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ORIGINAL RESEARCH

PLXDC1 Can Be a Biomarker for Poor Prognosis and Immune Evasion in Gastric Cancer

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Background: Research has revealed that Plexin domain containing 1 (PLXDC1) is correlated with the prognosis of a variety of tumors, but its role in the tumor microenvironment (TME) of gastric cancer has not been reported.

Methods: In this study, we analyzed PLXDC1 expression in gastric cancer using the Oncomine and the Cancer Genome Atlas (TCGA) databases and immunohistochemical staining experiments, and performed prognostic assessment with data from the TCGA and Kaplan–Meier Plotter databases. The immunomodulatory role of PLXDC1 in the gastric cancer TME was analyzed by signaling pathway enrichment, immune cell correlation analysis, immunomodulator risk model construction and immunohistochemical staining experiments of immune cells.

Results: The results indicated that PLXDC1 was overexpressed in gastric cancer and that its overexpression was associated with poor prognosis. Multivariate Cox analysis revealed that PLXDC1 could be an independent biomarker of the risk of gastric cancer. Signaling pathway enrichment revealed that high PLXDC1 expression was involved in signaling pathways related to immune activation and stromal activation, and Tumor Immune Dysfunction and Exclusion (TIDE) assessment indicated that high PLXDC1 expression was associated with a significantly higher risk of immune evasion than low PLXDC1 expression. A Cox risk model based on PLXDC1-associated immunomodulators also presented poor prognosis, and immune evasion was significantly higher in the high-risk group than in the low-risk group. In addition, immunohistochemical staining of CD8/CD3/$CD4^+$ T cells in the high and low PLXDC1 expression groups also observed immune cell distribution characteristics of immune evasion.

Conclusion: This study analyzed PLXDC1 from multiple biological perspectives and revealed that PLXDC1 can be a biomarker for poor prognosis and immune evasion in gastric cancer.

Keywords: PLXDC1, biomarker, tumor microenvironment, immune evasion, prognosis, immunotherapy

Introduction

Stomach cancer is one of the most common malignancies in the world and the fourth leading cause of cancer-related death.[1] According to the pathological staging of gastric cancer, stomach adenocarcinoma (STAD) accounts for 95% of all gastric cancer cases.[2] Patients diagnosed at an early stage with disease limited to the mucosa and submucosa and who undergo surgical treatment have five-year survival rates of 70% to 95%.[3] However, western surgical and population-based series show five-year survival rates of 20% to 30% for most patients with advanced tumors that have penetrated the submucosa.[4] Therefore, identifying effective biomarkers to help detect gastric cancer at an early stage and provide effective therapeutic targets is of great importance for patient prognosis.

Immunotherapy is a landmark clinical approach for cancer treatment, and immunotherapy primarily functions by modulating the immune system to kill tumor cells.[5,6] Programmed death-ligand 1 (PD-L1)/programmed death-1 (PD-1) inhibitors, which are immune checkpoint inhibitors (ICIs), kill tumor cells by binding to PD-L1 on the surface of tumor

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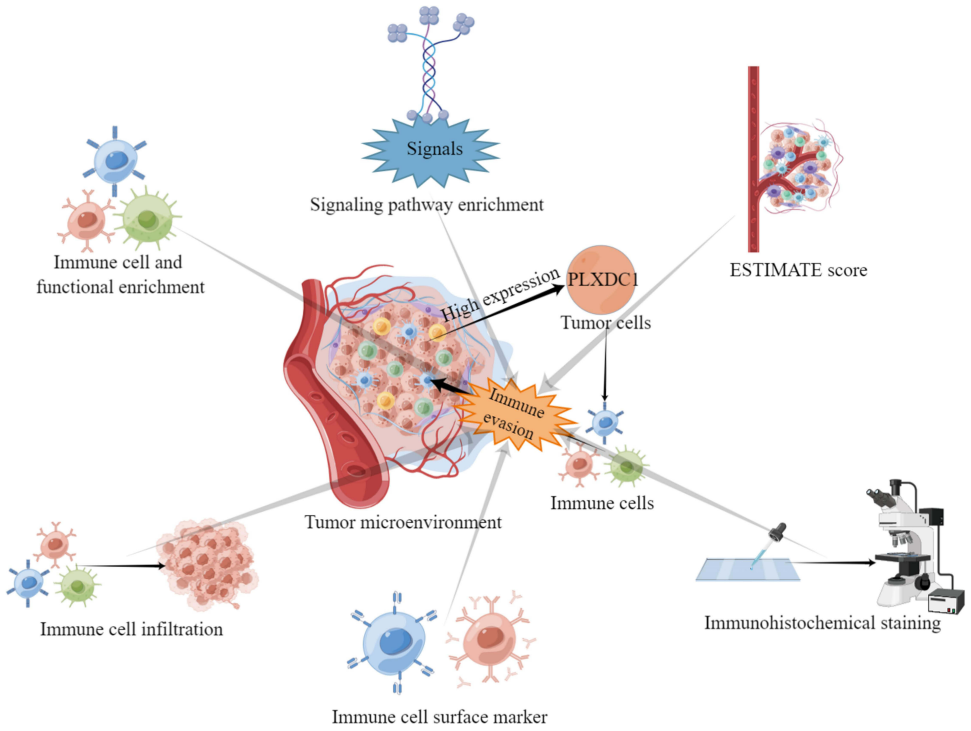
Graphical Abstract



cells or PD-1 on the surface of T cells to disrupt the ability of the tumor to evade the immune response.[7–10] Currently, immunotherapy has yielded promising results in the clinical treatment of non-small-cell lung cancer, providing a new direction for the treatment of gastric cancer and other malignant tumors.[11] However, with the application of immunotherapy in the clinic, researchers have found that only some patients benefit, and most patients do not have satisfactory responses to immunotherapy.[12]

