

***GABRP* is a potential prognostic biomarker and correlated with immune infiltration and tumor microenvironment in pancreatic cancer**

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Background: Pancreatic cancer is one of the most commonly diagnosed and lethal malignancies worldwide and has few good biomarkers and therapeutic targets. *GABRP* is the π subunit of the gamma-aminobutyric acid (GABA) A receptor, which is expressed in a number of non-neuronal tissues. *GABRP* is significantly upregulated in pancreatic cancer, but its biological and immunological role as well as its clinical diagnostic and prognostic value in pancreatic cancer is still incompletely known.

Methods: In this study, pancreatic adenocarcinoma (PAAD) cohorts from TCGA and GEO datasets were used to compare *GABRP* mRNA levels in cancerous and normal tissues and protein expression was evaluated using immunohistochemistry. The Kaplan-Meier plotter and GEPIA2 database were used to analyze the correlation between *GABRP* expression, overall survival, and disease-free survival in pancreatic cancer patients. Gene set enrichment analysis (GSEA) was performed with the Linked Omics database to explore the molecular mechanisms of *GABRP* in pancreatic cancer. And the correlation between *GABRP* expression and immune infiltration was explored using the TIMER database, CIBERSORT database and ESTIMATE algorithm.

Results: *GABRP* mRNA was significantly overexpressed in TCGA-PAAD cohorts ($P < 0.0001$) and enhanced *GABRP* expression predicted poorer overall survival according to Kaplan-Meier plotter database ($P = 0.0024$) and GEPIA2 ($P = 0.038$). Hypomethylation of promoter ($P < 0.01$) and the regulation of hsa-miR-3655 may contribute to the overexpression of *GABRP* in pancreatic cancer. GSEA analysis revealed that *GABRP* played an important role in the immune response. *GABRP* expression was also correlated with immune infiltration and immune cell markers. Higher *GABRP* expression was significantly associated with greater infiltration of immune cells and stromal cells into pancreatic cancer microenvironments as well as higher expression of six important immune check point genes including *PDCD1* ($P < 0.05$), *CD274* ($P < 0.05$), *CTLA4* ($P < 0.01$), *PDCD1LG2* ($P < 0.01$), *TIGIT* ($P < 0.01$) and *TIM3* ($P < 0.01$).

Conclusions: *GABRP* is a potential prognostic biomarker and is correlated with immune infiltration and tumor microenvironment in pancreatic cancer. This suggests that *GABRP* may serve as a potential prognostic biomarker and therapeutic target in pancreatic cancer as well as a possible regulator of tumor microenvironment affecting the efficacy of immunotherapy. Further studies are needed to elucidate the molecular mechanism of the immunoregulatory role of *GABRP*.

Keywords: *GABRP*; pancreatic cancer; immune infiltration; tumor microenvironment

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Introduction

Pancreatic cancer, primarily pancreatic ductal adenocarcinoma (PDAC), is one of the most commonly diagnosed malignancies which ranks 14th among all cancers and the 7th leading cause of cancer-related death worldwide. Global cancer statistics reveal that there were 495,773 new cases and 466,003 deaths of pancreatic cancer in 2020 (1). Pancreatic cancer is a fatal malignancy with a 5-year survival of about 5% when diagnosed and a median survival of <11 months (2). Surgical resection is the only potential curative treatment for pancreatic cancer at present and adjuvant chemotherapies have improved patient outcomes, but at the cost of a higher incidence of adverse effect (3,4). However, just a minority of pancreatic cancer patients are eligible for surgical therapy since the disease lacks early symptoms until developed into advanced stages (5). In recent years, targeted therapy and immunotherapy have brought therapeutic benefit in many solid tumors. Several targeted therapies such as *VEGF* inhibitors and *KRAS* inhibitors have been evaluated in pancreatic cancer, but received minimal effect. The same can be seen in immunotherapy, which shows promising efficacy in several solid tumor especially melanoma including *PD-1* inhibitors and *CTLA-4* inhibitors while the response rate is unsatisfactory in pancreatic cancer (6-8). Thus, it remains imperative to identify potential prognostic biomarkers and therapeutic targets to facilitate the clinical treatment and discovery of clinical candidate compounds and to exploit possible strategies of immunotherapy for pancreatic cancer.

GABRP is the π subunit of the gamma-aminobutyric acid (GABA) A receptor, which is a hetero-pentameric and ligand-gated chloride channel composed of five subunits. These subunits are encoded by 19 genes in 8 subclasses based on sequence homology including $\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, δ , ϵ , θ , π and $\rho 1-3$. *GABAA* receptors mainly distributed in the central nervous system which frequently consists of at least two α , two β subunits and one γ subunit. The γ subunit can be replaced by a δ , ϵ , π subunit, but the function of the π subunit is still unclear (9-11). Unlike other *GABAA* receptors, *GABRP* is detectable in multiple non-neuronal normal human tissues including uterus, prostate, ovaries,

small intestine and lung. It is reported that *GABRP* may play an important role in endometrial receptivity and control receptor sensitivity to endogenous steroids and pregnenolone (12-14).

Although the function of *GABRP* in normal tissues has not been fully elucidated, several studies have revealed dysregulation of *GABRP* expression in solid tumors including breast, ovarian, cervical and pancreatic cancer (15-18). Jiang *et al.* proposed that *GABRP* promoted pancreatic tumorigenesis via *KCNN4*-mediated Ca^{2+} signaling to regulate chemokine and macrophage recruitment, which indicated the significance and potential immunomodulatory role of *GABRP* in pancreatic cancer (18). However, there has been no study to comprehensively explore the expression, prognostic value and regulatory role of *GABRP* in pancreatic cancer and whether *GABRP* was associated with immune infiltration or could affect the tumor microenvironment. Here, we integrated multiple ‘omics’ data about pancreatic cancer from multiple authoritative databases and used bioinformatics analysis to comprehensively and systematically analyze the expression profile, clinicopathological characteristics and potential molecular mechanism of *GABRP* in pancreatic cancer. For the first time, we elucidated the potential mechanism of *GABRP* dysregulation and showed how *GABRP* might participate in regulating pancreatic tumor infiltrating cells and tumor microenvironment, which further confirmed the important role of *GABRP* as a prognostic biomarker and therapeutic target in pancreatic cancer as well as a possible regulator of tumor microenvironment affecting the efficacy of immunotherapy (Figure 1A). We present the following article in accordance with the REMARK reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2021/rc>).

Methods

Data collection

The data on *GABRP* mRNA expression in pancreatic adenocarcinoma (PAAD) tissues and normal tissues and corresponding clinical data were retrieved from the TCGA

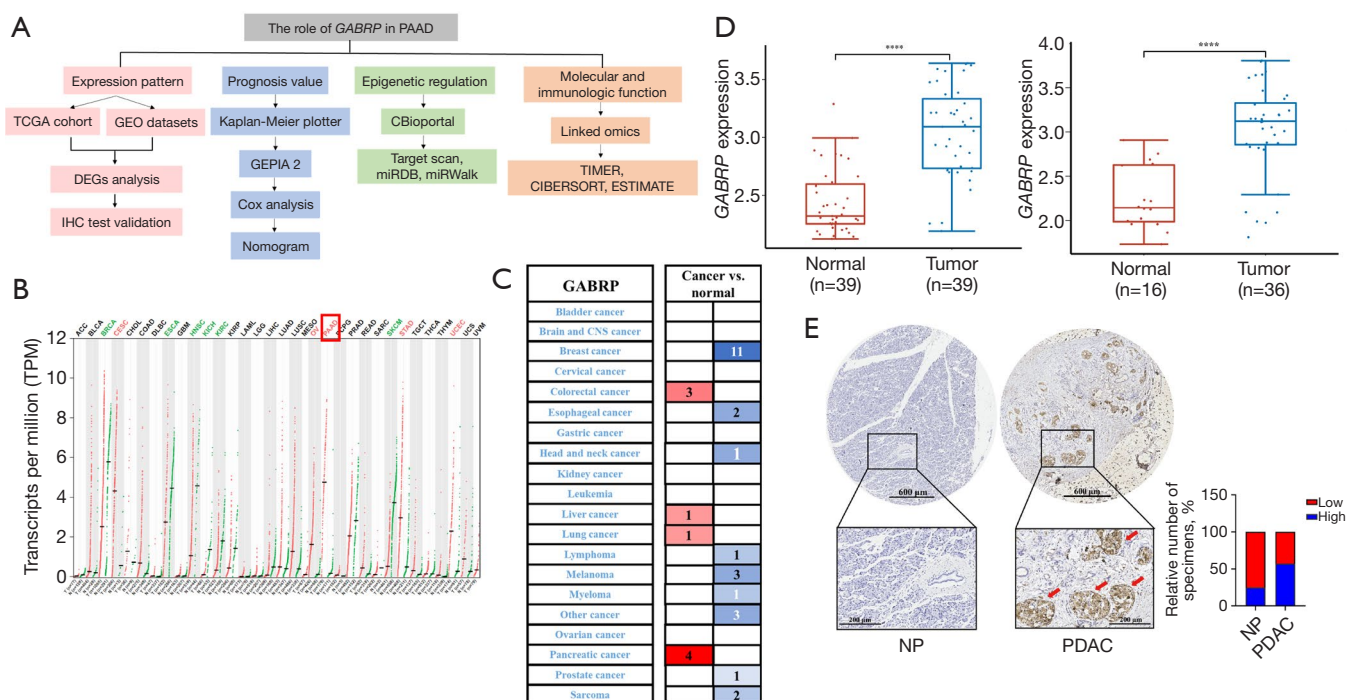


Figure 1 *GABRP* mRNA expression was upregulated in pancreatic cancer. (A) The flowchart of study procedures; (B) the expression level of *GABRP* was measured in different types of tumor tissues and compared with normal tissues in the GEPIA2 database; (C) the expression level of *GABRP* was measured in different types of tumor tissues and normal tissues using the Oncomine database (P value is 0.001, fold change is 2, and gene rankings apply to all); (D) *GABRP* expression in tumors and matching normal tissues using two independent cohorts (GSE15471, n=78; GSE16515, n=52) derived from Gene Expression Omnibus datasets; (E) immunohistochemical analysis of *GABRP* expression in a human PDAC tissue microarray. Representative *GABRP* images are shown in the left panel. The percentages of tissues displaying low and high staining in normal pancreas and PDAC tissues are shown in the middle panel. ****, $P < 0.0001$. TCGA, The Cancer Genome Atlas; GEO, Gene expression omnibus; DEGs, differential expressed genes; IHC, immunohistochemistry; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; STES, stomach and esophageal carcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; NP, normal pancreas; PDAC, pancreatic ductal adenocarcinoma.

database (<https://portal.gdc.cancer.gov/>). A total of 178 tumor tissues and 4 normal tissues obtained from The Cancer Genome Atlas (TCGA) database were included in the data analysis. The *GABRP* mRNA expression profiles of two pancreatic cancer datasets from the gene expression omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) were also included in the data analysis, which consisted of microarrays with accession number GSE15471

(GEO platform GPL570) which contained 39 tumor samples and 39 normal samples and the GSE16515 (GEO platform GPL570) which contained 36 tumor samples and 16 normal samples (19,20). The *GABRP* mRNA expression profiles of three pancreatitis datasets from the GEO database were also included in the data analysis including microarrays with the accession number, GSE15471 (GEO platform GPL6246), which contained six pancreatitis

samples and five normal samples, the GSE65146 (GEO platform GPL6246), which contained 64 pancreatitis samples and 9 normal samples, and the GSE41418 (GEO platform GPL1261) which contained 6 pancreatitis samples and 6 normal samples.

