, **3J**).

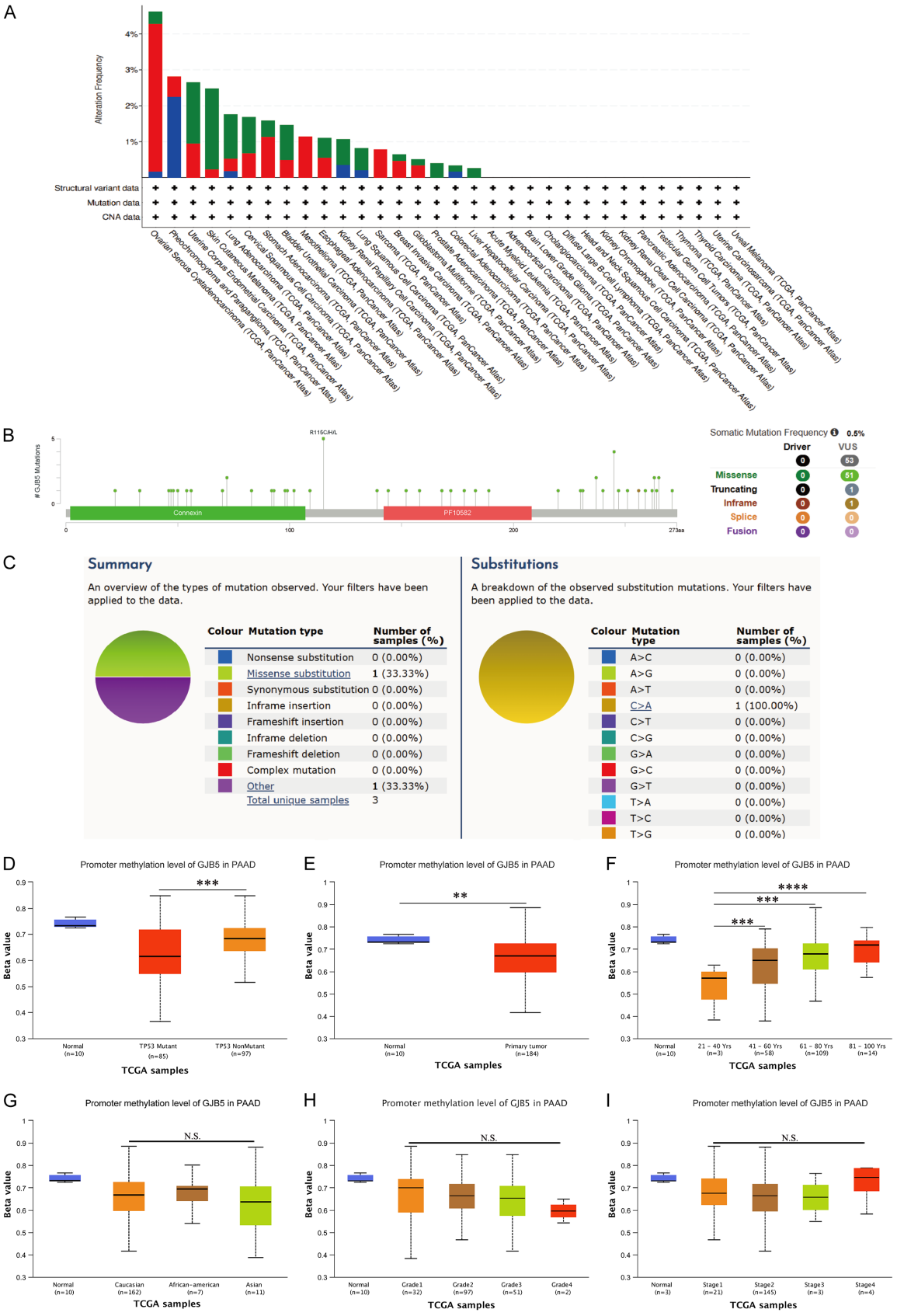


Figure 2. Genetic modifications and promoter methylation profiling of GJB5. Alteration of GJB5 in pan-cancers (A). Mutation site and type of GJB5 in pan-cancers (B). Overview of GJB5 mutative types and substitution in pan-cancers

(C). The potential correlation between clinical characteristics and the promoter methylation level of GJB5 (D-I). (E) Statistics performed by unpaired t-test with Welch's correction, the remaining statistics were performed by One-way analysis of variance (n.s. $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

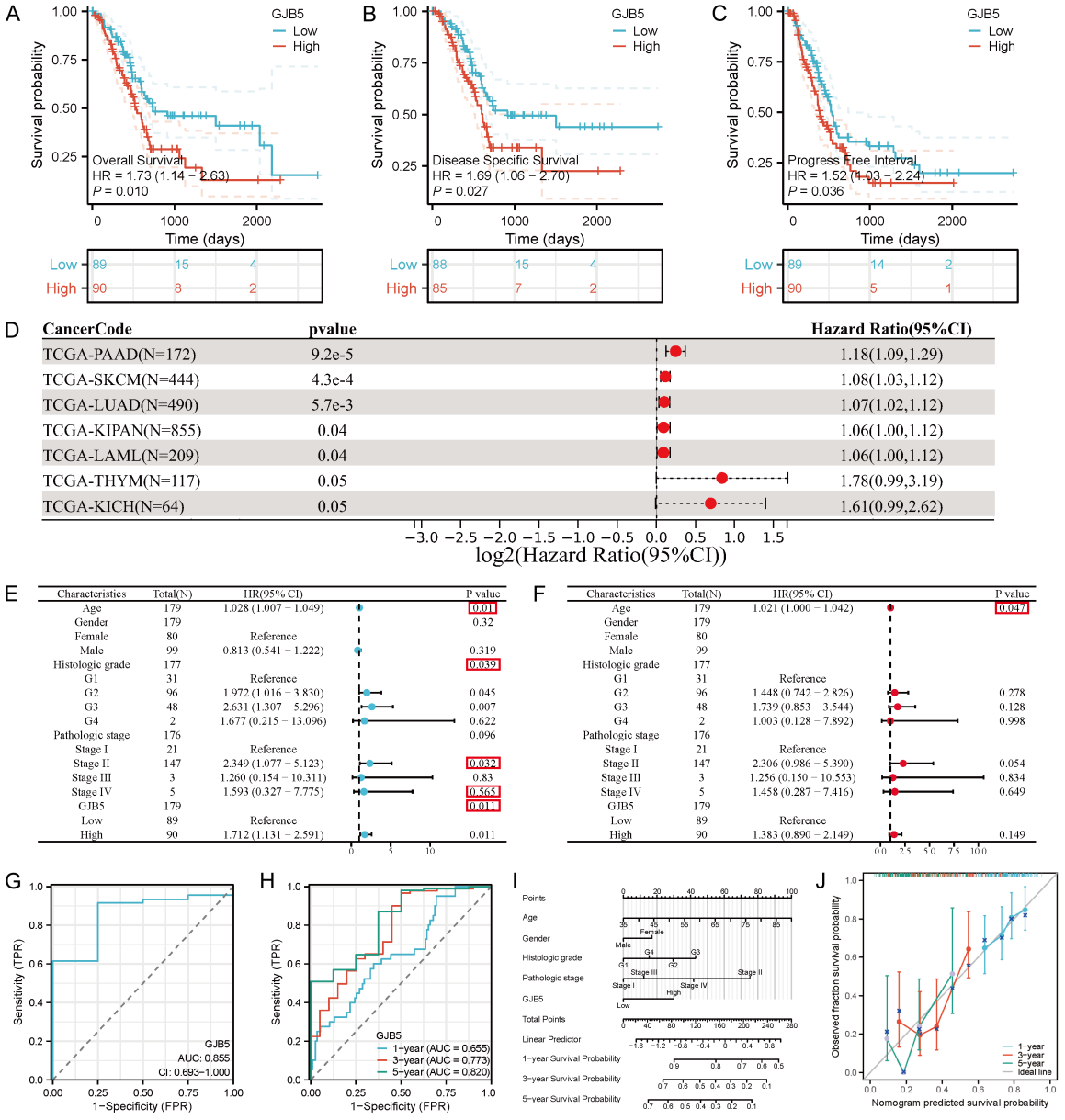


Figure 3. Prognostic value of GJB5 in PAAD. OS analysis in PAAD (A), DSS analysis in PAAD (B), and PFI analysis in PAAD (C). The forest plot of GJB5 in different cancers (D). Diagnostic ROC analysis with the AUC of GJB5 in PAAD (E). Time-dependent ROC of GJB5 in PAAD (F). Time-dependent AUC of GJB5 in PAAD (G). Nomogram for the prediction of patients with PAAD's survival (H). Nomogram for the prediction of patients with PAAD's survival (I). Calibration curves of the nomogram at 1-, 3-, and 5-year (J).