Tumor microenvironment (TME) refers to the surrounding microenvironment where tumor cells exist, including surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, various signaling molecules and extracellular matrix, and the immune cells within this microenvironment and the way they are regulated play an important role in tumorigenesis and development.[13,14] It has become a trend in modern tumor treatment to use the best treatment according to the different characteristics of the TME in tumor patients to improve the long-term survival of patients.[15] With the advent of the era of precision therapy, the TME has become a hot topic of research. Researchers have classified the characteristics of the TME in tumor patients into "immune inflammation", "immune evasion", and "immune desert" phenotypes, and the different phenotypes are associated with differences in the effectiveness of immunotherapy.[16–18] Therefore, it is necessary to identify suitable biological targets associated with the different characteristics of the TME in tumor patients to improve patient survival.

Plexin domain containing 1 (PLXDC1) encodes a protein associated with angiogenesis.[19] It was demonstrated that pigment epithelium-derived factor binds to PLXDC1 on the cell surface through its extracellular structural domain, thus exerting anti-neoangiogenesis effects and inhibiting tumor growth.[20] Currently, PLXDC1 is reported to be involved in the development of various cancers. For example, the glioblastoma endothelium drives bevacizumab-induced infiltrative growth through the regulation of PLXDC1.[21] In addition, studies have demonstrated that vascular abnormalities are a hallmark of most solid tumors and that abnormal angiogenesis in tumors contributes to immune evasion.[22–25] On the basis of such findings, researchers have proposed combining antiangiogenic therapy and immunotherapy in the treatment

of tumor patients to improve the effectiveness of immunotherapy in patients.[26,27] In this study, we explored the role of PLXDC1 in the prognosis and TME of gastric cancer.

Methods

Acquisition of PLXDC1 Expression Profiles in Gastric Cancer

We used the Oncomine database (https://www.oncomine.org/resource/login.html) and selected the "Gene Differential Expression" module to evaluate the expression of PLXDC1 in pan-cancer. Then, we chose gastric cancer as the subject of our study and further analyzed the differential expression of PLXDC1 in gastric cancer and its subtypes and in normal tissues using the dataset in the Oncomine database (P value<0.05). Next, we downloaded gastric cancer data (normal samples=32; cancer samples=375) from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/), and the paired and unpaired differential analysis of PLXDC1 expression in gastric cancer tissues and normal tissues were determined with the "limma" R package (P value<0.05; t-test).

Collection of Clinical Samples

Patients who underwent radical surgery for gastric cancer from January 2017 to December 2018 at the First Affiliated Hospital of Bengbu Medical College were selected. Inclusion criteria: clear primary gastric cancer before surgery and no chemotherapy and radiotherapy. Exclusion criteria: combination of other malignancies and death from an etiology other than gastric cancer. A total of 197 gastric cancer tissue samples and 30 paracancerous tissues were collected and conducted immunohistochemical staining experiments. All patients provided informed consent, and the ethics committee of the First Affiliated Hospital of Bengbu Medical College approved the study protocol.

Immunohistochemical Staining

Rabbit anti-human antibody PLXDC1 (50 μg) was obtained from Abcam. horseradish peroxidase (HRP)-coupled anti-rabbit antibody was provided by Jackson ImmunoResearch (WestGrove, PA, USA). Primary antibody against CD8+/CD3+/CD4+ T cell alpha rabbit monoclonal antibody was provided by Fuzhou Maishin Biotechnology (Fuzhou, China). All samples were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to 4 μm and adhered to slides. After de-affinity under different density gradients of xylene, the slides were rehydrated, and antigens were retrieved with citric acid buffer (pH 7.8, 0.1 M) at approximately 82°C for 24 min. The sections were evenly covered with endogenous peroxidase blocking solution for 15 min at room temperature to block the activity of endogenous peroxidase. After incubation with PLXDC1 primary antibody or overnight at 4°C, the slides were washed gently with phosphate-buffered saline and incubated with biotin-conjugated secondary antibody for 10 min at room temperature and incubated with streptavidin peroxidase for 5 min. All sections were stained with hematoxylin and then cleaned. After the sections were dried and cleaned, staining intensity was assessed by staining under a microscope by a pathologist.

Wax blocks of samples with staining intensity "+" and "+++" assessed by pathologists were stained for $CD8^+$/$CD3^+$/$CD4^+$ T cells to observe the distribution of immune cells in and around the tumor.