Differential expression analysis

Oncomine database analysis

The Oncomine database (<http://www.oncomine.org>) incorporated 86,733 samples and 715 gene expression datasets into a single comprehensive database allowing various analytical methods (21). We used the Oncomine database to evaluate the mRNA expression level of the *GABRP* gene among various cancer types. The threshold significance was determined by a P value of 0.001, fold-change of 2, gene rank of 'all' and data type of 'mRNA'.

GEPIA2 database analysis

The gene expression profiling interactive analysis 2 (GEPIA2) database (<http://GEPIA2.cancer-pku.cn/index.html>) was utilized to evaluate the expression of *GABRP* in different cancer types. Featuring 198,619 isoforms and 84 cancer subtypes, GEPIA2 (22) is an updated and enhanced version of GEPIA, a web resource for gene expression analysis based on tumor and normal samples from the TCGA and the GTEx databases. The gene expression profiles of *GABRP* among all tumor samples paired normal tissues were shown in a dot-plot.

Analysis of GEO microarray datasets

Two datasets, GSE15471 and GSE16515, were used to analyze the expression of *GABRP* in pancreatic cancer between normal tissues and tumor tissues. The data was processed by R software v4.0.3 and the plot was constructed using the R software package, ggplot2.

Survival analysis and construction of nomogram

The Kaplan-Meier plotter (www.kmplot.com) is a web-based tool for exploring the impact of 54,000 genes on patient survival covering 21 cancer types (23). The database is based on gene expression data, relapse-free and overall survival information downloaded from TCGA, GEO and the European Genome-Phenome Archive (EGA) database. The Kaplan-Meier plotter was used to perform prognostic analysis of *GABRP* and hsa-miR-3655 expression levels in PAAD, and the association between *GABRP* expression and

survival outcome based on different immune cell contents with auto-selected cutoff values. GEPIA2 was used to perform prognostic analysis of *GABRP* expression levels in PAAD based on *GABRP* expression with a specific cutoff value using the R software package 'survival'. The log rank P value, the hazard ratio (HR), and survival curves were calculated and annotated for each plot.

Univariate and multivariate Cox regression analysis was performed using the prognosis model from the Assistant for Clinical Bioinformatics (<https://www.aclbi.com/static/index.html#/prognosis>) to identify the appropriate terms to build the nomogram. The Forest was used to show the P value, HR and 95% CI of each variable through 'forestplot' in the R package. The variable that shows significance in multivariate Cox proportional analysis shows that it should be an independent indicator of prognosis. Based on the results of multivariate Cox proportional hazards analysis, a nomogram was developed to predict the X-year overall recurrence through the 'rms' R package.

Hypermethylation of GABRP promoter and prediction of GABRP regulatory miRNAs

The cBioPortal for cancer genomics was used to explore the relationship between *GABRP* DNA methylation and *GABRP* expression in TCGA-PAAD (24). TargetScan, miRDB and miRwalk were used to predict the regulatory miRNAs of *GABRP* (25-27). The common miRNAs predicted by the above three databases were included in the prediction cohort. The common miRNAs significantly downregulated in PAAD in the three cohorts were then regarded as potential regulatory miRNAs of *PARPBP* in HCC. UALCAN (28) (<http://ualcan.path.uab.edu/index.html>) is an interactive web-portal web resource to perform comprehensive analysis of TCGA gene expression data. We used UALCAN to assess the methylation levels of *GABRP* and the expression of miR-3655 in PAAD and its effect on prognosis.

LinkedOmics database analysis

LinkedOmics is a publicly available portal that includes multi-omics data from all 32 TCGA cancer types and provides a unique platform for molecular biologists and clinicians to access, analyze and compare multi-omics cancer data within and across tumor types (29). We searched for *GABRP* co-expressed genes in the PAAD dataset (n=178) based on the Pearson correlation coefficient. In the LinkInterpreter module, gene set enrichment analysis

(GSEA) module was used to assess the biological processes (BPs), cell components (CCs), molecular function (MFs), and KEGG pathways based on co-expressed genes. The GSEA program was run with 500 simulations. If the P value and false discovery rate (FDR) were both less than 0.05, the gene set was considered significantly enriched.

TIMER database analysis

The tumor immune estimation resource (TIMER, <https://cistrome.shinyapps.io/timer/>) is a web server designed for comprehensive analysis of tumor-infiltrating immune cells in 32 cancer types from TCGA (30). The TIMER database was used to analyze the relationship between the level of *GABRP* expression and six tumor-infiltrating immune cells (TIICs) and tumor purity in PAAD. The six TIICs include B cells, CD4⁺ T cells, CD8⁺ T cells, macrophages, neutrophils, and dendritic cells. The SCNA (somatic copy number alteration) module was used to compare tumor infiltration levels in PAAD with different somatic copy number alterations for *GABRP*. Then the correlation between *GABRP* and several immune cell markers were assessed by using the correlation module (31).

CIBERSORT database analysis

Cell type identification by estimating relative subsets of RNA transcripts (CIBERSORT, <https://cibersort.stanford.edu/>) is a deconvolution algorithm developed to characterize 22 types of immune cells composition in complex tissues (32). By integrating TCGA-PAAD datasets, GSE15471 and GSE16515 datasets, we analyzed the integrated mRNA expression matrix using the CIBERSORT R script acquired from the CIBERSORT website. After filtering samples with $P \geq 0.05$, 59 normal samples and 205 tumor samples were included in the study.

Estimation of tumor microenvironment components in PAAD

The ESTIMATE algorithm (33) uses gene expression signatures to estimate the fraction of stromal cells and immune cells and calculates the immune score and stromal score of PAAD tumor samples. The algorithm was implemented using the 'estimate' package by R software. Two subtypes of PAAD were classified based on *GABRP* expression (high *vs.* low) and compared using the Kruskal-Wallis test.

Immunohistochemistry (IHC) analysis

The PAAD chip (Cat. No. HPanA060CS03-M-109) was purchased from SuperChip in Shanghai, China. The chip contained two normal pancreatic tissues and 29 PAAD tumor and adjacent tumor tissues, which included phases I (3 cases), II (23 cases) and III (3 cases) PAAD tumor tissues. After routinely dewaxing and hydration, antigen retrieval was performed in 10 mM saline sodium citrate at sub-boiling temperature for 90 s. Then, the activity of endogenous peroxidases in specimens was blocked with 3% hydrogen peroxide. After permeabilization with 0.1% Triton X-100 and blocking with 5% bovine serum albumin (BSA), the chip was incubated with primary antibody against rabbit anti-*GABRP* antibody (1:200 dilution, PA5-46830, Invitrogen) at 4 °C overnight. The chip was balanced at room temperature for 30 min, washed with PBS and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (DAKO, Wuhan, China) for 60 min at room temperature. *GABRP* expression was visualized by DAB (DAKO, Wuhan, China) staining. The chip was scanned by Panoramic Scan (3DHISTECH Ltd., Hungary).

Statistical analysis

The Wilcoxon rank sum test was employed to explore the different expression pattern between PAAD tumor samples and normal samples. The PAAD cohorts were divided into two groups using the optimal threshold evaluated by X-tile 3.6.1 on the basis of *GABRP* expression levels (34). The relationship between *GABRP* expression and clinicopathological parameters was analyzed using Chi-squared test. The correlation between *GABRP* and TIICs was calculated by Spearman correlation. The Cox proportional hazards model was used to identify the independent indicators related to OS, and the hazard ratios (HRs) with 95% confidence intervals (CI) were also calculated. Nomograms were constructed based on the results of multivariate Cox analysis on the basis of OS to predict the X-year overall recurrence using the 'rms' R package. All statistical analyses were performed using R software (Version 4.0.2), and the threshold of statistical significance was set at $P < 0.05$.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional

Table 1 Association between *GABRP* expression and the clinical parameters in patients with PAAD in TCGA-PAAD cohort

Variables	Total (N=178)	<i>GABRP</i> expression		P value
		High (N=111)	Low (N=67)	
Age (years), n (%)				1
<65	82 (46.1)	51 (45.9)	31 (46.3)	
≥65	96 (53.9)	60 (54.1)	36 (53.7)	
Gender, n (%)				0.0809
Male	98 (55.1)	55 (49.5)	43 (64.2)	
Female	80 (44.9)	56 (50.5)	24 (35.8)	
Grade, n (%)				0.01938*
G1	31 (17.4)	14 (12.6)	17 (25.4)	
G2	95 (53.4)	61 (55.0)	34 (50.7)	
G3	48 (27.0)	35 (31.5)	13 (19.4)	
G4	2 (1.1)	0 (0)	2 (3.0)	
Unknown	2 (1.1)	1 (0.9)	1 (1.5)	
TNM stage, n (%)				0.4281
I	21 (11.8)	10 (9.0)	11 (16.4)	
II	147 (82.6)	96 (86.5)	51 (76.1)	
III	3 (1.7)	2 (1.8)	1 (1.5)	
IV	4 (2.2)	3 (2.7)	1 (1.5)	
Unknown	3 (1.7)	0 (0)	3 (4.5)	
Living status, n (%)				1
Alive	71 (39.9)	36 (32.4)	35 (52.2)	
Dead	107 (60.1)	75 (67.6)	32 (47.8)	

*, P<0.05. PAAD, pancreatic adenocarcinoma; TCGA, The Cancer Genome Atlas.

ethical approval and informed consent were waived.