Furthermore, univariate Cox regression analysis suggested that GJB5 level (P = 0.009), pathologic T stage (P = 0.045), N stage (P = 0.002), and histologic grade (P = 0.017) were all significantly associated with OS. Multivariate Cox regression analysis revealed that the N1 stage (P = 0.028) and R1 (P = 0.032) were significantly correlated with OS. These results indicated that GJB5 is an independent prognostic factor for patients with PAAD (**Table 2**).

Table 1. Relationship between GJB5 expression level and clinicopathological variables in patients with PAAD

Characteristics	Low GJB5 expression	High GJB5 expression	*P*-value	Statistic
n	89	90		
Pathologic T stage, n (%)			0.271	3.916
T1	5 (2.8%)	2 (1.1%)		
T2	15 (8.5%)	9 (5.1%)		
T3	66 (37.3%)	77 (43.5%)		
T4	1 (0.6%)	2 (1.1%)		
Pathologic N stage, n (%)			0.388	0.745
N0	27 (15.5%)	23 (13.2%)		
N1	58 (33.3%)	66 (37.9%)		
Pathologic stage, n (%)			0.0759	3.150
Stage I	14 (8.2%)	7 (4.1%)		
Stage III and Stage II	69 (40.4%)	81 (47.4%)		
Histologic grade, n (%)			0.0130	10.779
G1	22 (12.4%)	9 (5.1%)		
G2	45 (25.4%)	51 (28.8%)		
G3	18 (10.2%)	30 (16.9%)		
G4	2 (1.1%)	0 (0%)		
OS event, n (%)			0.062	3.486
Alive	49 (27.4%)	37 (20.7%)		
Dead	40 (22.3%)	53 (29.6%)		
DSS event, n (%)			0.147	2.104
No	55 (31.8%)	45 (26%)		
Yes	32 (18.5%)	41 (23.7%)		
PFI event, n (%)			0.714	0.134
No	38 (21.2%)	36 (20.1%)		
Yes	51 (28.5%)	54 (30.2%)		

GJB5: Gap junction protein 5; PAAD: Pancreatic adenocarcinoma.

Survival analysis of GJB5 expression in different subtypes of PAAD

The Kaplan-Meier survival curve was used to determine the prognostic value of GJB5 across different clinical characteristics. The results suggest that high GJB5 expression is a significant risk factor in 13 subtypes, including age > 65 ($P = 0.029$), age ≤ 65 ($P = 0.006$), male gender ($P = 0.034$), female gender ($P = 0.029$), T stage: T1 and T2 ($P = 0.011$), T3 ($P = 0.003$), N stage: N1 ($P = 0.046$), grade: G1 ($P = 0.014$), alcohol drinking history: yes ($P = 0.018$), pathologic stage: stage I ($P = 0.019$), stage II ($P = 0.016$), residual tumor: R1 ($P = 0.048$), and primary therapy outcome: CR ($P = 0.007$) (**Figure 4**).

Investigating the relationship between GJB5 expression and TIME

Immunity is a vital component of tumor initiation, growth, and treatment. Additionally, immunomodulators can significantly affect the function of infiltrating lymphocytes in TIME. Therefore, it is imperative to investigate the correlation between GJB5 and TIME.

Based on the TISIDB database, we performed multiple analyses to investigate the correlation between GJB5 expression and various factors within PAAD, including lymphocyte infiltration, chemokines, immune checkpoint inhibitors, and immune checkpoint stimulators. For each analysis, we highlighted the PAAD results in red boxes and presented four main results.

Table 2. Univariate and multivariate analyses of prognostic factors for patients with PAAD

Characteristics	Total (N)	Univariate analysis Hazard ratio (95% CI)	*P*-value	Multivariate analysis Hazard ratio HR (95% CI)	*P*-value 2
Age	179	1.028 (1.007-1.049)	0.010	1.021 (1.000-1.042)	0.047
Gender	179		0.320		
Female	80	Reference			
Male	99	0.813 (0.541-1.222)	0.319		
Histologic grade	177		0.039		
G1	31	Reference		Reference	
G2	96	1.972 (1.016-3.830)	0.045	1.448 (0.742-2.826)	0.278
G3	48	2.631 (1.307-5.296)	0.007	1.739 (0.853-3.544)	0.128
G4	2	1.677 (0.215-13.096)	0.622	1.003 (0.128-7.892)	0.998
Pathologic stage	176		0.096		
Stage I	21	Reference		Reference	
Stage II	147	2.349 (1.077-5.123)	0.032	2.306 (0.986-5.390)	0.054
Stage III	3	1.260 (0.154-10.311)	0.830	1.256 (0.150-10.553)	0.834
Stage IV	5	1.593 (0.327-7.775)	0.565	1.458 (0.287-7.416)	0.649
GJB5	179		0.011		
Low	89	Reference		Reference	
High	90	1.712 (1.131-2.591)	0.011	1.383 (0.890-2.149)	0.149

The results demonstrated that GJB5 was positively correlated with various chemokines, including CCL7 (Cor = 0.227, $P = 0.002$), CCL13 (Cor = 0.261, $P < 0.001$), and CXCL14 (Cor = 0.215, $P = 0.004$); however negatively correlated with CCL14 (Cor = -0.21, $P = 0.005$) (**Figure 5A**, **5B**). Additionally, GJB5 expression was positively correlated with certain immunoinhibitors, including LGAL59 (Cor = 0.251, $P < 0.001$) and IL10RB (Cor = 0.307, $P < 0.001$), and negatively correlated with CD160 (Cor = -0.367, $P < 0.001$) and KDR (Cor = -0.283, $P < 0.001$) (**Figure 5C**, **5D**). Notably, GJB5 exhibited positive correlation with multiple immunostimulators, including TMEM173 (Cor = 0.343, $P < 0.001$), TNFSF9 (Cor = 0.316, $P < 0.001$), RAET1E (Cor = 0.294, $P < 0.001$), and CD276 (Cor = 0.289, $P < 0.001$) (**Figure 5E**, **5F**). Furthermore, GJB5 was positively correlated with the infiltration of most immunocytes, including CD56 bright (Cor = 0.264, $P < 0.001$) and CD56 dim (Cor = 0.300, $P < 0.001$) natural killer cells (NKs), CD4 positive central memory T cells (Tcm) (Cor = 0.365, $P < 0.001$), and activated CD4+ T cells (Cor = 0.292, $P < 0.001$) (**Figure 5G**, **5H**).

Using the ssGSEA algorithm in the R-Gene set variation analysis package (1.46.0), we calculated the infiltration levels of 24 immunocyte types between high- and low-GJB5 expression groups. The results revealed that high GJB5 expression was associated with high enrichment scores for activated dendritic cells (aDC), DC, macrophages, NK CD56 bright cells, NK CD56 dim cells, T regulatory cells, Th2 cells, and Th1 cells. However, Th17 cells were significantly enriched in the low-GJB5 expression group (**Figure 6A**). The Spearman correlation coefficient between GJB5 expression and enrichment of immunocytes is presented in **Figure 6B-J**.

In summary, these results suggest that high GJB5 expression levels are associated with increased infiltration of immune effector cells and immunostimulators in the TME. This observation supports the hypothesis that GJB5 can improve the TME's sensitivity to immunostimulatory factors, thereby promoting the recruitment and activation of immune cells to support an effective antitumor response.