Survival Analysis of PLXDC1 Expression in Gastric Cancer

Using the Kaplan–Meier Plotter online website (https://kmplot.com/analysis/), we selected the "Gastric Cancer" module to classify high- and low-risk groups according to the cutoff value of PLXDC1 expression. Then, we compared survival differences in overall survival (OS) (n=875), first progression (FP) (n=640) and post-progression survival (PPS) (n=498) between high and low PLXDC1 expression groups in gastric cancer patients (P value<0.05; Log rank test). Next, we performed univariate Cox analyses of PLXDC1 expression with clinical factors such as age, gender, grade, stage, T stage, N stage and M stage using TCGA data (P value<0.05). Then, we used receiver operating characteristic (ROC) curves to assess the accuracy of the prognostic prediction of these clinical factors, and the larger the area under the curve (AUC) was, the more accurate the prediction was. In addition, using multivariate Cox analysis, we identified independent risk factors for prognosis (P value<0.05). Finally, PLXDC1 was integrated with clinicopathological parameters to construct a nomogram survival prediction system to predict 1-, 3-, and 5-year patient survival by using the "rms" R package.

Gene Set Enrichment Analysis (GSEA) Pathway Enrichment

We used GSEA version 4.1.0, a tool that allows the analysis of genome-wide expression profiling microarray data and that compares genes with predefined gene sets,[28] to identify pathways associated with PLXDC1 in gastric cancer. The gene expression matrix in gastric cancer was processed by Perl software to obtain the input file related to the target gene. Then, the upregulated gene set was defined as phenotype h (h=101/178), the downregulated gene set was defined as phenotype l (l=77/78), and "h-versus-l" was selected to enrich for PLXDC1-related pathways. Gene sets associated with pathways with |normalized enrichment score (NSE) |>1, nominal (NOM) p value < 0.05 and false discovery rate (FDR) q-value < 0.25 are generally considered significant.

Analysis of PLXDC1 Expression in the TME of Gastric Cancer

High (n=149) and low (n=226) expression groups were classified according to the mean value of PLXDC1 expression (2.378) in gastric cancer samples obtained from the TCGA database and the Hallmark pathway gene set obtained from the Molecular Signatures Database (MSigDB) online website (http://www.gsea-msigdb.org/gsea/login.jsp). By using the "GSVA" R language package, gene set variation analysis (GSVA) pathway enrichment analysis was performed with the PLXDC1 high- and low-expression groups in the Hallmark pathway gene set, and single-sample GSEA (ssGSEA) enrichment was used to analyze the distribution of immune cells and functional gene sets in the PLXDC1 high- and low-expression groups (P value<0.05). In addition, an analysis of the stromal and immune cells in malignant tumor tissues using the expression data (ESTIMATE) algorithm was performed to assess the immune cell score, stromal score and tumor purity score of the groups with high and low PLXDC1 expression in the gastric cancer TME. Finally, the risk of immune evasion in the PLXDC1 high and low expression groups was assessed with the Tumor Immune Dysfunction and Exclusion (TIDE) website (http://tide.dfci.harvard.edu/login/).

Then, we analyzed the correlation between the expression of PLXDC1 and infiltration of immune cells in the gastric cancer immune microenvironment using the TISIDB online website (P value<0.05) (http://cis.hku.hk/TISIDB/index.php), which is an integrated repository portal for tumor-immune system interactions.[29] Based on the "Subtype" panel, the immune cells present in gastric cancer tissues were classified into six subtypes, C1 (wound healing; n=129), C2 (IFN-gamma dominant; n=210), C3 (inflammatory; n=36), C4 (lymphocyte depleted; n=9), C5 (immunologically quiet), and C6 (TGF-b dominant; n=7), in order to explore the correlation between PLXDC1 expression and the C1/2/3/4/6 subtypes.

Analysis of PLXDC1-Associated Immunomodulators in the TME of Gastric Cancer

Using the "Immunomodulators" panel in the TISIDB, we analyzed the correlation between the levels of 23 immunoinhibitors and 44 immunostimulators produced in the TME of gastric cancer and the expression of PLXDC1 (P value<0.05). Then, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) website (https://www.string-db.org/online) was used to construct a protein interaction network of the immunomodulators whose expression was associated with PLXDC1 expression. Next, we performed a functional analysis of PLXDC1-related immunomodulators with the Metascape online website (http://metascape.org/gp/index.html/main/step1).

Based on immunomodulators associated with the expression of PLXDC1, we used the least absolute shrinkage and selection operator regression (LASSO) algorithm with penalty parameter tuning conducted by 10-fold cross-validation with the "glmnet" and "survival" R packages. Then, the immunomodulators identified by LASSO were subjected to stepwise multivariate Cox proportional hazards regression analysis to identify the optimal candidates and construct an immune-related prognosis prediction model. The formula for calculating the risk score was as follows:

$$Riskscore = \sum_{i=1}^{n} coefi \times Xi$$

where "coefi" and "Xi" represent the coefficient and expression level of each PLXDC1-related immunomodulator, respectively. Patients were classified into high-risk and low-risk groups based on the median score as the risk cutoff point. The survival curves of the two groups were plotted using the Kaplan–Meier method and compared by the Log rank test using the "survival" and "survminer" packages in R, with a P value< 0.05 indicating significance. ROC curve analysis was conducted, and the AUC values were determined to evaluate the prognostic model's predictability using the

"survivalROC" package. Finally, we integrated the risk score obtained from the model with clinical data such as age, gender, grade, stage, T stage, N stage, and M stage downloaded from TCGA and performed univariate and multivariate Cox analyses to explore whether the risk score was an independent prognostic risk factor.