Results

GABRP expression was upregulated in pancreatic cancer

The mRNA expression of *GABRP* in different cancers and normal tissues was evaluated by analyzing the GEPIA2 and Oncomine databases (Figure 1). In GEPIA2, the expression of *GABRP* was obviously elevated in PAAD compared to normal tissues (Figure 1B). In the Oncomine database, the expression of *GABRP* was significantly higher in PAAD than normal tissues (Figure 1C). Next, the expression of *GABRP* in pancreatic cancer was further validated by analyzing two

GEO cohorts (GSE15471 and GSE16515) (19,20,35,36). In GEO cohort, GSE15471 and GSE16515 (Figure 1D), *GABRP* was significantly overexpressed in tumor samples compared to normal pancreas tissues. To further check the expression pattern of *GABRP* protein in PDAC, immunohistochemical (IHC) analysis was carried out in tissue microarrays of human PDAC. It was shown in Figure 1E that *GABRP* expression is higher in PDAC than in normal pancreas, while commonly detected in the PDAC cells (Figure 1E). However, there was no significant change in the expression of *GABRP* in pancreatitis (Table S1). In summary, *GABRP* was highly expressed in pancreatic cancer.

Association between GABRP expression, clinicopathological characteristics and survival outcomes

The clinicopathological data of 178 PAAD patients were obtained from the TCGA database and separated into low or high *GABRP* expression based on the optimal threshold of OS. As shown in Table 1, there was no significance in age, gender, TNM stage and living status while grade was significantly associated with the expression level of *GABRP* (P<0.05). The correlation between *GABRP* expression and survival outcome of PAAD was next investigated using the Kaplan-Meier plotter. Results from Kaplan-Meier (KM) survival analysis showed that elevated expression of *GABRP* was linked to poor prognosis in PAAD. Patients with higher *GABRP* expression had shorter overall survival than those with lower *GABRP* expression (Figure 2A, HR =1.91, P=0.0024). Similarly, statistically significant lower disease-free survival (DFS) was observed in patients with higher *GABRP* expression (Figure 2B, HR =5, P=0.00073). GEPIA2 was used to confirm the effect of *GABRP* expression on pancreatic cancer prognosis (Figure 2C,2D). Similar results from GEPIA2 disclosed that patients with high *GABRP* expression had worse prognosis in TCGA PAAD cohorts.

Expression of GABRP is an independent prognostic indicator of PAAD

The prognostic value of *GABRP* in PAAD was first assessed by using the Kaplan-Meier plotter and GEPIA2. In the next step, Cox regression analysis was performed to explore independent risk factors of OS by using data from the TCGA PAAD cohort. In the univariate model, *GABRP* expression, age and grade were significantly related to OS in PAAD (Figure 3A, all P<0.05). Multivariate analysis further

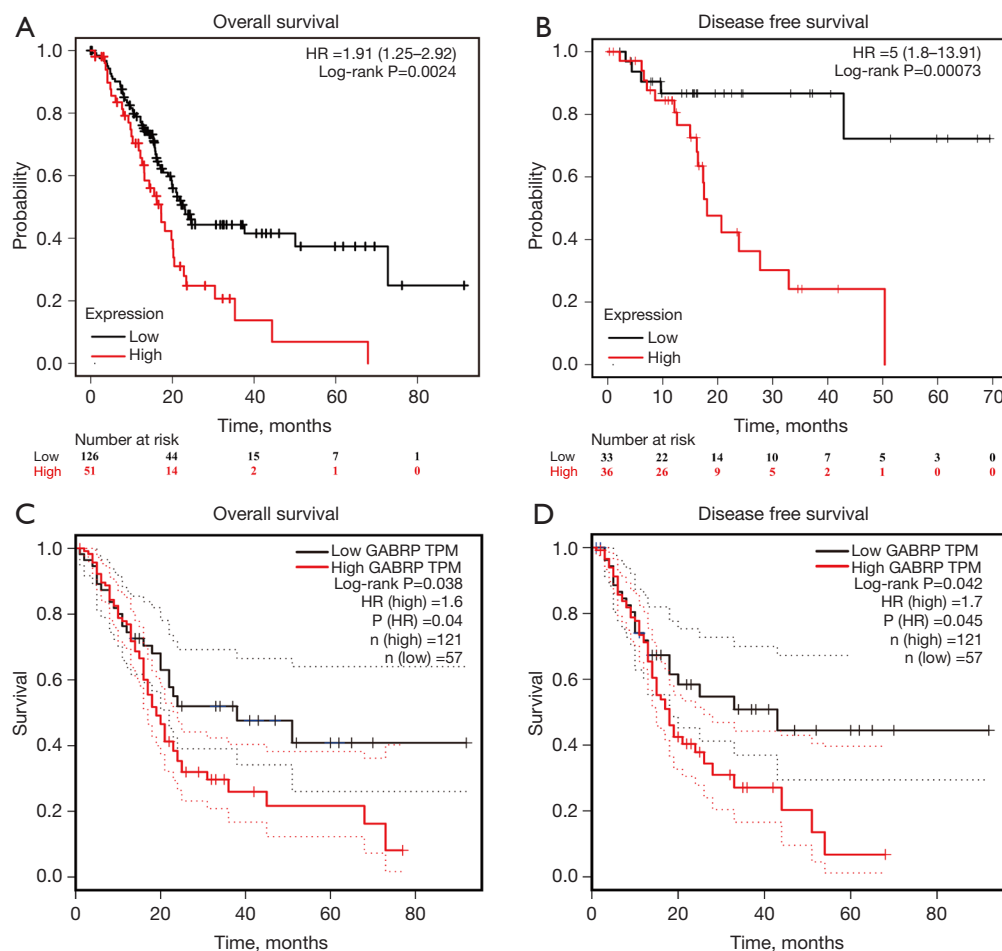


Figure 2 Overexpressed *GABRP* independently predicts poor OS in PAAD. (A,B) Correlation between *GABRP* and prognosis of pancreatic cancer in Kaplan-Meier plotter database; (C,D) correlation between *GABRP* and prognosis of pancreatic cancer in GEPIA2 database. OS, overall survival; PAAD, pancreatic adenocarcinoma.

confirmed that *GABRP* expression was an independent risk factor of OS in PAAD [Figure 3B, HR = 1.09879 (1.00705, 1.19888), P = 0.03419]. Finally, a prognostic nomogram for PAAD was constructed to further predict the prognostic value of *GABRP* in PAAD based on mRNA expression and *GABRP* expression was identified as an independent indicator of OS. As shown in Figure 3C, a nomogram was generated to assess the performance of the risk score in combination with the *GABRP* expression of PAAD patients in predicting the OS. The concordance index (c-index) value was 0.541 for PAAD, which measures the level of agreement between prediction by nomogram and the observed survival status. The calibration curves for 1-, 2-, 3- and 5-year survival were also plotted for PAAD (Figure 3D).

Promoter hypomethylation and hsa-miR-3655 downregulation may contribute to *GABRP* upregulation in PAAD

The potential mechanism of *GABRP* overexpression in PAAD was explored from the perspective of epigenetic regulation. Increasingly, studies have revealed abnormal DNA methylation in cancers and many non-neoplasm diseases. Promoters with cancer-associated hypomethylation are always linked to the activation of genes that were silent in normal tissues (37). The analysis via cBioportal revealed a significant negative correlation between *GABRP* methylation and *GABRP* expression (Figure 4A, Spearman's $r = -0.77$, $P = 6.52 \times 10^{-37}$, Pearson's $r = -0.66$, $P = 1.40 \times 10^{-23}$).

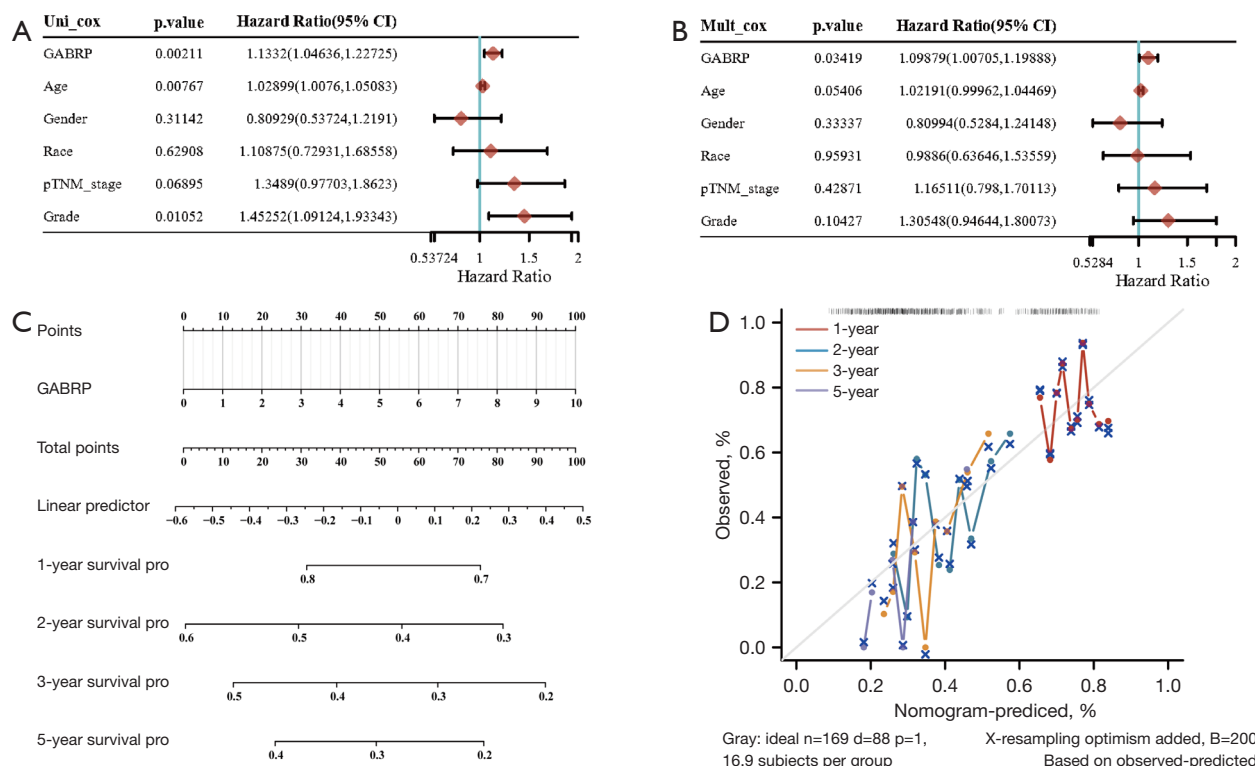


Figure 3 Overexpressed *GABRP* independently predicts poor OS in PAAD. (A) Univariate Cox proportional hazards regression model analysis based on overall survival in PAAD; (B) multivariate Cox proportional hazards regression model analysis based on overall survival in PAAD; (C) postoperative prognostic nomogram for patients with PAAD; (D) the calibration curve of the nomogram for predicting overall survival at 1, 2, 3 and 5 years. Observed OS was plotted on the y-axis and nomogram predicted probability of OS was plotted on the x-axis. OS, overall survival; PAAD, pancreatic adenocarcinoma.