GSEA

The hallmarks of GSEA provided insights into potential downstream mechanisms regulated by GJB5 overexpression. It was observed that overexpression of GJB5 is associated with the activation of several signaling pathways including G2M checkpoint (NES = 2.273, adjusted *P*

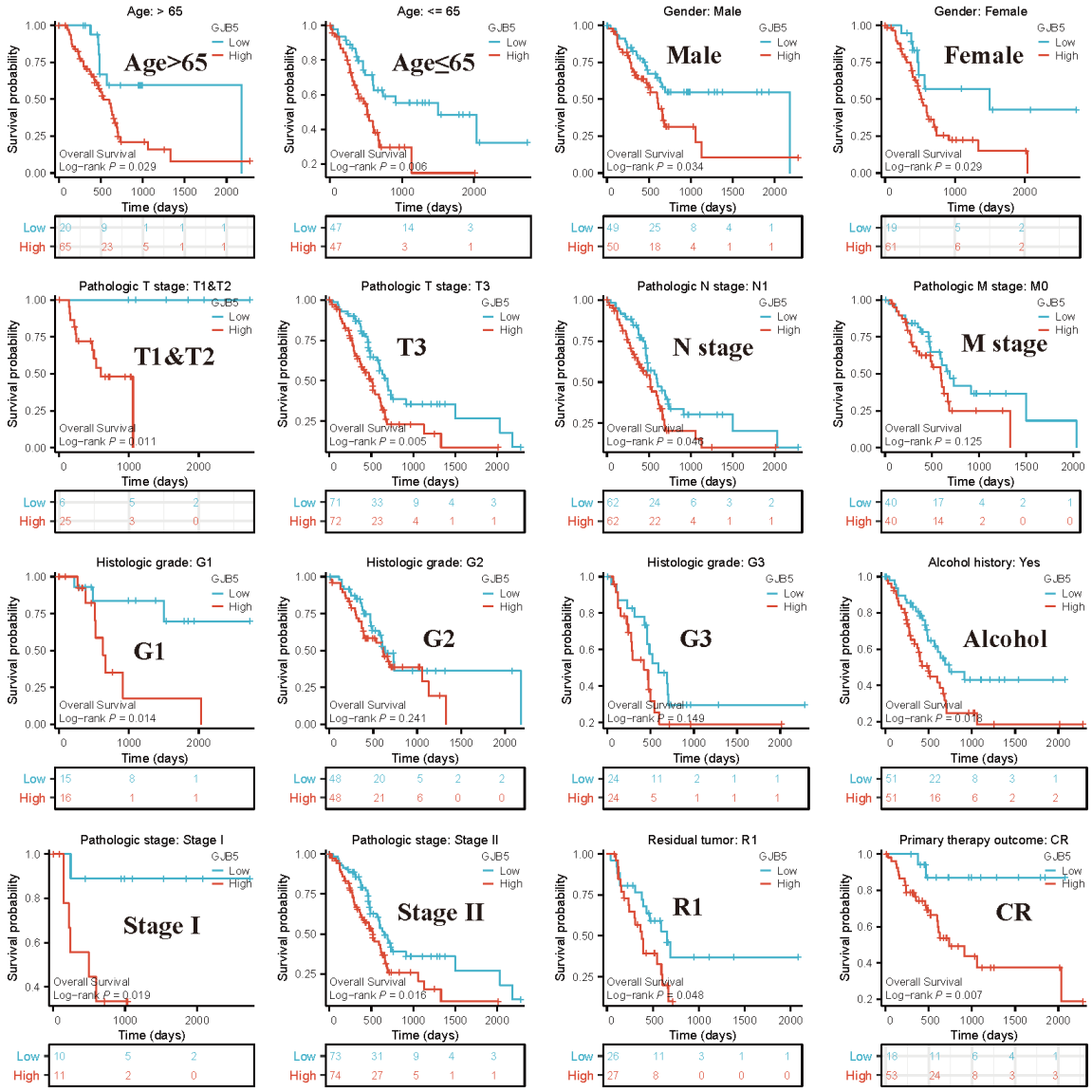
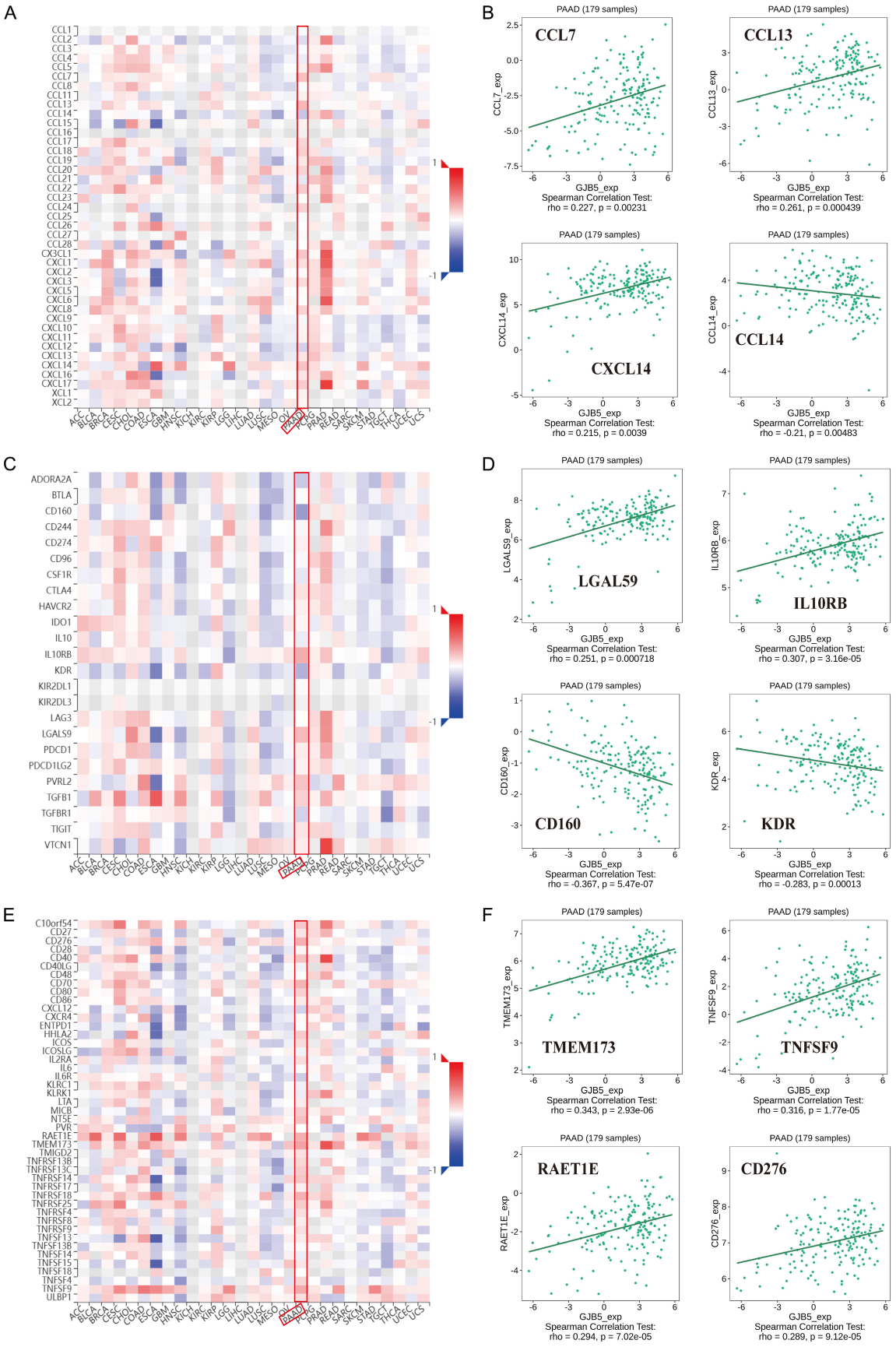


Figure 4. Kaplan-Meier analysis was used to determine the correlation between GJB5 expression and clinical variables.

< 0.001), Tumor Necrosis Factor-alpha signaling through NF-κB pathway (NES = 2.330, adjusted $P < 0.001$), E2F targets (NES = 2.340, adjusted $P < 0.001$), epithelial mesenchymal transition (EMT) (NES = 1.570, adjusted $P < 0.001$), and P53 pathway (NES = 1.848, adjusted $P < 0.001$) (**Figure 7A-F**). The observed activation of the G2M checkpoint pathway suggests that GJB5 is involved in cell cycle progression and DNA damage response. TNFα signaling through the NF-κB pathway activation indicates that GJB5 can modulate the immune response, inflammation, and cell survival. Furthermore, activation of E2F targets suggests that GJB5 can regulate cell proliferation and differentiation. The observed activation of the EMT pathway indicates that GJB5 can play a role in cancer metastasis and invasion. Additionally, activation of the p53 pathway suggests that GJB5 can influence DNA damage response, cell cycle arrest, and apoptosis.