Statistical Analysis

Statistical analyses were performed using R software version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria). For the analysis of quantitative data, the significance of normally distributed variables was analyzed using Student's *t*-test, and nonnormally distributed variables were analyzed using the Wilcoxon rank-sum test. The Log rank test was used to compare data between two groups, and the Kruskal–Wallis test was performed to compare data among more than two groups. A p value < 0.05 was considered significant.

Results

Expression of PLXDC1 in Gastric Cancer

Pan-cancer differential expression analysis in the Oncomine database showed that PLXDC1 was highly expressed in a variety of cancers, including gastric cancer (Figure 1A). Analysis of gastric cancer and its subtypes revealed that PLXDC1 was overexpressed in gastric cancer tissues compared with normal tissues according to Wang's data.[30] Based on the data from Cho,[31] PLXDC1 was highly expressed in diffuse gastric adenocarcinoma, gastric mixed adenocarcinoma and gastric intestinal type adenocarcinoma (Table 1). Next, paired difference analysis and unpaired difference analysis using the TCGA database indicated that PLXDC1 was significantly overexpressed in gastric cancer tissues compared with normal tissues (Figure 1B and C). In addition, immunohistochemical staining revealed that PLXDC1 was overexpressed in gastric cancer tissues (Figure 1D). Overall, we demonstrated PLXDC1 overexpression in gastric cancer.

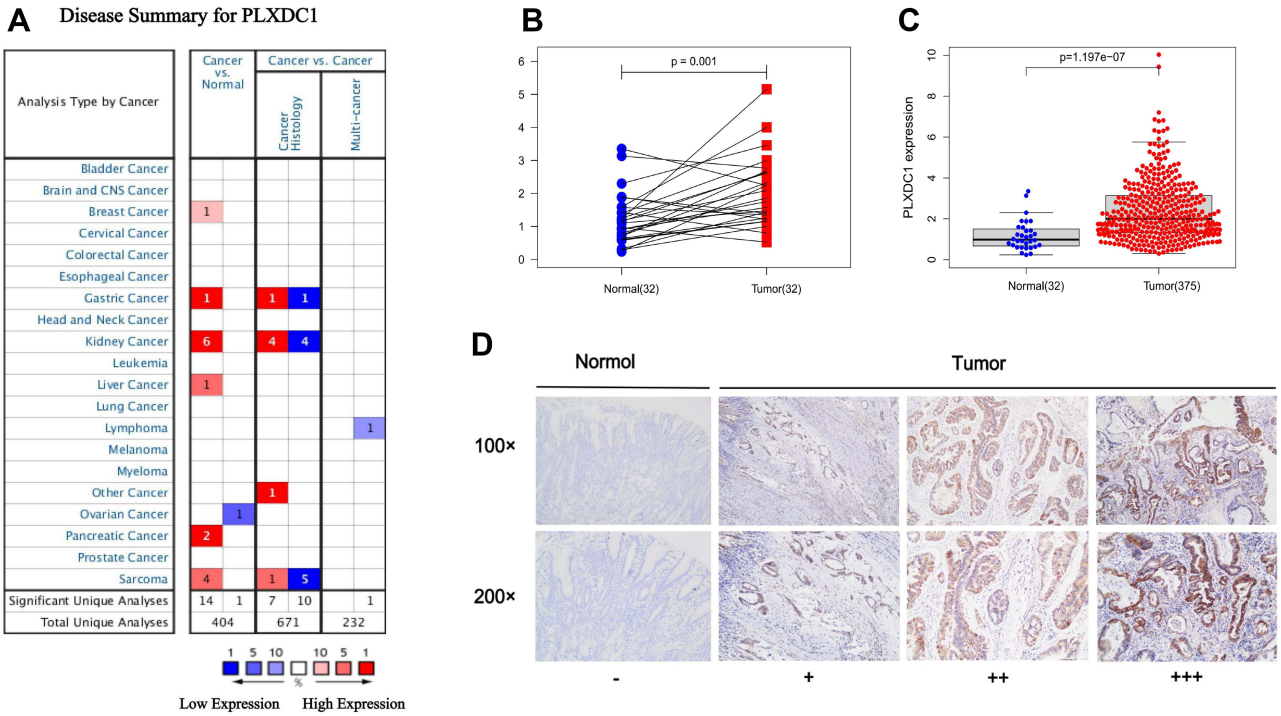


Figure 1 Expression of PLXDC1 in tumors. (**A**) The expression of PLXDC1 in pan-cancer in the Oncomine database. Figures represent the number of studies. Red represents high expression. Blue represents low expression. (**B**) Paired difference analysis and (**C**) unpaired difference analysis of PLXDC1 expression in tumor tissues and paraneoplastic tissues in TCGA database. (**D**) Immunohistochemical staining analysis of PLXDC1 expression in gastric cancer. -: No staining in normal paracancerous tissue; +: Weak staining in tumor tissues; ++: Moderate staining in tumor tissues; +++: Strong staining in tumor tissues.

Table 1 Transcription Levels of PLXDC1 in Gastric Cancer Subtypes in the Oncomine Database

Types of Gastric Cancer Tissues vs Normal Tissues	Fold Change	*P* value	*t*-Test	Reference
Gastric Cancer vs Normal	2.048	0*	6.306	Wang[30]
Diffuse Gastric Adenocarcinoma vs Normal	1.621	0.023*	2.511	Cho[31]
Gastric Mixed Adenocarcinoma vs Normal	2.040	0.025*	3.022	Cho[31]
Gastric Intestinal Type Adenocarcinoma vs Normal	1.335	0.004*	2.784	Cho[31]

Note: **P* value < 0.05.