In addition, UALCAN was used to verify the promoter methylation of *GABRP* in PAAD, and the level of *GABRP* promoter methylation in PAAD was significantly lower than in normal tissues (Figure 4B). Patients with PAAD were stratified based on the stage, grade, nodal metastasis status and TP53 mutant status. The results showed that lower levels of *GABRP* promoter methylation were associated with higher-grade tumors, advanced individual cancer stages, lymph node metastasis and TP53 mutant status (Figure S1A-S1D).

We next explored the potential regulatory miRNAs of *GABRP* in PAAD using miRDB, TargetScan and MirWalk. Seventeen common miRNAs were selected as candidates containing *GABRP*-binding sequences (Figure 4; Table S2). After evaluation with UALCAN, hsa-miR-3655 was identified as a potential regulatory miRNA of *GABRP*. Hsa-miR-3655 was significantly downregulated in PAAD compared with normal tissues in the TCGA-PAAD dataset (Figure 4D, $P=0.0067$). KM analysis revealed that low

expression of hsa-miR-3655 was associated with worse survival (Figure 4E, $P=0.015$).

Analysis of co-expressed genes of *GABRP* in PAAD

LinkedOmics was used to explore the co-expressed genes of *GABRP* in PAAD which should shed light on the biological meaning of *GABRP* in the process of tumorigenesis and development. A total of 6,647 genes had co-occurrences in PAAD (false discovery rate, FDR < 0.01). A total of 4,100 genes (red dots) were significantly positively correlated with *GABRP* expression and 2,547 genes (green dots) were significantly negatively correlated with *GABRP* expression in PAAD (Figure S2A). The top 50 ranked genes significantly correlated with *GABRP* expression were visualized in a heatmap (Figure S2B,S2C). *GABRP* expression showed a strong negative association with expression of *PENT* ($r=-0.5772$, $P=3.34E-17$), *ATP6AP1* ($r=-0.5402$, $P=7.08E-15$), *HDAC5* ($r=-0.5217$, $P=8.17E-14$),

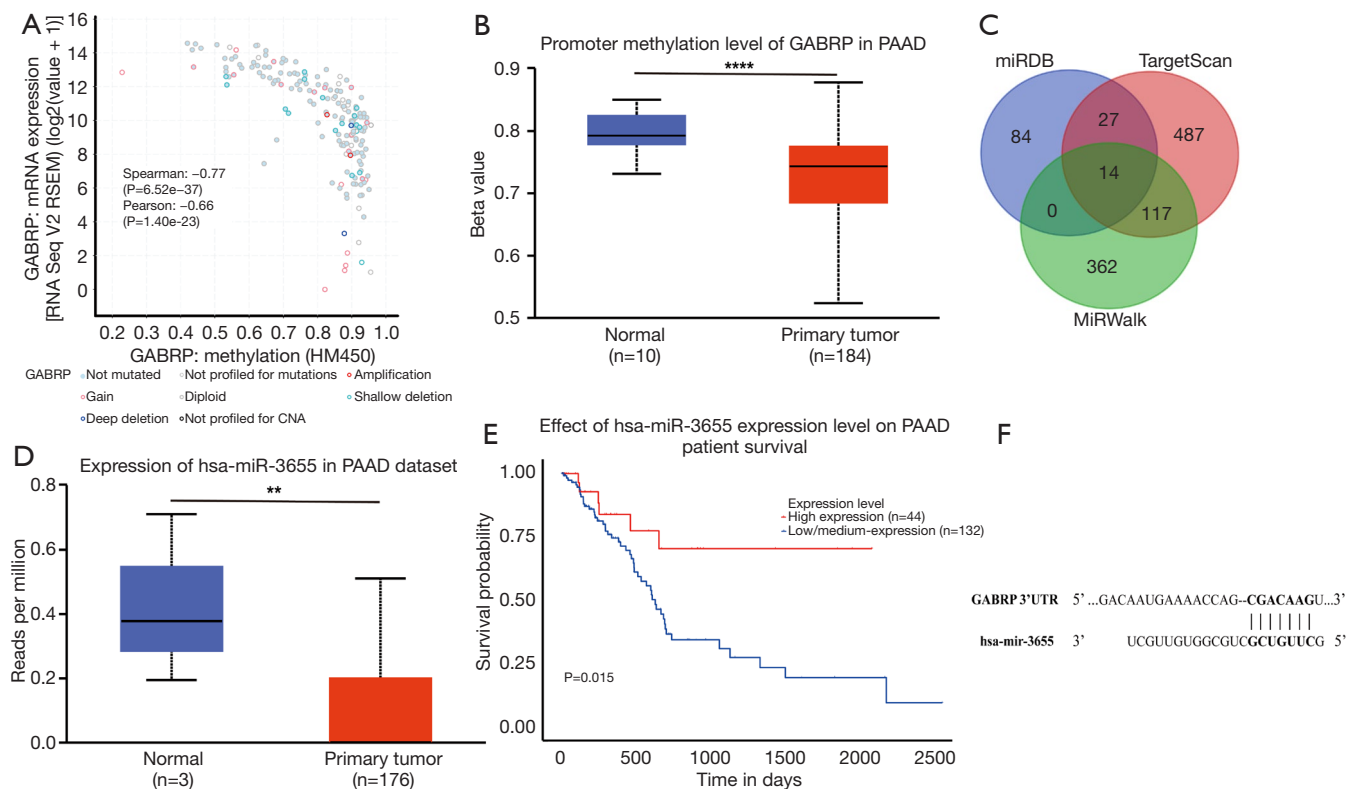


Figure 4 Promoter hypomethylation and miR-3655 downregulation contributed to *GABRP* upregulation in PAAD (A) Correlation analysis between *GABRP* expression and *GABRP* methylation; (B) comparison of *GABRP* promoter methylation level in tumor tissues compared with normal tissues; (C) common miRNAs containing *GABRP*-binding sequences using miRDB, TargetScan and MirWalk databases; (D) expression of miR-3655 in PAAD tissues compared with normal tissues; (E) low miR-3655 predicts worse survival in PAAD; (F) putative binding site of *GABRP* 3'-UTR by miR-3655. **, $P < 0.01$; ****, $P < 0.0001$. PAAD, pancreatic adenocarcinoma.

etc. (Figure S2B). *GABRP* expression also showed a strong positive association with expression of *GTB4* ($r=0.6364$, $P=1.33 \times 10^{-21}$), *MMP7* ($r=0.6193$, $P=3.14 \times 10^{-20}$), *LOC284578* ($r=0.6085$, $P=2.06 \times 10^{-19}$), etc. (Figure S2C). GSEA of co-expressed genes of *GABRP* was performed to explore the underlying biological functions of *GABRP* overexpression in PAAD. Gene ontology (GO) term annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis by GSEA are presented in Figure 5. In biological processes (BPs), *GABRP* co-expressed genes were correlated with regulation of leukocyte activation, immune response-regulating signaling pathway, adaptive immune response, etc. (Figure 5A). In cellular components (CCs), *GABRP* co-expressed genes were related to immunological synapse, cell substrate junction, MHC protein complex, etc. (Figure 5B). In molecular functions (MFs), *GABRP* co-expressed genes were associated with collagen binding, cytokine receptor activity, cytokine binding, etc. (Figure 5C). KEGG pathway

analysis showed enrichment in the NOD-like receptor signaling pathway, NF-kappa B signaling pathway, cytokine-cytokine receptor interaction, etc. (Figure 5D). The GO analysis and KEGG analysis results revealed that *GABRP* co-expressed genes had a widespread impact on immune responses.

Differential immune infiltration landscape of pancreatic cancer and normal tissues

We systematically depicted the pattern of immune cells in pancreatic cancer by extracting and processing the signature gene expression profiles from TCGA-PAAD and GEO cohorts (GSE15471 and GSE16515) performed by CIBERSORT. After filtering the samples for $P \geq 0.05$, 59 normal samples and 205 tumor samples were included in the analysis. The differential immune infiltration landscape of pancreatic cancer and normal tissues was displayed