Subsequent correlation analysis revealed that GJB5 expression was significantly associated with TP53, which is a key regulator of the p53 pathway, as well as other factors, including vimentin, CDK1, Snail1, and Bax (critical regulators of the cell cycle), EMT, and apoptosis


A
B
C
D
E
F
PAAD (179 samples)
CCL7
CCL13
CXCL14
CCL14
LGAL59
IL10RB
CD160
KDR
TMEM173
TNFSF9
RAET1E
CD276
GJB5_exp
Spearman Correlation Test:
rho = 0.227, p = 0.00231
rho = 0.261, p = 0.000439
rho = 0.215, p = 0.0039
rho = -0.21, p = 0.00483
rho = 0.251, p = 0.000718
rho = 0.307, p = 3.16e-05
rho = -0.367, p = 5.47e-07
rho = -0.283, p = 0.00013
rho = 0.343, p = 2.93e-06
rho = 0.316, p = 1.77e-05
rho = 0.294, p = 7.02e-05
rho = 0.289, p = 9.12e-05

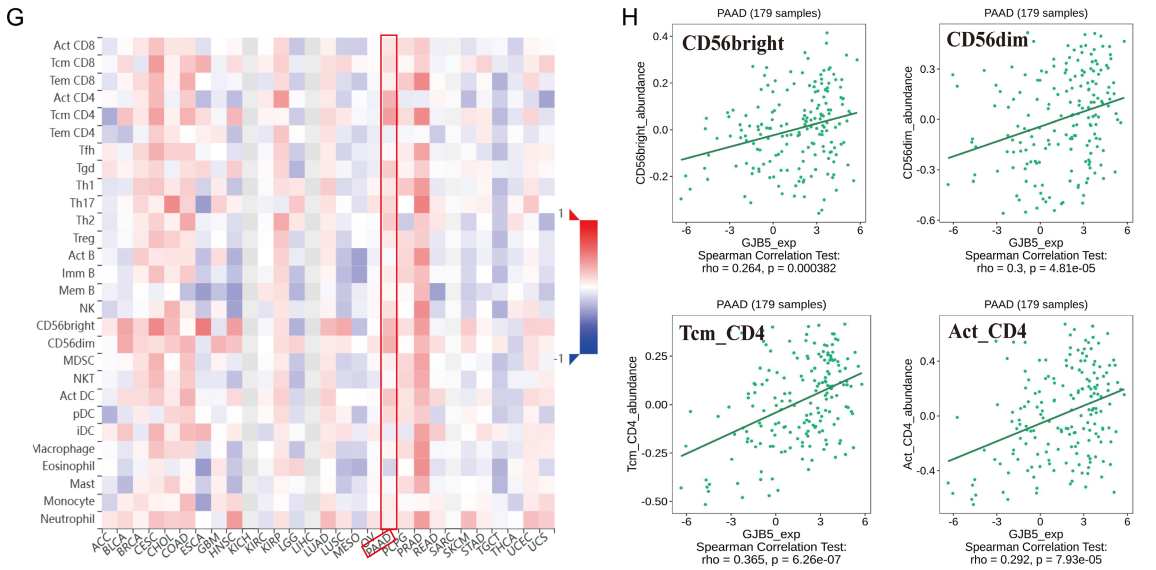


Figure 5. Correlation analysis of GJB5 and TIME. Correlation with chemokines in cancers (A), and the top four results in PAAD (B). Correlation with immunoinhibitors in cancers (C), and four main results in PAAD (D). Correlation with immunostimulators in cancers (E), and the four top results in PAAD (F). Correlation with infiltration of immunocytes in cancers (G), and four main results in PAAD (H).

(**Figure 7G-K**). These findings suggest that GJB5 can regulate multiple cellular processes and have potential therapeutic value as a target for PAAD.

Analysis of drug efficacy targeting GJB5

We obtained data on both pharmacologic efficacy and mRNA levels from the CellMiner database. The correlation between drug activity z-scores and GJB5 gene expression levels was analyzed. GJB5 levels exhibited significant negative correlation with six drugs including carmustine (Cor = -0.32, P = 0.013), bortezomib (Cor = -0.32, P = 0.012), arsenic trioxide (Cor = -0.32, P = 0.013), rapamycin (Cor = -0.31, P = 0.017), midostaurin (Cor = -0.32, P = 0.013), and cisplatin (Cor = -0.30, P = 0.02) (**Figure 8**). These results are highly significant for optimizing personalized treatment guidance for patients with PAAD and high GJB5 expression levels.

Identification of GJB5-related genes and their biological functions

Using the STRING tool, we identified 10 GJB5-binding proteins that have been previously verified in other studies. These included GJA1, GJA4, GJB3, GJB4, GJC1, GJC3, GJD2, GJD3, GJD4, and GJE1 (**Figure 9A**). Additionally, we identified the top 50 genes positively correlated with GJB5 expression in PAAD using the GEPIA2 tool. GO/KEGG analysis of the aforementioned genes (**Figure 9B**) indicated that GJB5-related genes are associated with lipase activity regulation, positive protein processing regulation, the connexin complex, and proteasome processes. Furthermore, Metascape analysis exhibited similar results, suggesting that the 50 genes were associated with the cell cycle and positively regulated protein processing and cytokine signaling in the immune system (**Figure 9C**).

Furthermore, we used the top 10 related genes from GEPIA2 and 10 genes from the PPI results to conduct co-expression analysis of GJB5 across various cancers using TIMER2. The findings were visualized in a heatmap (**Figure 9D**, **9E**), which indicated that these 20 genes exhibited significant co-expression with GJB5 among most cancer types.

A prognostic value analysis of GJB5-related genes

Based on Kaplan-Meier survival analyses, we observed positive outcomes in seven of the 20 genes listed above. These genes included *ANXA2* (HR = 1.97, 95% CI: 1.29-2.99, P = 0.002), *GJB3* (HR = 2.08, 95% CI: 1.36-3.17,