Survival Analysis of PLXDC1 in Gastric Cancer

Survival analysis from the Kaplan–Meier Plotter online website showed that high expression of PLXDC1 in gastric cancer was associated with poor OS (cutoff value= 324), FP (cutoff value= 322), and PPS (cutoff value= 315) (*P* value<0.05; hazard ratio (HR) > 1; Figure 2A–C). Then, we integrated PLXDC1 expression with clinicopathological parameters (age, gender, grade, stage, T stage, N stage and M stage) and performed univariate and multivariate Cox analyses. Univariate Cox analysis showed correlations of PLXDC1 expression, age, gender, grade, stage, T stage, N stage and M stage with poor OS (*P* value<0.05; HR>1; Table 2). Furthermore, the ROC curves showed high predictive accuracy for PLXDC1 expression, age, grade, stage, N stage and M stage in the univariate Cox prognostic analysis (Figure 2D). Multivariate Cox analysis indicated that PLXDC1 and age were independent prognostic risk factors for gastric cancer (*P* value<0.05; Figure 2E). Finally, we integrated PLXDC1 expression with clinicopathological parameters

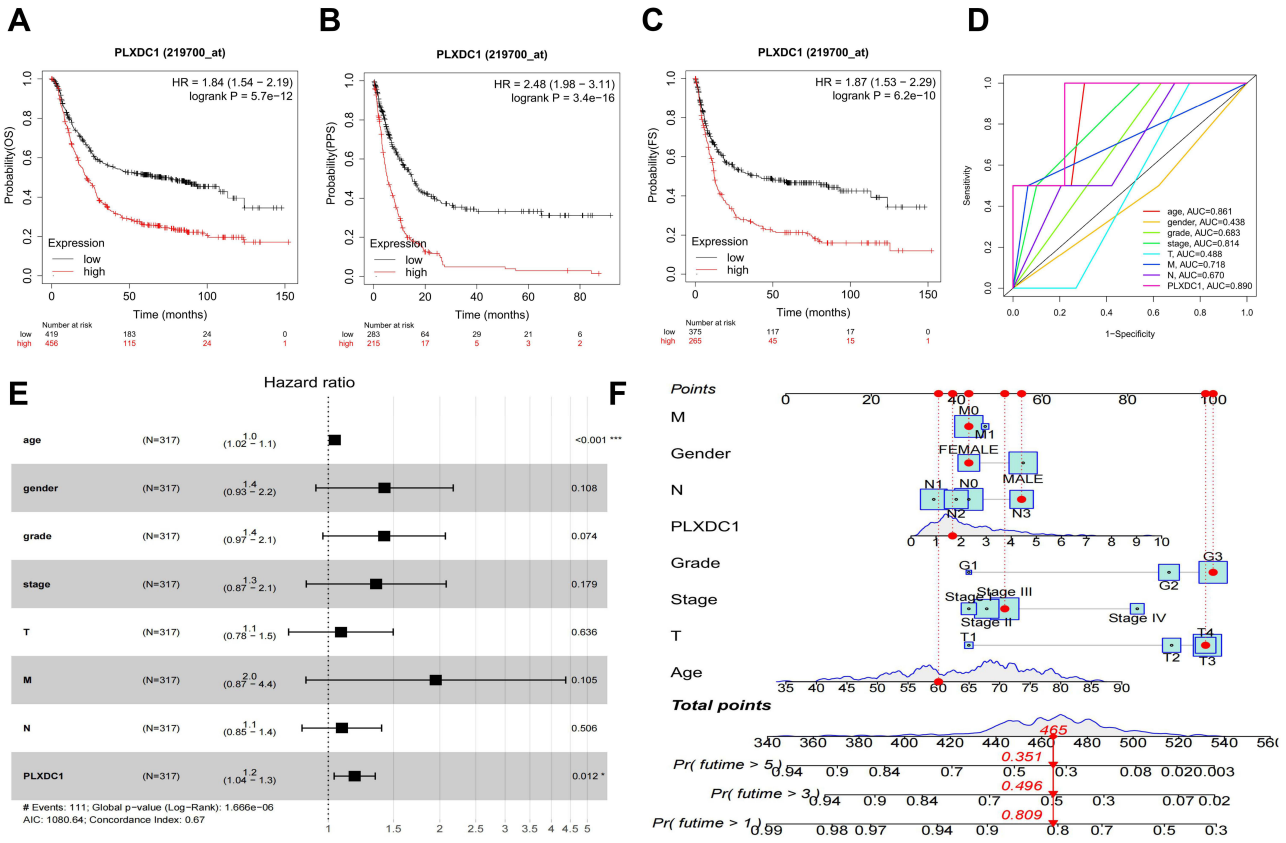


Figure 2 Prognostic analysis of PLXDC1 in gastric cancer. (**A**) OS, (**B**) FP and (**C**) PPS analysis of PLXDC1 in gastric cancer in the Kaplan-Meier Plotter database. (**D**) The accuracy of PLXDC1 expression and clinicopathological parameters in univariate Cox analysis for the prediction of gastric cancer prognosis as assessed by using ROC curves. (**E**) Multivariate Cox survival analysis of PLXDC1 expression and clinicopathological parameters. (**F**) PLXDC1 integrates clinicopathological parameters to build a nomogram survival prediction system to predict 1-, 3 -and 5-year patient survival. In the nomogram survival prediction system, each clinicopathological parameter of the patient corresponds to a score, and all scores are summed to obtain an overall score for the patient, which predicts the 1-, 3-, and 5-year survival rates for the patient. **P* value < 0.05; ****P* value < 0.001.