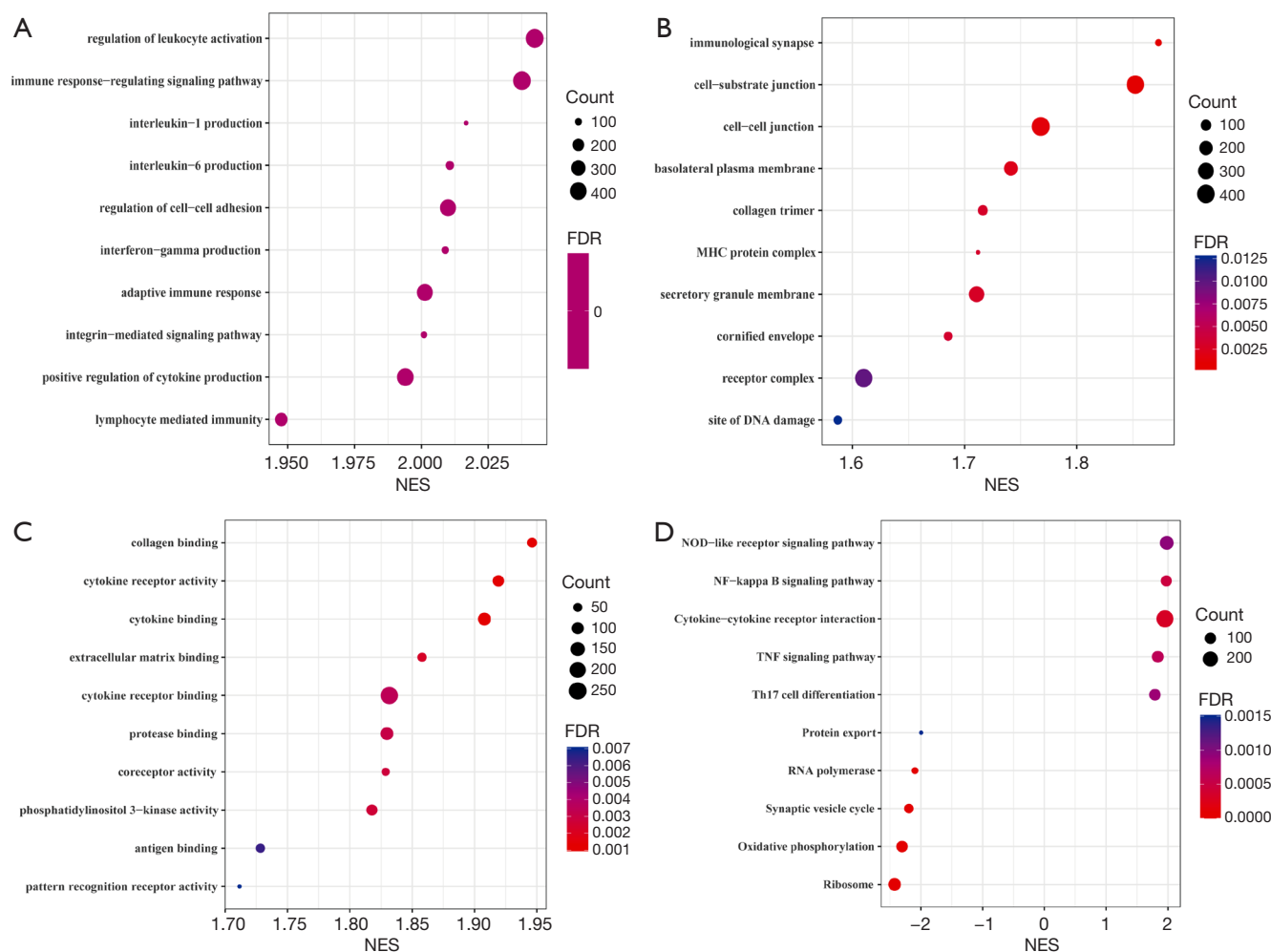


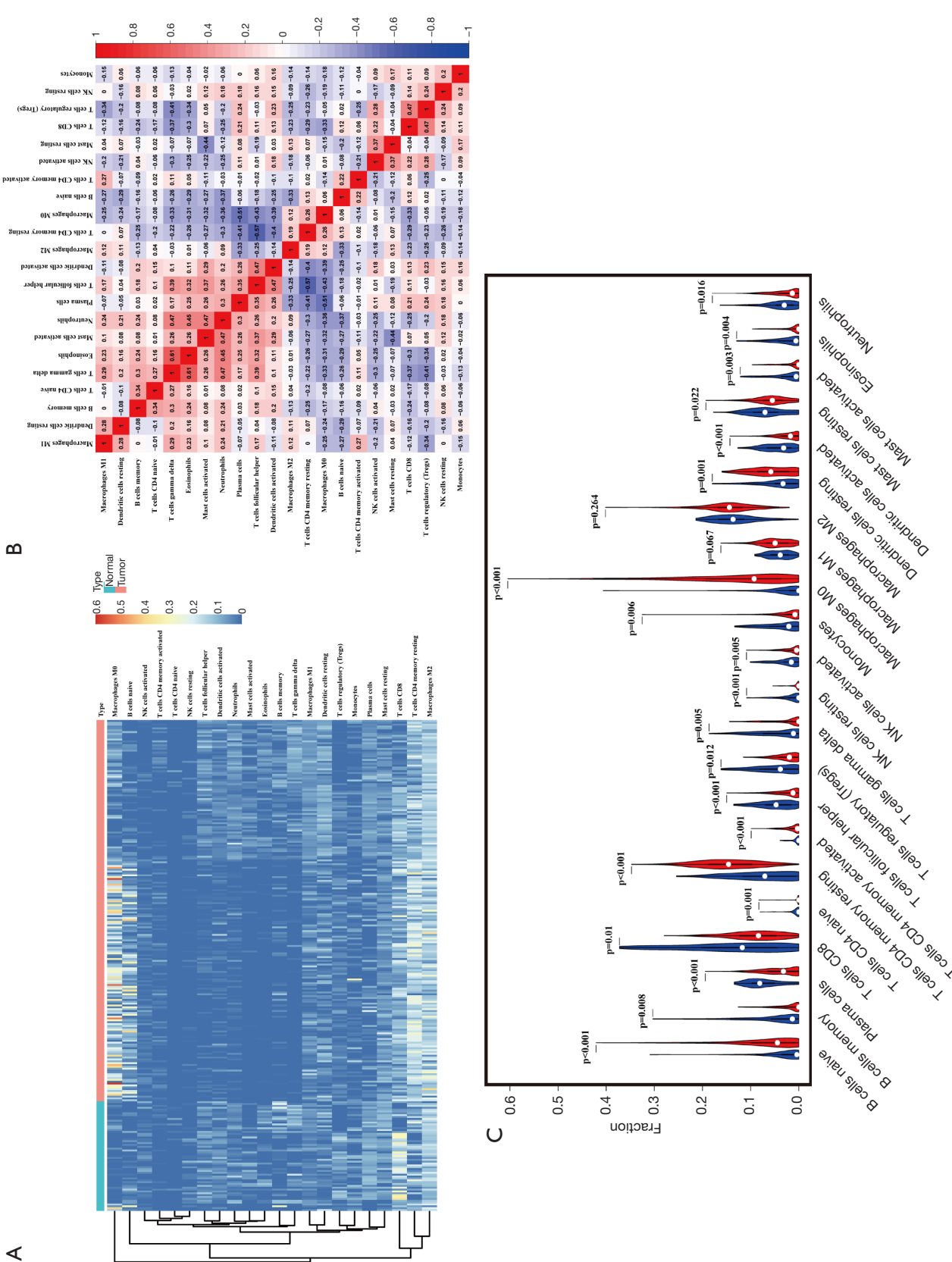
Figure 5 Analysis of genes co-expressed with *GABRP* in PAAD. The bubble plot for (A) GO-BP, (B) GO-CC and (C) GO-MF and (D) KEGG pathway analysis by GSEA. NES, normalized enrichment score; PAAD, pancreatic adenocarcinoma; GO, Gene ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; FDR, false discovery rate.

in Figure 6. There was a different pattern of infiltrating immune cells between normal tissues and pancreatic cancer tissues (Figure 6A). Moreover, different correlation patterns among 22 immune cells were found in pancreatic cancer and normal tissues (Figure 6B). In the study cohort, the fractions of 22 immune cells were correlated with each other in different degrees suggesting a complex tumor infiltrating environment in pancreatic cancer. The violin plot of 22 immune cells showed that the proportion of different infiltrating immune cells significantly varied between tumor and normal tissues except for macrophages M1 and M2 (Figure 6C). Compared to normal tissues, the fractions of naïve B cells, CD4⁺ T cells and macrophage M0 were relatively higher in tumor tissues while CD8⁺ T cells

and NK cells were significantly lower in tumor tissues.

Correlation between *GABRP* expression and immune infiltration in pancreatic cancer

Based on the results of GSEA analysis, we first assessed the relationship between *GABRP* and six types of infiltrating immune cells including B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils and dendritic cells by accessing the TIMER database (Figure 7A). The results suggested that the expression level of *GABRP* was positively correlated with the infiltration degree of B cells ($r=0.22$, $P=3.86E-03$), CD8⁺ T cells ($r=0.298$, $P=7.73E-05$), macrophages ($r=0.194$, $P=1.10E-02$), neutrophils ($r=0.277$, $P=2.41E-04$)



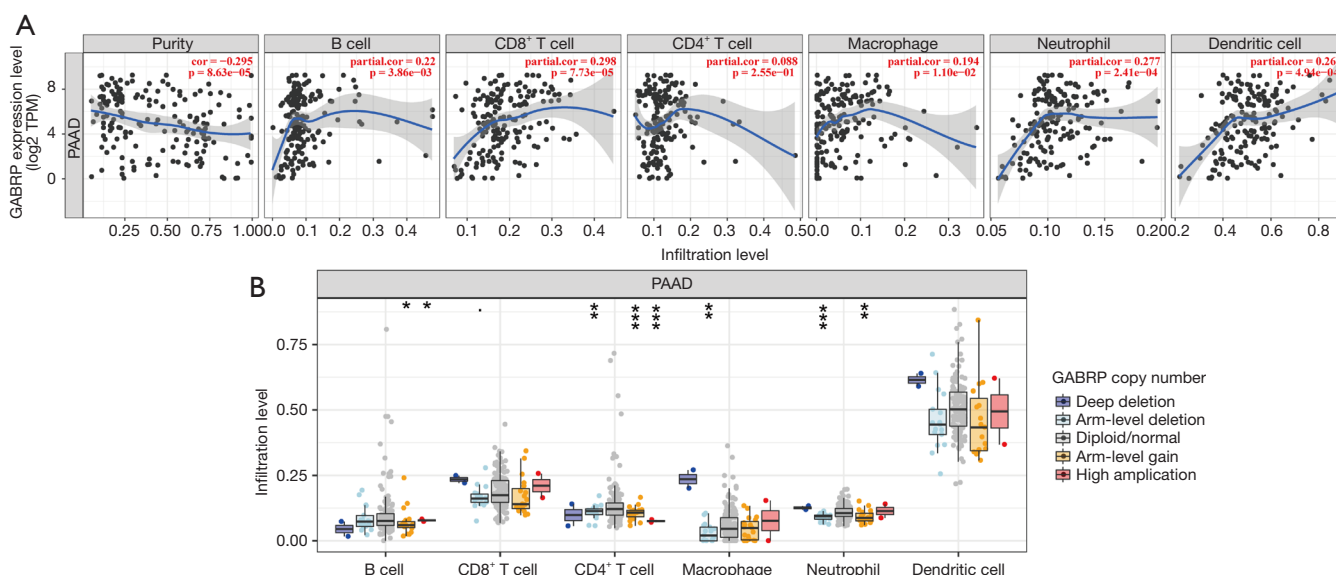


Figure 7 Correlation between *GABRP* expression and immune infiltration in pancreatic cancer. (A) Association between *GABRP* expression and immune cell infiltration in PAAD; (B) associations between *GABRP* gene copy numbers and immune cell infiltration in PAAD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. PAAD, pancreatic adenocarcinoma.

and dendritic cells ($r = 0.264$, $P = 4.94E-04$) in PAAD. In addition, a higher *GABRP* expression level was associated with lower tumor purity ($r = -0.295$, $P = 8.63E-05$), which may contribute to worse clinical features and outcomes (38). Genes with somatic copy number alterations (sCNAs) could influence tumorigenesis, progression and immunotherapy response (39). *GABRP* copy number alterations were related to immune cell infiltration by B cells, CD4⁺ T cells, macrophages, and neutrophils in PAAD (Figure 7B).