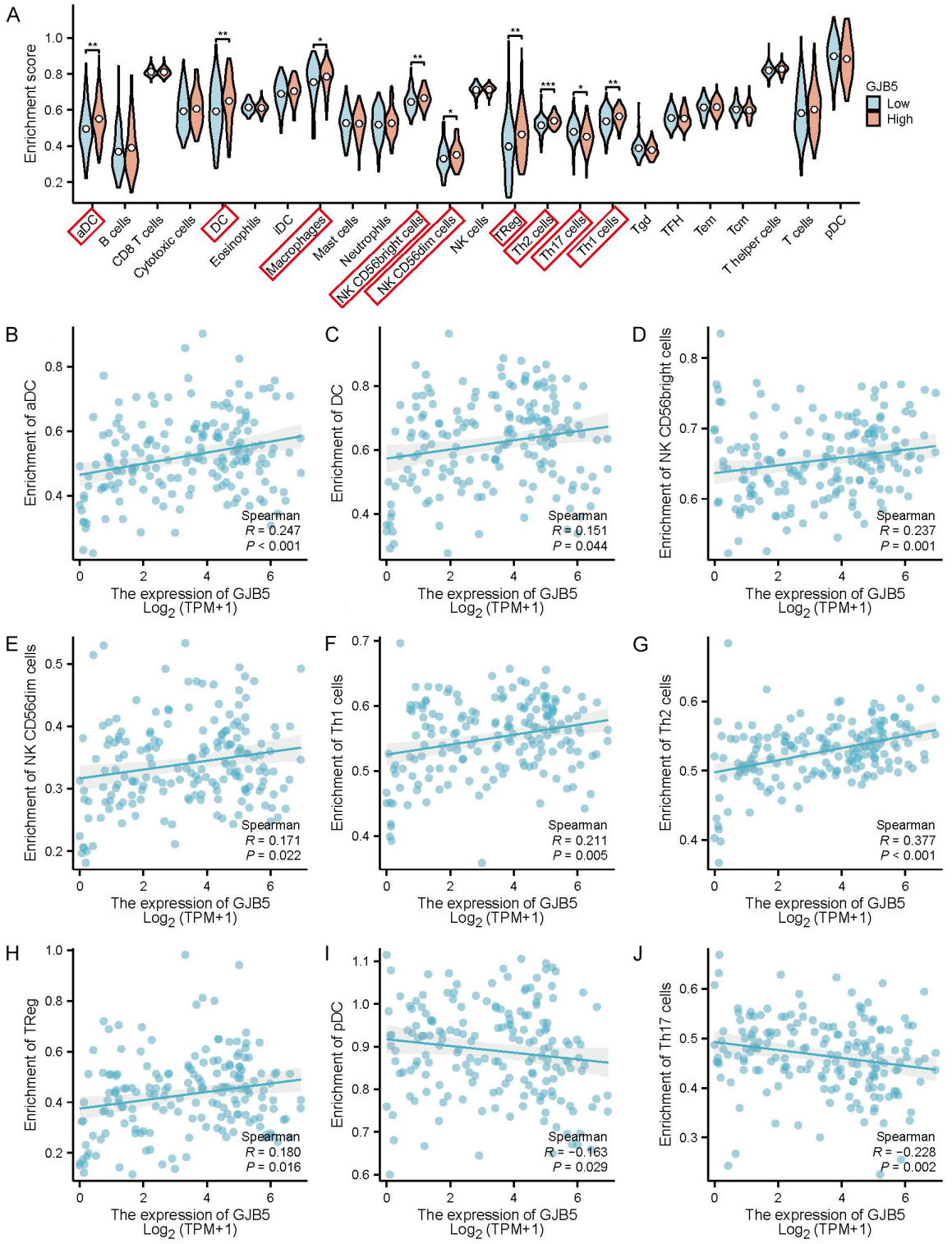


Figure 6. ssGSEA analysis of GJB5 in PAAD. Infiltration of 24 types of immune cells between high- and low-GJB5 expressed groups (A). Correlation of the expression of GJB5 and various immune cells (B-J). Statistics performed by Pearson correlation (*P < 0.05; **P < 0.01; ***P < 0.001).

P < 0.001), *GJB4* (HR = 1.74, 95% CI: 1.15-2.64, P = 0.009), *GNA15* (HR = 1.74, 95% CI: 1.15-2.63, P = 0.009), *KCNN4* (HR = 2.10, 95% CI: 1.38-3.20, P < 0.001), *KRT19* (HR = 1.89,

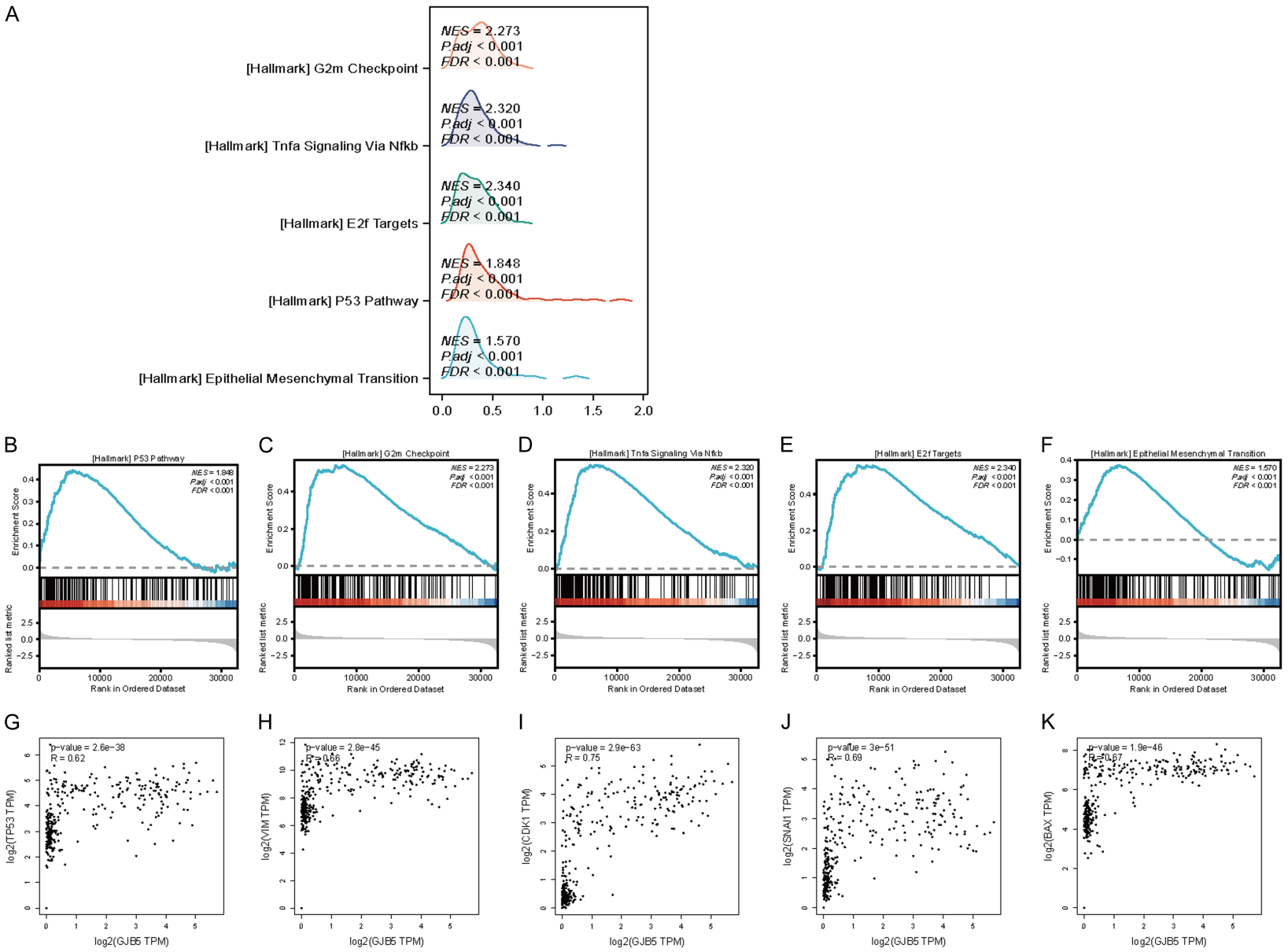


Figure 7. GSEA analysis of hallmark genes (A-F). Correlation analysis between GJB5 and TP53, vimentin, CDK1, Snail1, and Bax (G-K).

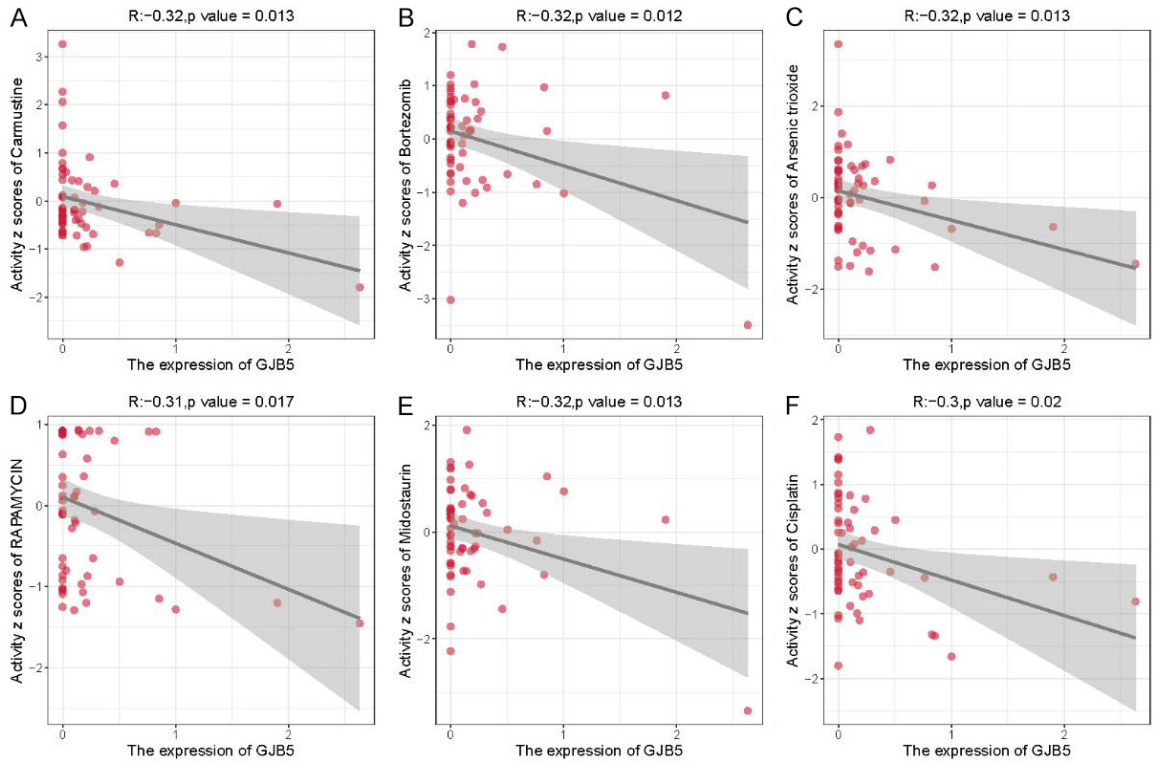


Figure 8. Scatter plots of the correlation between GJB5 expression and the activity z-score of drugs. The correlation between GJB5 gene expression levels and drug activity of carmustine, bortezomib, arsenic trioxide, rapamycin, midostaurin, and cisplatin (A-F).