Table 2 Univariate Cox Survival Analysis of PLXDC1 Expression and Clinical Parameters

Gene	HR	HR.95L	HR.95H	*P* value
PLXDC1	1.150	1.030	1.290	0.0171*
Age	1.030	1.010	1.050	0.0056*
Gender	1.480	0.980	2.250	0.0624
Grade	1.370	0.950	1.980	0.0954
Stage	1.540	1.220	1.930	0.0002*
T	1.300	1.020	1.650	0.0315*
N	2.050	1.100	3.830	0.0246*
M	1.270	1.070	1.500	0.0064*

Note: *P value < 0.05.
Abbreviations: HR, Hazard Ratio; L, Low; H, High.

to construct a nomogram survival prediction system, which could accurately predict 1-, 3-, and 5-year patient survival (Figure 2F). The above analysis indicated that PLXDC1 can be used as a biomarker of the poor prognosis of gastric cancer.

GSEA Enrichment Analysis of PLXDC1 in Gastric Cancer

To further explore the oncogenic relevance of PLXDC1 in gastric cancer, we performed GSEA analysis. Since PLXDC1 is overexpressed in gastric cancer, we focused on the signaling pathways activated by PLXDC1. The results showed that PLXDC1 promotes immune activation, such as the chemokine signaling pathway, JAK/STAT signaling pathway and Toll-like receptor signaling pathway. Interestingly, PLXDC1 also promotes the activation of mesenchymal signaling, such as the TGF-β signaling pathway. In addition, PLXDC1 is involved in tumor progression, such as via the Hedgehog signaling pathway (Figure 3 and Table 3).

Effect of PLXDC1 Expression on the TME of Gastric Cancer

Hallmark pathway gene set analysis revealed that PLXDC1 promotes immune activation in the groups with high expression in gastric cancer; for example, PLXDC1 promotes the interferon α/β response, allograft rejection signaling, IL6-JAK-STAT3 signaling, IL2-STAT5 signaling, inflammatory response and TFNA-NFKB signaling (Figure 4A). The ssGSEA enrichment analysis of the immune cells and functional gene set files revealed that the immune cells and functional enrichment of the high PLXDC1 expression group was significantly higher than that of the low PLXDC1 expression group (Figure 4B). However, a previous survival analysis of the high PLXDC1 expression group did not reveal a survival advantage compared to the low PLXDC1 expression group (Figure 2A), which became the focus of our attention. Further analysis revealed that high PLXDC1 expression activates mesenchymal signaling, such as epithelial mesenchymal transition (EMT) signaling, TGF-β signaling and angiogenesis (Figure 4A). Studies have demonstrated that tumors with an immune evasion phenotype in the TME are characterized by the accumulation of large numbers of immune cells in the stroma surrounding the tumor, but these immune cells are unable to penetrate the stroma to reach the parenchymal components of the tumor.[32] We scored immune cells and stromal cells in the high and low PLXDC1 expression groups by the ESTIMATE method, and the results showed that the immune and stromal cell contents were significantly higher in the high PLXDC1 expression group than in the low PLXDC1 expression group (Figure 4D), which was consistent with the results of previous analyses. Analysis of the TME characteristics showed high TGF-β expression, bone marrow inflammation and tumor neovascularization as microenvironmental features of immune-evasive tumors.[22,33,34] Interestingly, the results of our analysis showed that TGF-β, angiogenesis and EMT levels were significantly higher in the high PLXDC1 expression group than in the low PLXDC1 expression group (Figure 4C). Therefore, we proposed that high PLXDC1 expression may promote immune evasion characteristics of the TME of gastric cancer. Finally, the risk of immune evasion in the high and low PLXDC1 expression groups was compared by the TIDE index. The results were consistent with our conclusion that the risk of TME immune evasion mediated by high