Correlation between *GABRP* expression and immune marker expression in pancreatic cancer

In order to clarify the reason why *GABRP* was associated with immune infiltration, we further explored the correlations between *GABRP* expression and a series of immune markers related to various types of immune cells using the TIMER database. Several immune cells were characterized by immune markers including B-cells, CD8⁺ T cells, neutrophils, macrophages, dendritic cells, Th1 cells, Th2 cells and Treg cells, and their correlation with *GABRP* expression was determined using the TIMER database (40,41). As shown in Table 2, *GABRP* expression had a significantly positive correlation with immune makers of B cells, CD8⁺ T cells, neutrophils, dendritic cells, Th1 cells, Th2 cells and Treg cells, without regard to age or

tumor purity. The macrophage markers were significantly correlated with *GABRP* expression, except for *CD84* and *CD163* in the condition adjusted by tumor purity optionally. The above analysis laid the foundation for the important role of *GABRP* in immune infiltration.

Prognostic analysis of *GABRP* based on different immune cell contents in pancreatic cancer

Given the effect of *GABRP* on prognosis and immune cell infiltration in PAAD, we determined whether *GABRP* expression could affect the prognosis of pancreatic cancer due to immune cell infiltration. We performed a prognostic analysis of *GABRP* based on different immune cell contents in PAAD using the Kaplan-Meier plotter. The results indicated that higher *GABRP* expression related to worse prognosis in terms of enriched B cells, decreased CD4⁺ T cells, enriched CD8⁺ T cells, decreased macrophages, enriched NK cells, and decreased Treg cells compared to decreased B cells, enriched CD4⁺ T cells, decreased CD8⁺ T cells, enriched macrophages, decreased NK cells, and enriched Treg cells (Figure 8). In addition, the different profiles of CD4⁺ T cells and macrophages could considerably alter the effect of *GABRP* expression on survival outcome of PAAD (Figure 8C, 8D, 8G, 8H). The above results suggested that higher expression of *GABRP* may affect survival of

Table 2 Correlation analysis between *GABRP* and immune cell type markers in PAAD using TIMER database

Cell type	Gene markers	PAAD					
		None		Purity		Age	
		Cor	P	Cor	P	Cor	P
B cells	<i>CD19</i>	0.263	3.73E-04	0.214	4.94E-03	0.264	3.76E-04
	<i>CD79A</i>	0.26	4.60E-04	0.201	8.24E-03	0.262	4.12E-04
CD8 ⁺ T cells	<i>CD8A</i>	0.325	1.02E-05	0.279	2.15E-04	0.323	1.10E-05
	<i>CD8B</i>	0.332	6.45E-06	0.282	1.87E-04	0.331	6.44E-06
Neutrophils	<i>CEACAM3</i>	0.372	3.00E-07	0.311	3.50E-05	0.37	3.72E-07
	<i>CSF3R</i>	0.295	6.59E-05	0.202	7.99E-03	0.293	7.26E-05
	<i>FCGR3B</i>	0.24	1.26E-03	0.158	3.85E-02	0.238	1.37E-03
	<i>CCR7</i>	0.236	1.54E-03	0.19	1.28E-02	0.225	2.51E-03
Macrophages	<i>CD68</i>	0.268	3.01E-04	0.182	1.72E-04	0.26	4.58E-04
	<i>CD84</i>	0.2	7.45E-03	0.108	1.58E-01	0.2	7.59E-03
	<i>CD163</i>	0.233	1.73E-03	0.13	8.91E-02	0.229	2.03E-03
	<i>MS4A4A</i>	0.256	5.60E-04	0.153	4.53E-02	0.225	5.87E-04
Dendritic cells	<i>CD1C</i>	0.271	2.47E-04	0.221	3.60E-03	0.258	4.95E-04
	<i>ITGAX</i>	0.297	5.89E-05	0.222	3.51E-03	0.291	7.96E-05
	<i>NRP1</i>	0.265	3.62E-04	0.201	8.36E-03	0.248	8.60E-04
Th1 cells	<i>STAT1</i>	0.393	6.66E-08	0.336	6.99E-06	0.391	6.73E-08
	<i>TBX21</i>	0.226	2.39E-03	0.184	1.60E-02	0.226	2.38E-03
Th2 cells	<i>GATA3</i>	0.27	2.72E-04	0.248	1.09E-03	0.263	3.97E-04
	<i>STAT6</i>	0.42	6.59E-09	0.413	2.02E-08	0.417	6.81E-09
Treg	<i>CCR8</i>	0.3	4.56E-05	0.24	1.58E-03	0.291	8.20E-05
	<i>FOXP3</i>	0.296	6.36E-05	0.229	2.64E-03	0.287	1.03E-04

PAAD, pancreatic adenocarcinoma.

pancreatic cancer partly due to immune infiltration.

Evaluation of tumor microenvironment in pancreatic cancer based on *GABRP* expression

As one of the most lethal solid tumors worldwide, pancreatic cancer is characterized by an immunosuppressive and obstructive tumor microenvironment as well as a highly malignant phenotype (42). Thus, gaining insight into the tumor environment is critical to improving immunotherapy. Given the impact of *GABRP* expression on immune infiltration in pancreatic cancer, we speculated whether the expression level of *GABRP* was associated with the status of the tumor microenvironment. ESTIMATE was used to

evaluate the composition and infiltration of immune cells and stromal cells in the tumor microenvironment using gene expression signatures based on *GABRP* expression. Compared to the cohorts with lower *GABRP* expression, higher *GABRP* expression was significantly correlated with higher immune score, stromal score and lower tumor purity (*Figure 9A-9D*). Furthermore, we examined the expression of six immune checkpoint genes including *PDCD1*, *CD274*, *CTLA4*, *PDCD1LG2*, *TIGIT* and *TIM3*, which participated in immune escape and affected immunotherapy response. The results showed that expression of the above immune checkpoint genes was significantly upregulated in the cohort with higher *GABRP* expression (*Figure 9E-9J*, $P < 0.05$).

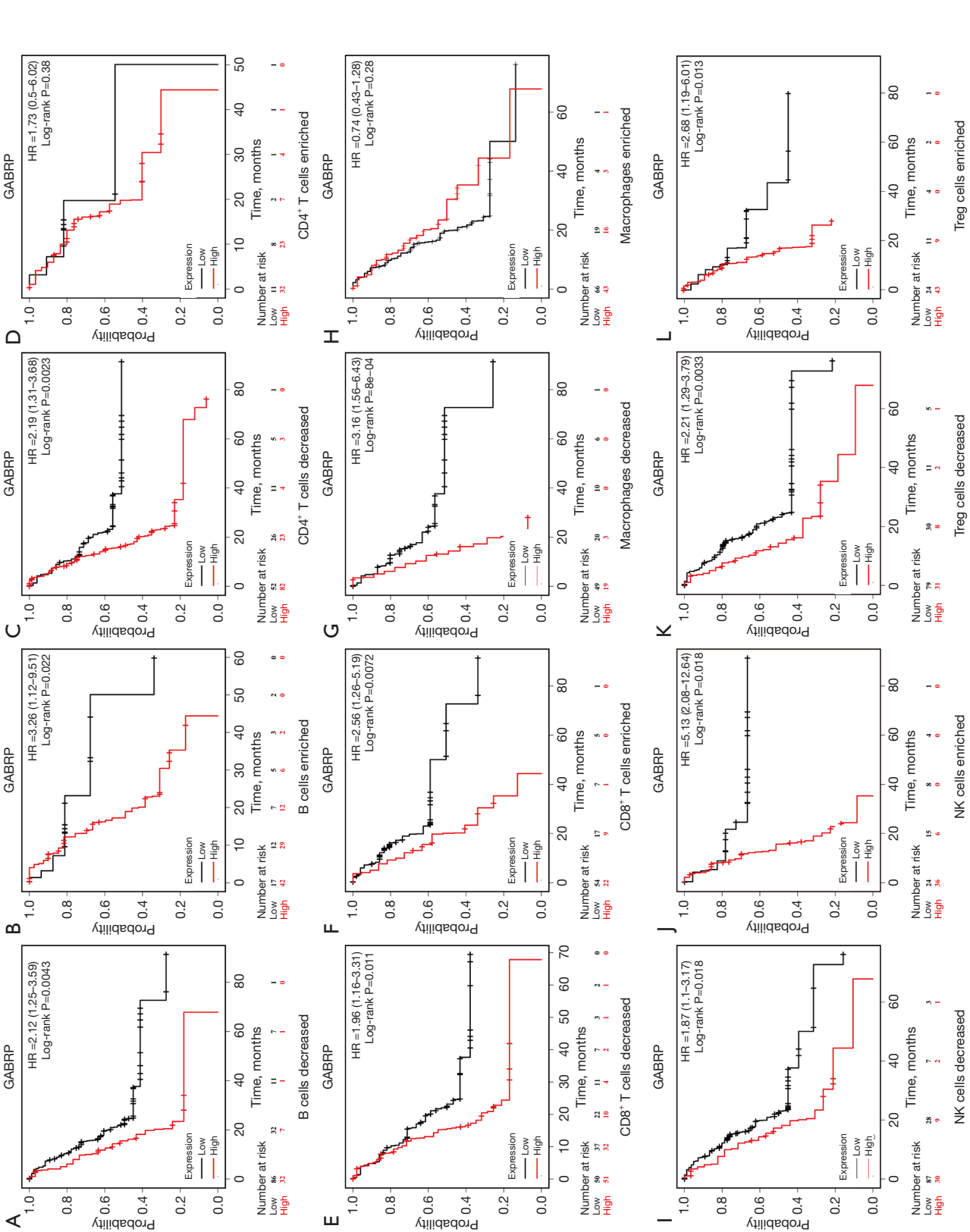


Figure 8 Prognostic analysis of GABRP based on different immune cell levels in pancreatic cancer. (A-L) Relationships between GABRP of different immune cell subgroups and PAAD prognosis. PAAD, pancreatic adenocarcinoma.