95% CI: 1.25-2.87, *P* = 0.003), and *PROM2* (HR = 1.70, 95% CI: 1.11-2.60, *P* = 0.014) (**Figure 10A-G**). Notably, these findings regarding GJB5-related genes suggest that these molecules could collectively contribute to tumor progression.

Function analysis of top-related genes

Using the GSCALite (http://bioinfo.life.hust.edu.cn/web/GSCALite/) tool, we analyzed the co-functionality of the previously mentioned seven genes and GJB5 in PAAD. The results suggested that these genes primarily induced activation of the EMT and RAS/MAPK pathways while simultaneously activating the cell cycle pathway, ultimately resulting in tumor growth (**Figure 11A**, **11B**).

Inhibition of cell proliferation, migration, and invasion by downregulating GJB5 expression

The multifaceted findings described above suggested that GJB5 functions as an oncogene in PADD, driving tumor initiation and progression. Furthermore, its activation of cell proliferation and ability to regulate the immune response can ultimately result in a poor prognosis. Therefore, we conducted a series of experiments to determine whether inhibiting GJB5 at the cellular level could effectively suppress malignant biological behaviors.

We evaluated the knockdown efficiency of three siGJB5s through Western blotting and observed that siGJB5-2 exhibited the highest efficacy (**Figure 12A**, **12B**). Therefore, siGJB5-2 was selected for further analysis. Cell counting kit-8 (CCK-8) assay and colony formation assay were performed to determine the effects of GJB5 knockdown on cell proliferation. Compared to the NC group, the number of PANC-1 and SW1990 colonies was significantly decreased in the siRNA group (**Figure 12C-E**). The results of the wound healing assay exhibited that, compared with the NC group, GJB5 knockdown groups exhibited wider wounds in both PANC-1 and SW1990 cells at the same 48-h interval (**Figure 12F**). Furthermore, transwell assays were performed to determine the effects of GJB5 silencing on cell migration

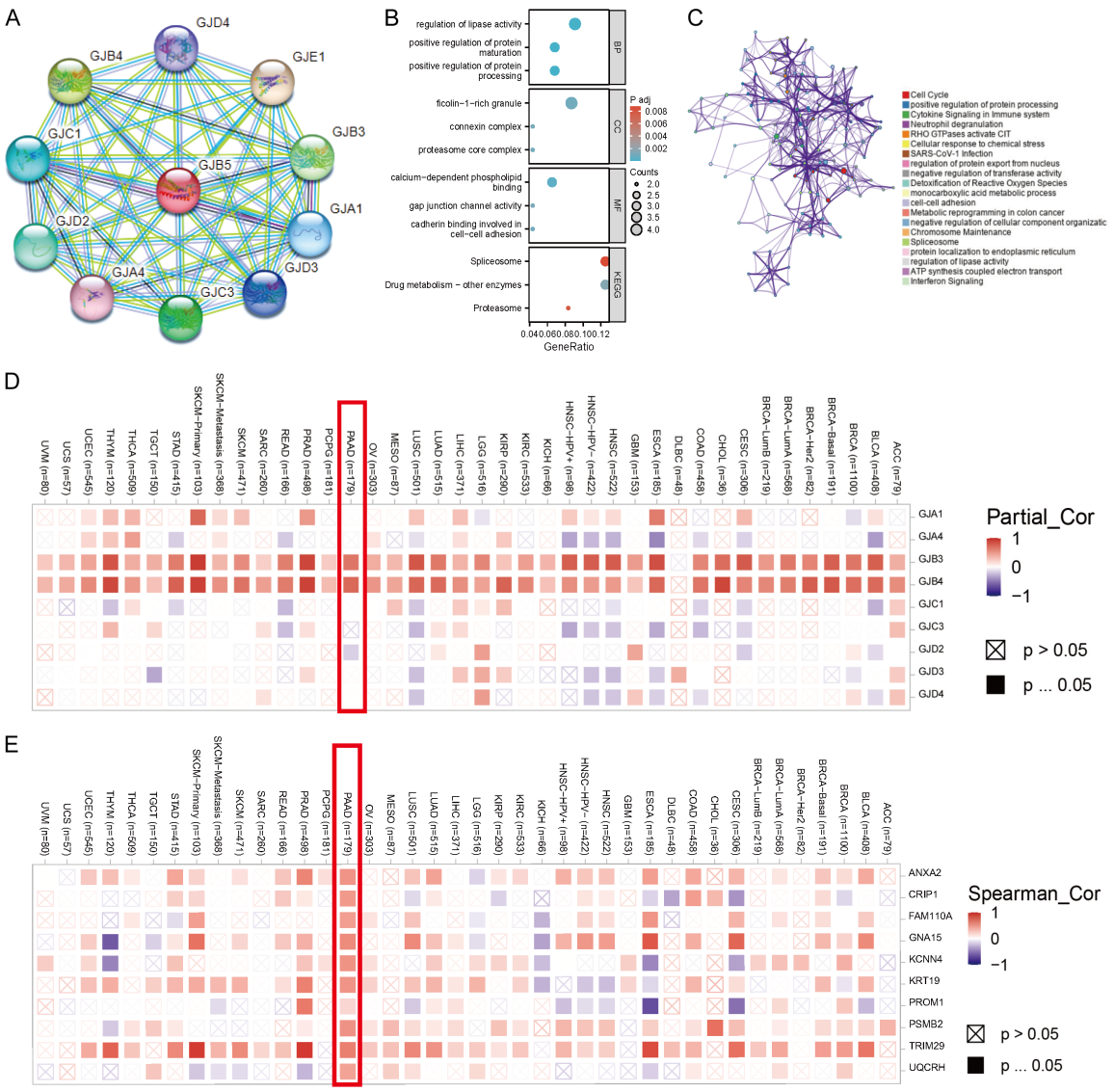


Figure 9. Exploration of GJB5-related genes. PPI network of the GJB5 protein through the STING tool (A). GO and KEGG analysis of the top 50 GJB5-related genes (B). Network of GO-enriched processes through Metascape (C). The expression heatmap for the genes from the STRING tool with the TIMER2 tool in cancer (D). Heatmap of the top 10 GJB5-related genes from GEPIA2, using the TIMER2 tool in cancers (E).

and invasion. The results exhibited that GJB5 knockdown significantly inhibited the migration and invasion of PANC-1 and SW1990 cells (**Figure 12G**). These results indicate that knocking down GJB5 in PANC-1 and SW1990 cells significantly inhibited their proliferation, migration, and invasion, strongly supporting our earlier findings.

Discussion

Pancreatic cancer continues to be one of the most aggressive forms of cancer and poses a significant public health challenge. This underscores the need to conduct further research on potent biomarkers and therapeutic targets to address this pressing issue. Therefore, we investigated the role of the GJB5 gene in PAAD.