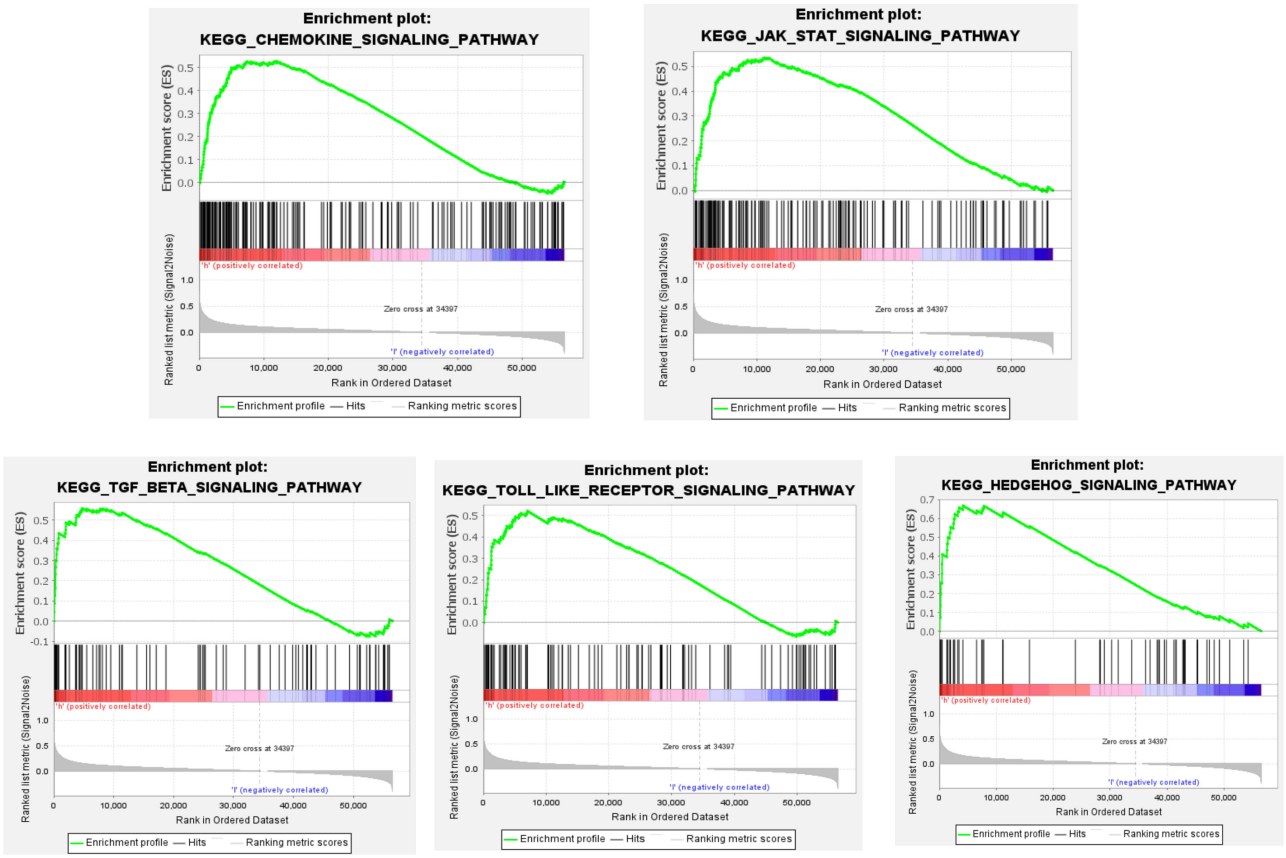


Figure 3 GSEA enrichment analysis of PLXDC1 in gastric cancer.

PLXDC1 expression was higher than that under conditions of low PLXDC1 expression (Figure 4E). Overall, we demonstrated from multiple biological perspectives that high PLXDC1 expression mediates the immune evasion state of the TME in gastric cancer.

Correlation Analysis of PLXDC1 Expression and Immune Cell Infiltration in the TME of Gastric Cancer

To further validate our findings, we analyzed the correlation between PLXDC1 expression and immune cell infiltration in gastric cancer. The heatmap demonstrates that PLXDC1 expression is positively correlated with the infiltration of multiple immune cells in pan-cancer (Figure 5A). In the TME of gastric cancer, we found that PLXDC1 expression was significantly positively correlated with the infiltration of most immune cells in the gastric cancer TME, such as Tcm_CD8, Tem_CD8, Tcm_CD4, Tem_CD4, Tfh, Tgd, Th1, Th17, NK, MDSC, NKT, Act_DC, pDC, iDC, macrophages, eosinophils, mast, and

Table 3 Results of PLXDC1 Enrichment Upregulation of GSEA Pathways in Gastric Cancer

GeneSet	NES	NOM *p* value	FDR *q* value
CHEMOKINE_SIGNALLING_PATHWAY	1.78	0.012*	0.036*
TOLL_LIKE_RECEPTOR_SIGNALLING_PATHWAY	1.79	0.008*	0.037*
TGF_BETA_SIGNALLING_PATHWAY	1.84	0.006*	0.025*
JAK_STAT_SIGNALLING_PATHWAY	1.97	0*	0.009*
HEDGEHOG_SIGNALLING_PATHWAY	2.16	0*	0.002*

Note: *P value < 0.05.
Abbreviations: GSEA, Gene Set Enrichment Analysis; NSE, normalized enrichment score; NOM, nominal; FDR, false discovery rate.