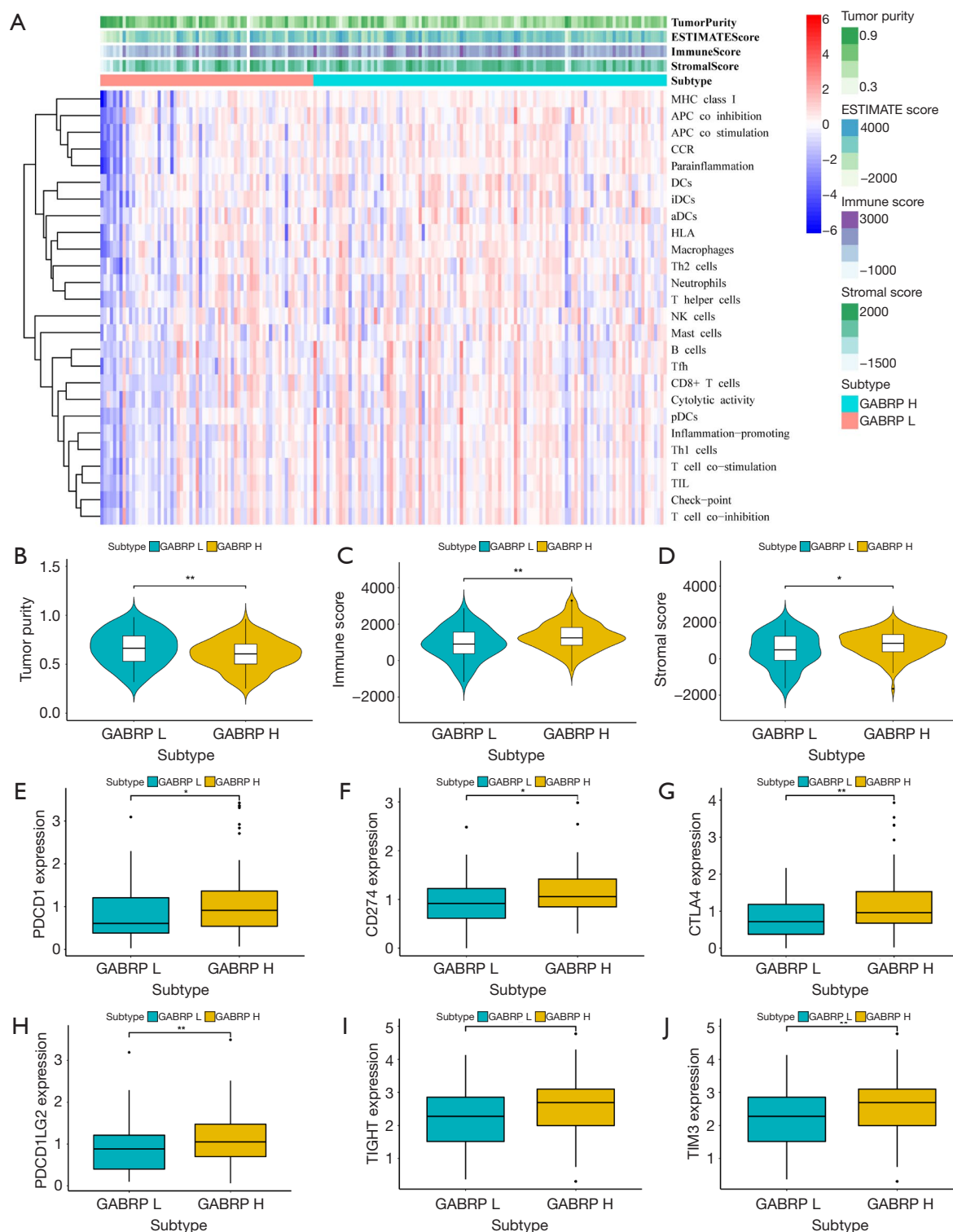


Figure 9 Evaluation of tumor microenvironment in pancreatic cancer based on *GABRP* expression. (A) Heatmap of two different immune subtypes based on *GABRP* expression; (B-D) evaluation of immune scores, tumor purity and stromal scores for the two immune subtypes. Correlation between expression of (E) *PDCD1*, (F) *CD274*, (G) *CTLA4*, (H) *PDCD1LG2*, (I) *TIGIT* and (J) *TIM3* evaluated by Mann-Whitney U-test; bars indicate medians. *, $P < 0.05$; **, $P < 0.01$.

Discussion

Pancreatic cancer is one of the most fatal malignancies worldwide with the worst survival of all carcinomas (43). In recent years, the incorporation of immunotherapy has greatly revitalized and achieved great therapeutic advances in the treatment of many solid tumors (44). However, pancreatic cancer remains almost completely resistant to immunotherapy and the majority of clinical trials related to immunotherapy failed to prove clinical efficacy in pancreatic cancer (7). Thus, it is worthwhile to identify novel prognostic biomarker and therapeutic targets as well as to understand the factors affecting the efficacy of immunotherapy for pancreatic cancer. In this study, we performed a systematic bioinformatics analysis of *GABRP* in pancreatic cancer integrating the mRNA expression profiles from TCGA-PAAD and GEO cohorts. In pancreatic cancer, enhanced *GABRP* expression was correlated with a worse prognosis and was an independent predictor of survival. We found that the expression level of *GABRP* was associated with the number of infiltrating immune cells and several immune cell markers indicating the role of *GABRP* in immunological interactions. We found a potential link between *GABRP* expression and the tumor microenvironment, highlighting the need for further research to elucidate the immunological role of *GABRP* in pancreatic cancer.

We assessed *GABRP* expression in pancreatic cancer compared with normal tissues and found that *GABRP* was significantly upregulated in pancreatic cancer using the independent Oncomine and GEPIA2 databases, which was further validated in two GEO datasets (GSE15471 and GSE16515). We observed a remarkable correlation between *GABRP* and relatively poor prognosis in pancreatic cancer related to OS and DFS. Multivariate analysis revealed that the expression level of *GABRP* was an independent risk factor for unfavorable survival in pancreatic cancer. However, *GABRP* expression was not correlated with TNM stage, but grade, in the TCGA cohort, which may be due to the limited number of cases with metastasis. To further validate the prognostic value of *GABRP* in pancreatic cancer, nomograms were constructed based on *GABRP* expression. Even if the nomograms reached a moderate prognostic accuracy (C index =0.541), they may not be precise enough due to the lack of enough independent risk factors identified in multivariate Cox analysis. However, the above results suggested that *GABRP* holds promise as a valuable prognostic biomarker for pancreatic cancer to

improve clinical decisions.

We further explored the potential mechanism of *GABRP* overexpression in pancreatic cancer from the perspective of epigenetics. DNA methylation plays an important role in regulating gene expression and aberrant DNA methylation is involved in cancer progression (45). DNA hypomethylation is frequently observed in human tumors and contributes to the aberrant activation of specific genes related to cancer progression (46). In pancreatic cancer, there exists a significantly negative correlation between *GABRP* methylation and *GABRP* mRNA expression. Moreover, methylation of the *GABRP* promoter was significantly upregulated in pancreatic tumor tissues. The above analysis implied that the enhanced expression of *GABRP* might be partially attributed to DNA hypomethylation. Micro-RNAs (miRNAs) can regulate the expression of target genes in many processes and have been identified as significant participants in the progression of pancreatic cancer (47). In the present study, hsa-miR-3655 was identified as a candidate regulatory miRNA for *GABRP*, and we showed that the expression of hsa-miR-3655 was significantly downregulated in pancreatic cancer compared with normal controls, and its downregulation was related to poor prognosis. The putative binding between the *GABRP* 3'-UTR site and miR-3655 further confirmed that miR-3655 could be a potential upstream regulator of *GABRP* in pancreatic cancer. The above findings suggested that epigenetic regulation might contribute to the dysregulation of *GABRP*.

To further clarify the underlying biological function of *GABRP* in pancreatic cancer, GSEA was performed to determine *GABRP* co-expressed genes. GO analysis showed consistent enrichment of genes in several immunologically relevant biological processes and pathways, which indicated that *GABRP* might participate in immune responses. An inflammatory microenvironment is now recognized to be an integral factor contributing to tumorigenesis (48). KEGG pathway analysis showed that several inflammation-related pathways were enriched such as the NOD-like receptor pathway, NF kappa B pathway, and TNF pathway, all of which are crucial inflammatory regulators involved in tumor initiation and progression (49-51). The results of GSEA analysis suggested that the role of *GABRP* in pancreatic cancer was closely connected with an inflammatory immune response.

Immune checkpoint therapy (ICT) has demonstrated great clinical benefit in several cancer treatments in recent years, although pancreatic cancer studies reported limited

clinical response to ICT (52-54). Thus, a comprehensive and in depth understanding of tumor immune environment and immune infiltration in pancreatic cancer could greatly assist in the development of effective immunotherapeutic strategies for pancreatic cancer. We compared the immune infiltration landscape between pancreatic cancer and normal controls and found an obvious increase in macrophages, especially M0 macrophages, and CD4⁺ T cells, and a decrease in CD8⁺ T cells. The infiltration of macrophages into solid tumors was associated with poor survival outcomes while the infiltration of CD8⁺ T cells was correlated with better prognosis (55,56). We further explored this subject and found a relationship between *GABRP* expression and immune infiltration. The expression of *GABRP* was positively correlated with the infiltration of B cells, CD8⁺ T cells, macrophages, neutrophils and dendritic cells, and *GABRP* copy number alteration was associated with B cells, CD4⁺ T cells, macrophages, and neutrophil infiltration in PAAD. In addition, *GABRP* expression was associated with different immune cell markers, and the differences in numbers of infiltrating immune cells could significantly affect the prognosis of pancreatic cancer patients. All the above results indicated that *GABRP* was correlated with immune infiltration which affected the prognosis of pancreatic cancer.