Our extensive results indicated that GJB5 was highly expressed across various tumor types, including PAAD. Furthermore, data from three cohorts in GEO confirmed its anomalous expression. GJB5 levels exhibited a significant correlation with higher tumor grade and TP53 mutation in patients with PAAD. Promoter DNA methylation is a common epigenetic modification observed in human cancers. This modifica-

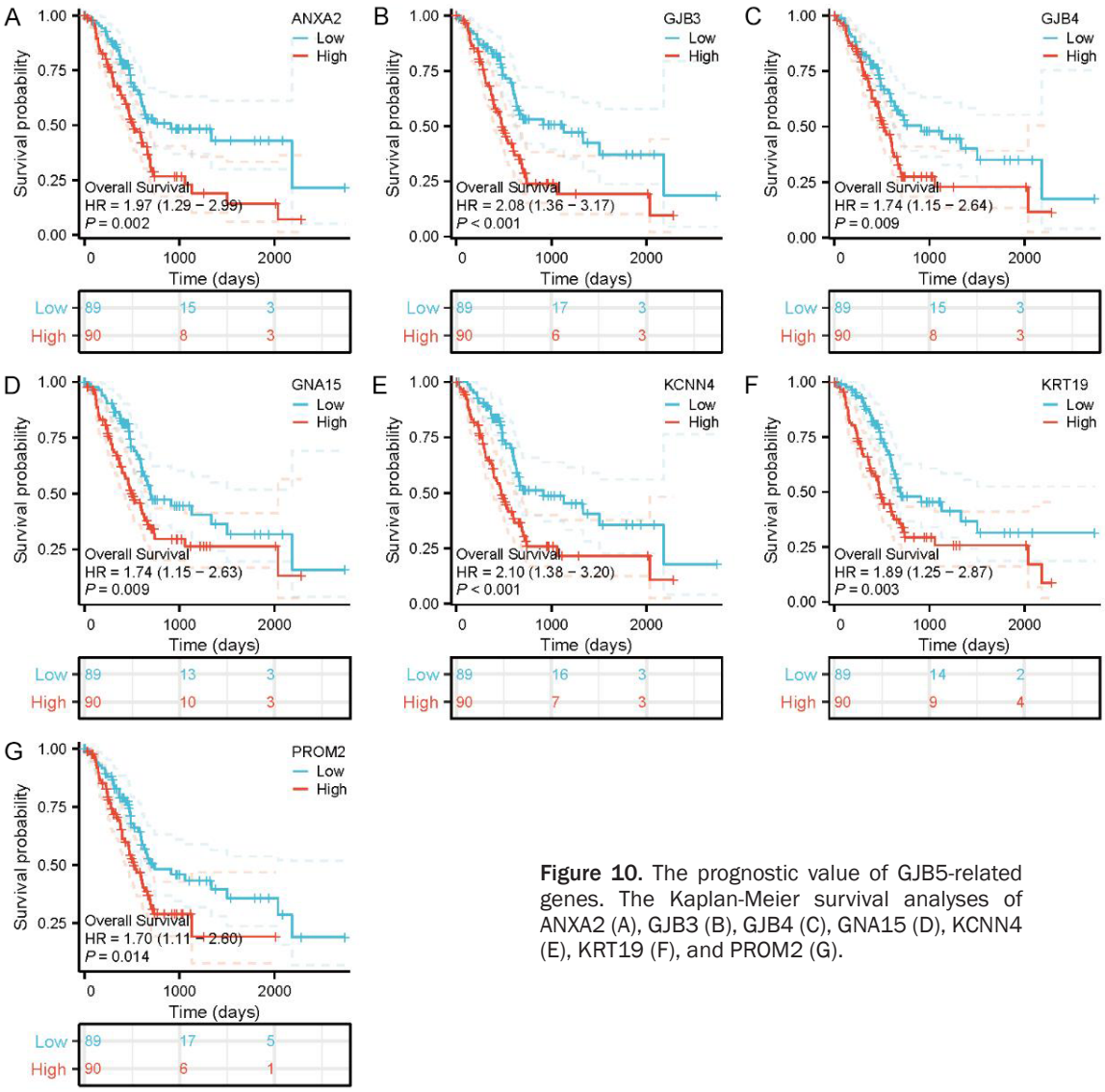


Figure 10. The prognostic value of GJB5-related genes. The Kaplan-Meier survival analyses of ANXA2 (A), GJB3 (B), GJB4 (C), GNA15 (D), KCNN4 (E), KRT19 (F), and PROM2 (G).

tion occurs on cytosine nucleotides across CpG islands and frequently results in gene silencing, representing a significant mechanism for the loss of gene function [33]. Furthermore, oncogenes are frequently hypomethylated in tumors. The upregulation and increased activity of these oncogenes play a significant role in promoting tumorigenesis. This phenomenon highlights the significance of epigenetic regulation in gene expression and cancer development [34-36]. Our results suggest that minimal genetic alterations were observed in the GJB5 gene in PAAD; however, tumor tissues exhibited a significant reduction in promoter methylation compared with adjacent normal tissues.

The study findings demonstrate that GJB5 can have prognostic implications in PAAD. Specifically, survival analysis revealed that high GJB5 expression in patients with PAAD was correlated with shorter OS, DSS, and PFI, as determined from TCGA data. Notably, Cox regression analysis of pan-cancer data identified GJB5 as a significant risk factor across multiple cancers, including PAAD. The promoter hypomethylation of the GJB5 gene can contribute to its abnormal expression in pancreatic cancer. Additionally, a correlation analysis between clinical drug efficacy and GJB5 expression suggested the potential value of GJB5 as a biomarker for personalized treatment in patients with PAAD, with six significantly negative corre-

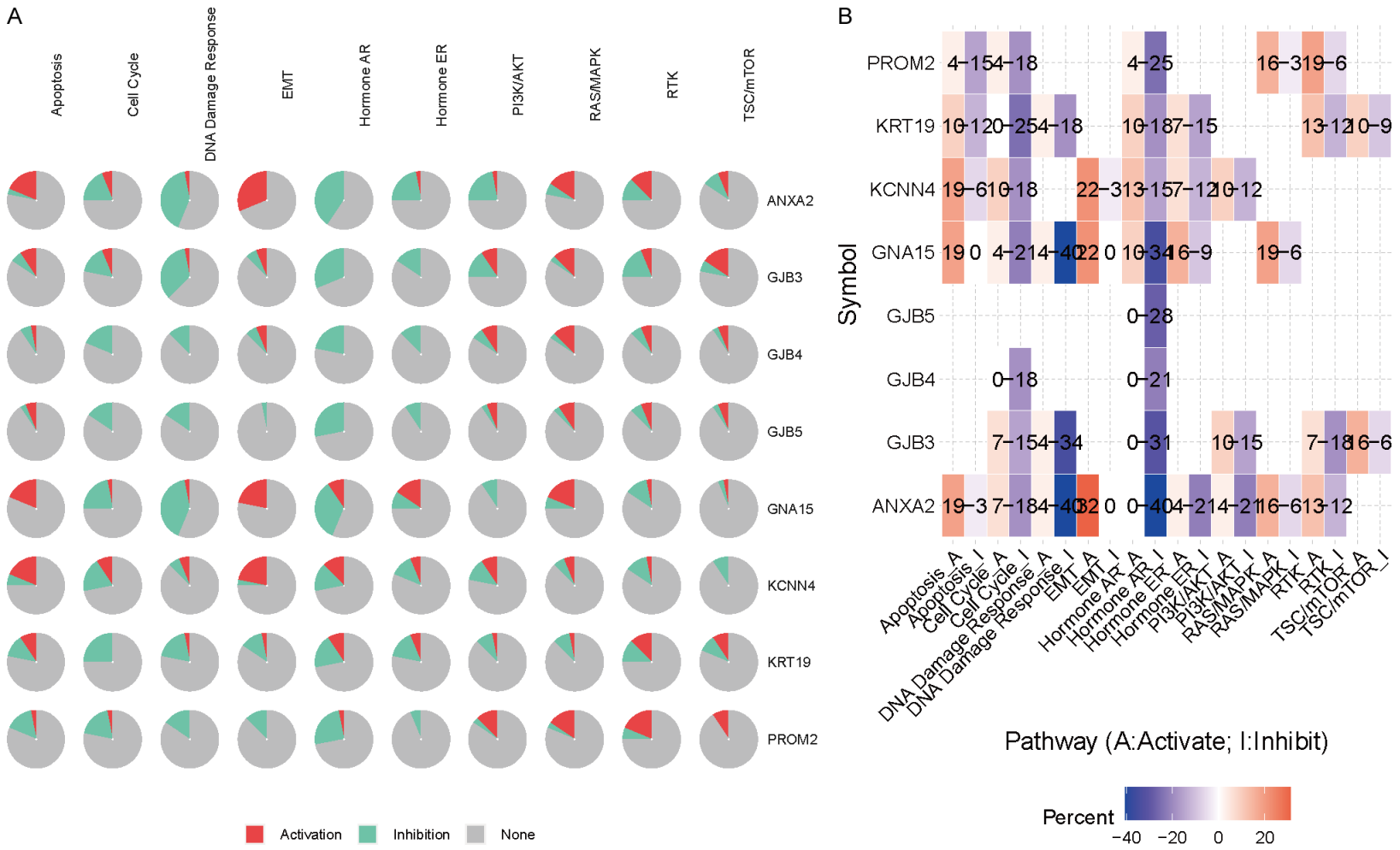


Figure 11. Function analysis of GJB5-related genes in PAAD. Signaling pathways analysis of GJB5-related genes (A, B).

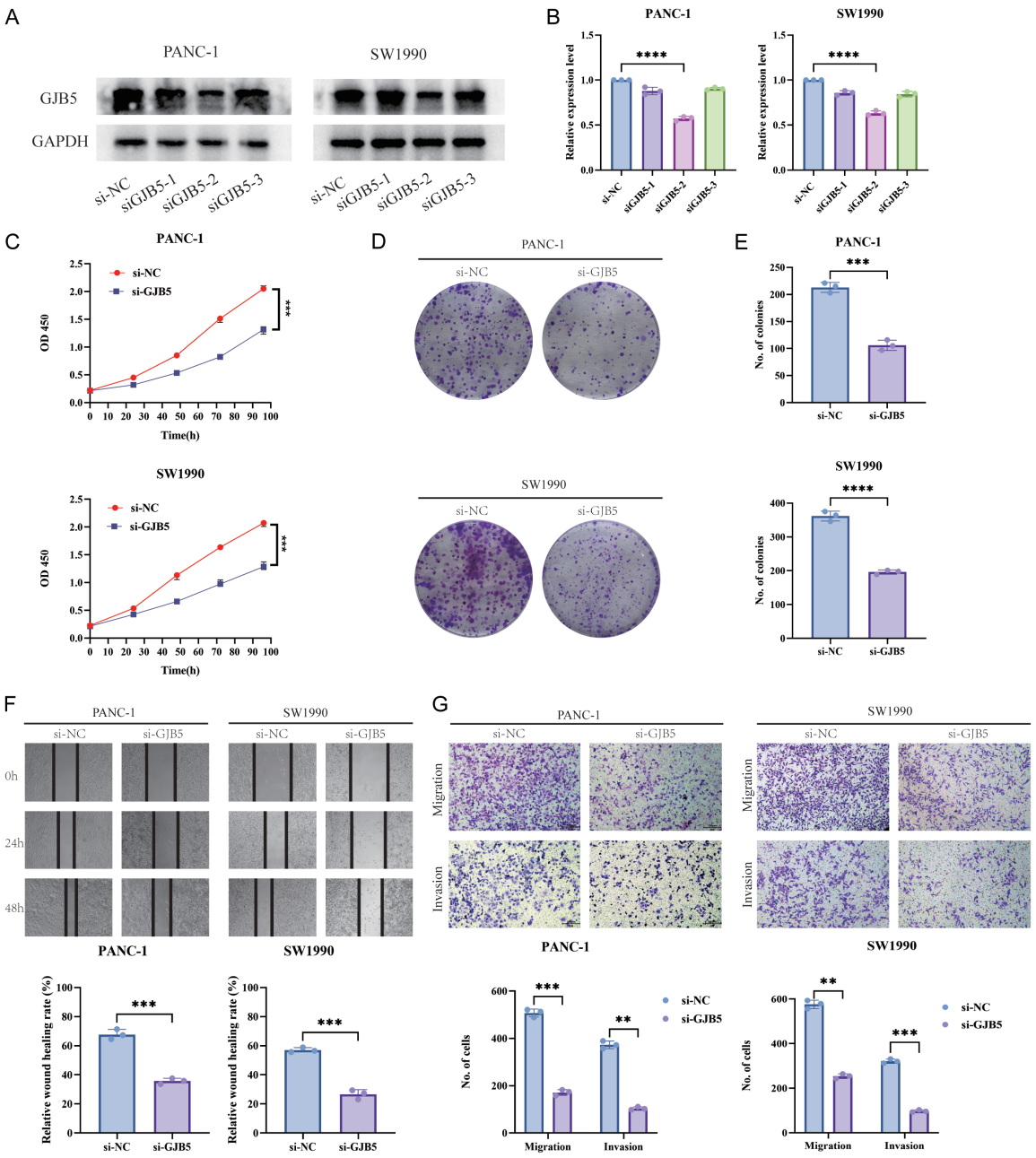


Figure 12. Knockdown of GJB5 markedly suppresses cell proliferation, migration, and invasion. The efficiency of siRNA was evaluated through WB in PANC-1 and SW1990 (A, B). CCK-8 assay and colony assay demonstrated that the proliferative ability of PANC-1 and SW1990 was inhibited in the GJB5 knockdown group (C-E). Wound healing assay exhibited that the wound widths of the GJB5 knockdown groups were significantly wider than the siNC group (F). The Transwell assay exhibited that migration and invasion of PANC-1 and SW1990 in the GJB5 knockdown group were significantly inhibited (G). (A, B) statistics performed by One-way analysis of variance, (C-G) statistics performed by unpaired t-test with Welch's correction (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

lations (Cor ≤ 0.3, P < 0.05). Overall, these results highlight the importance of GJB5 as an overexpressed gene and a significant marker of poor prognosis in PAAD.

Furthermore, multiple studies support the hypothesis that both innate and adaptive immune cells are essential for promoting tumorigenesis and tumor progression within the TME [37, 38]. The relationship between GJB5 and the TIME was analyzed, revealing positive correlations between GJB5 expression and the infiltration of chemokines (CCL7 and CCL13), immune checkpoint stimulators (TMEM173 and TNFSF9), and

immune cells (NK cells, active CD4+ T cells, and CD4+ Tcm). The results indicated that GJB5 can modulate the effectiveness of tumor immunotherapy by improving immune activity. The identification of suitable techniques to capitalize on this discovery, such as drugs that suppress GJB5 to increase immune cell infiltration, could have significant implications for future cancer research. This finding provides a foundation for potential therapeutic strategies to improve the immune response within the TME. It underscores the importance of developing new treatments to improve patient outcomes in oncology.

Furthermore, GSEA exhibited significant activation of key pathways involved in tumor proliferation and progression in high-GJB5 expression samples. Specifically, G2M checkpoint, EMT, E2F targets, TNFα signaling pathway, and p53 signaling pathways were significantly activated. These results are consistent with existing reports that underscore the role of these pathways in promoting tumor growth and progression [39-42]. Furthermore, the analysis of GJB5-related genes suggested their involvement in EMT activation, thereby promoting the initiation and development of tumors to a certain extent through synergistic effects.

To provide credible evidence for the role of GJB5 in PAAD, we selected PANC-1 and SW1990 cells and performed a series of cell experiments. Following GJB5 knockdown, the migration and invasion assay exhibited a significant impairment in cell migration. The wound healing assay and colony assay demonstrated a wider wound and fewer colonies in GJB5 knockdown groups compared to the siNC groups after the same time interval. Furthermore, the proliferative, migrative, and invasive competence of pancreatic cancer cells was significantly impaired according to the results of the colony assay, wound healing assay, and transwell assay. These findings provided additional evidence to support the promotive role of GJB5 in developing tumor cells in PAAD.

There are certain limitations of our study. The comprehensive identification of downstream molecules regulated by GJB5 expression remains a challenge, given current experimental limitations and the limited number of PAAD samples available for analysis. Furthermore, rigorous *in vivo* experiments are required to validate existing findings and identify novel aspects of GJB5's role in PAAD. Additionally, ongoing research endeavors are designed to address these aspects in future investigations and provide more conclusive insights into the subject matter.

The present study provides compelling evidence from multi-omics analysis, experimental studies, and clinical samples, establishing GJB5 as a significant contributor to tumor proliferation and migration. GJB5, a member of the Connexin family of proteins, is identified as a significant factor in PAAD. Identifying GJB5 as a promoter of tumor progression underscores its potential value as a novel biomarker. It provides promising opportunities to target GJB5 in developing innovative treatment strategies for PAAD.

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Disclosure of conflict of interest

None.

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