The immune cells in the pancreatic cancer microenvironment play a significant role in tumorigenesis and progression (57). Given the impact of *GABRP* on immune infiltration, we further explored the relationship between *GABRP* expression and the components of the pancreatic cancer microenvironment using ESTIMATE to evaluate 22 types of immune cells and stromal cells in tumor samples. We found that higher *GABRP* expression was significantly correlated with higher immune score and stromal score and lower tumor purity, which was in accordance with the analysis of immune infiltration via TIMER. Inflammation is intimately associated with immune responses, and inflammatory infiltration into the pancreatic cancer microenvironment contributed to tumor growth and metastasis (58). Pancreatic cancer is characterized by a pathological hallmark of stroma desmoplasia, which creates a mechanical barrier that reduces chemotherapy efficacy and results in low immune cell infiltration (57). According to our results, higher *GABRP* expression was correlated with increased density of immune cells and stromal cells, which resulted in lower tumor purity and complexity of the tumor microenvironment. The above results indicated an underlying connection between *GABRP* and tumor

microenvironment. Higher *GABRP* expression was markedly associated with higher expression of six immune checkpoint genes, indicating that *GABRP* expression might potentially affect the efficacy of immunotherapy (59). The above correlation might provide new insights into immunological treatment for pancreatic cancer patients who received limited benefit from immune checkpoint inhibitors.

In summary, *GABRP* is a potential prognostic marker for pancreatic cancer and correlated with immune infiltration and tumor microenvironment. However, the present study still has some limitations. While the conclusions of this study were based on *GABRP* mRNA and protein expression, our results only indicated an overall relationship between *GABRP* and immune infiltration and tumor microenvironment. Further studies are needed to verify the epigenetics regulation of *GABRP* expression level and the underlying mechanism of the specific interactions between *GABRP*, immune infiltration and tumor environment.

Conclusions

In the present study, we demonstrated that *GABRP* was overexpressed in pancreatic cancer and that enhanced *GABRP* expression correlated with poor prognosis. Promoter hypomethylation and the regulation of hsa-miR-3655 might contribute to the overexpression of *GABRP* in pancreatic cancer. *GABRP* expression was correlated with immune infiltration and microenvironment. Further studies are needed to elucidate the molecular mechanism of the regulatory role of *GABRP* on the immune response.

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Footnote

Reporting Checklist: The authors have completed the

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2021/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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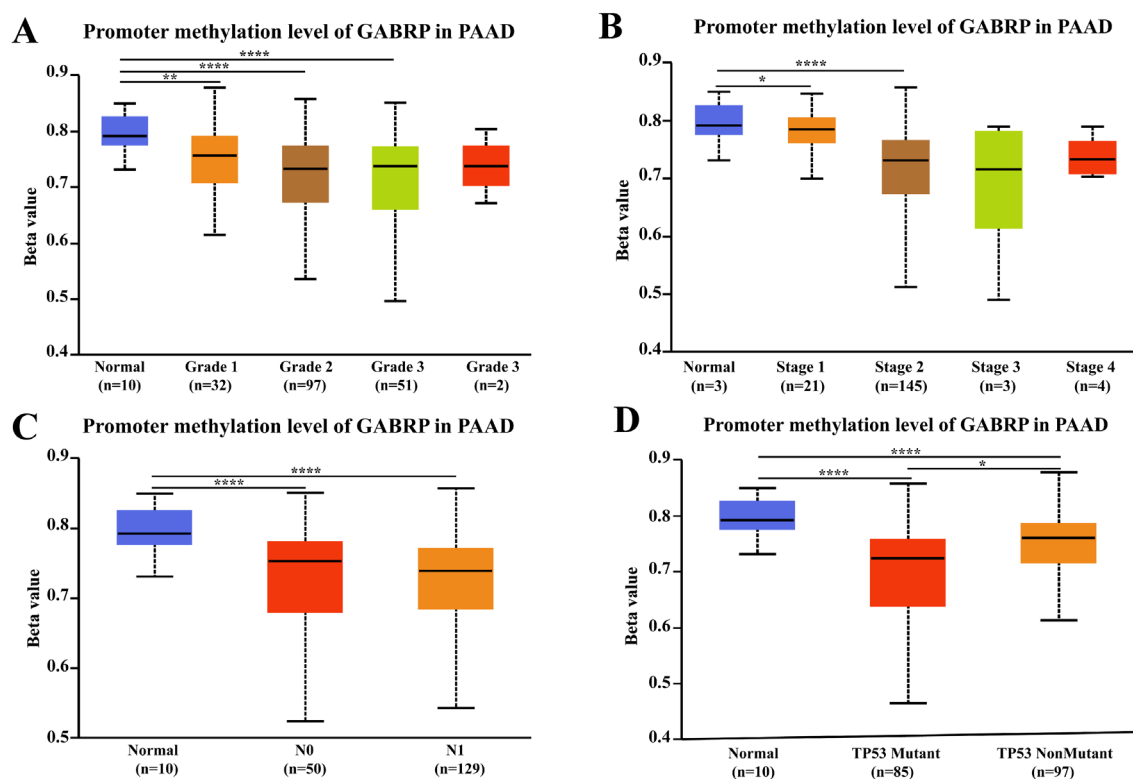


Figure S1 The promoter methylation levels of GABRP in PAAD. The promoter methylation levels of GABRP in PAAD based on (A) grade, (B) stage, (C) nodal metastasis status and (D) TP53 mutant status. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

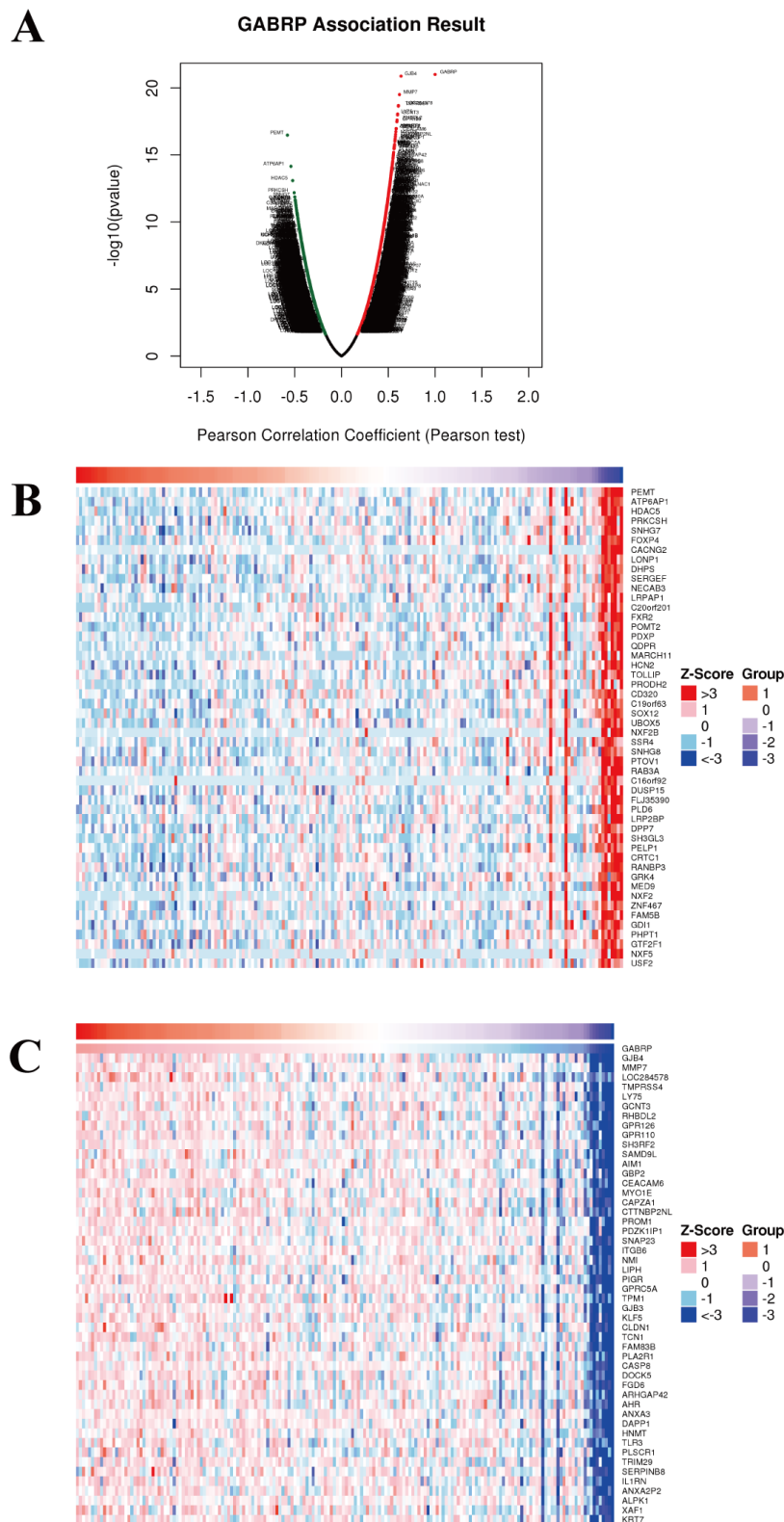


Table S1 The expression status of GABRP in pancreatitis vs. normal tissues

Dataset	Gene	Design	logFC	P value	adj.P.Val	Expression status
GSE109227	<i>GABRP</i>	Normal vs. pancreatitis	0.045112	0.535	0.63	No change
GSE65146	<i>GABRP</i>	Normal vs. pancreatitis	0.038486	0.257713	0.5091	No change
GSE41418	<i>GABRP</i>	Normal vs. pancreatitis	-0.00411	3.68E-01	0.55502	No change

Table S2 Common miRNA containing GABRP-binding sequences predicted by miRDB, TargetScan and MirWalk databases and corresponding expression level (P value) in TCGA pancreatic cancer and normal tissues

Predicted targeted miRNA	Normal vs. tumor (P value)
hsa-miR-4773	0.9488
hsa-miR-4429	N/A
hsa-miR-320c	0.5620
hsa-miR-320b	0.5729
hsa-miR-3655	0.006662
hsa-miR-3660	N/A
hsa-miR-4430	N/A
hsa-miR-3652	N/A
hsa-miR-4252	N/A
hsa-miR-4448	N/A
hsa-miR-4447	N/A
hsa-miR-3163	N/A
hsa-miR-5682	N/A
hsa-miR-8087	N/A