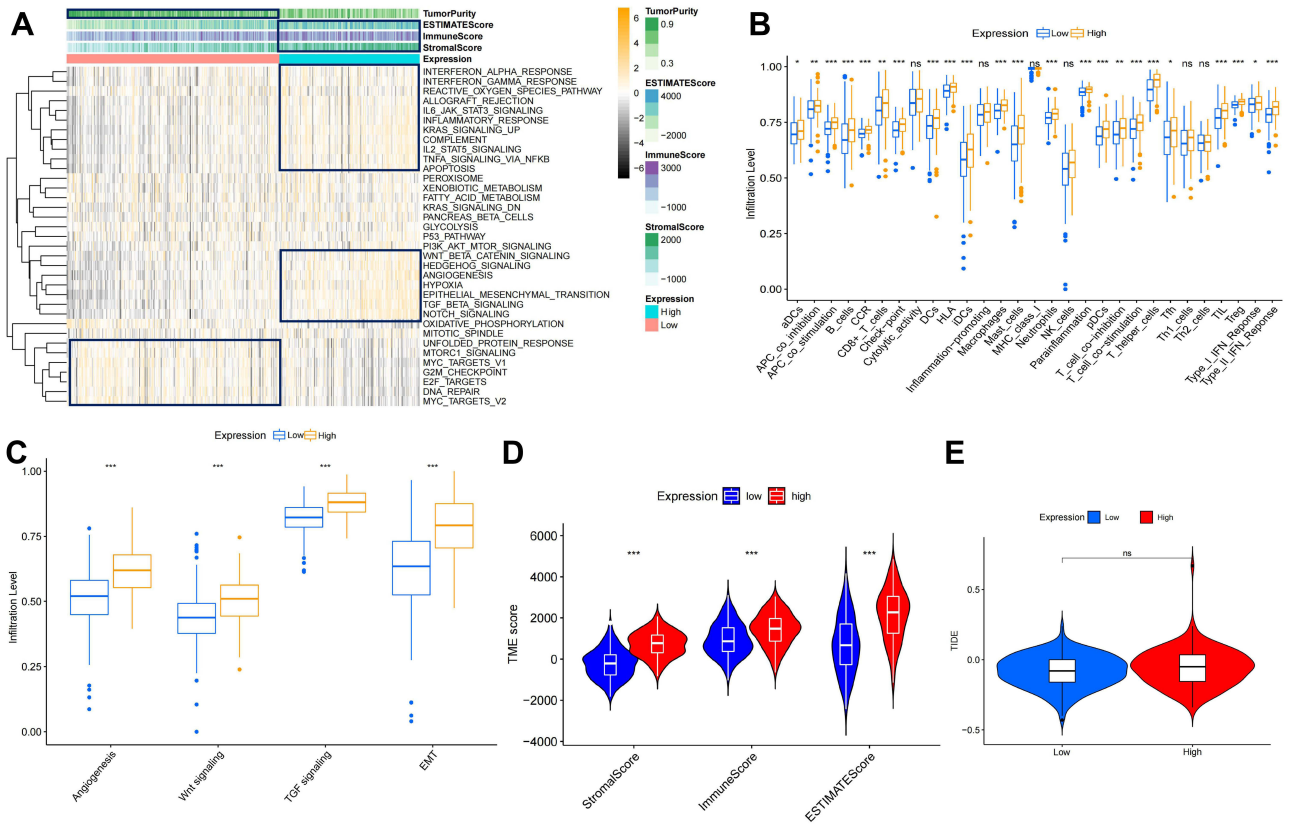


Figure 4 Analysis of the role of PLXDC1 in the TME of gastric cancer. (**A**) Hallmark pathway gene set enrichment in the high and low PLXDC1 expression groups in gastric cancer. (**B**) Differential analysis of immune cell infiltration and functional gene sets in gastric cancer in the high and low PLXDC1 expression groups. (**C**) Differential analysis of stromal activation signaling pathways in gastric cancer in the high and low PLXDC1 expression groups. (**D**) Differential analysis of immune cell, stroma and ESTIMATE scores in the high and low PLXDC1 expression groups in gastric cancer. (**E**) Differential analysis of TIDE in high and low PLXDC1 expression groups in gastric cancer. **P* value < 0.05; ***P* value < 0.01; ****P* value < 0.001.

Abbreviation: ns, no significance.

neutrophil (rho>0, *P* value<0.05), while it was negatively correlated with the infiltration of Act_CD4 (rho<0, *P* value<0.05; Figure 5B). However, high PLXDC1 expression was associated with poor prognosis, suggesting that with increased PLXDC1 expression, immune cell infiltration increased but did not enhance the long-term prognosis of patients. In addition, PLXDC1 expression was significantly correlated with gastric cancer type (C 1/2/3/4/6) (*P* value<0.05) and was higher in the stromal subtype (C 6) than in the immune subtype (C 2/3/4) (Figure 5C). Therefore, this further supports our conclusion in (Figure 4) that the increased stromal cell contents in the high PLXDC1 expression group inhibits the function of immune cells, thus leading to the immune evasion phenotype of gastric cancer.

Establishment of a PLXDC1-Related Immune Prognostic Model and Predictive Assessment

Immunomodulators are an important component of the TME, and their alterations are closely related to the prognosis of patients.[35] TISIDB website analysis revealed 13 immunoinhibitors (ADORA2A, BTLA, CD96, CSF1R, CTLA4, HAVCR2, IL10, KDR, PDCD1LG2, PVRL2, TGFB1, TGFBR1, and TIGIT) (*P* value<0.05; Table 4) and 35 immunostimulators (C10orf54, CD27, CD28, CD40, CD40LG, CD48, CD80, CD86, CD267, CXCL12, CXCR4, ENTPD1, ICOS, ICOSLG, IL2RA, IL6, KLRC1, KLRK1, LTA, NT5E, PVR, TMEM173, TNFRSF4, TNFRSF8, TNFRSF9, TNFRSF13B, TNFRSF13C, TNFRSF14, TNFRSF17, TNFRSF25, TNFSF4, TNFSF13B, TNFSF14, TNFSF15, and TNFSF18) (*P* value<0.05; Table 5) in the TME of gastric cancer were significantly correlated with PLXDC1 expression. The protein–protein interaction network constructed by the STRING website revealed that 48 immunomodulators associated with PLXDC1 expression have complex interactions (Figure 6A). Functional enrichment analysis revealed

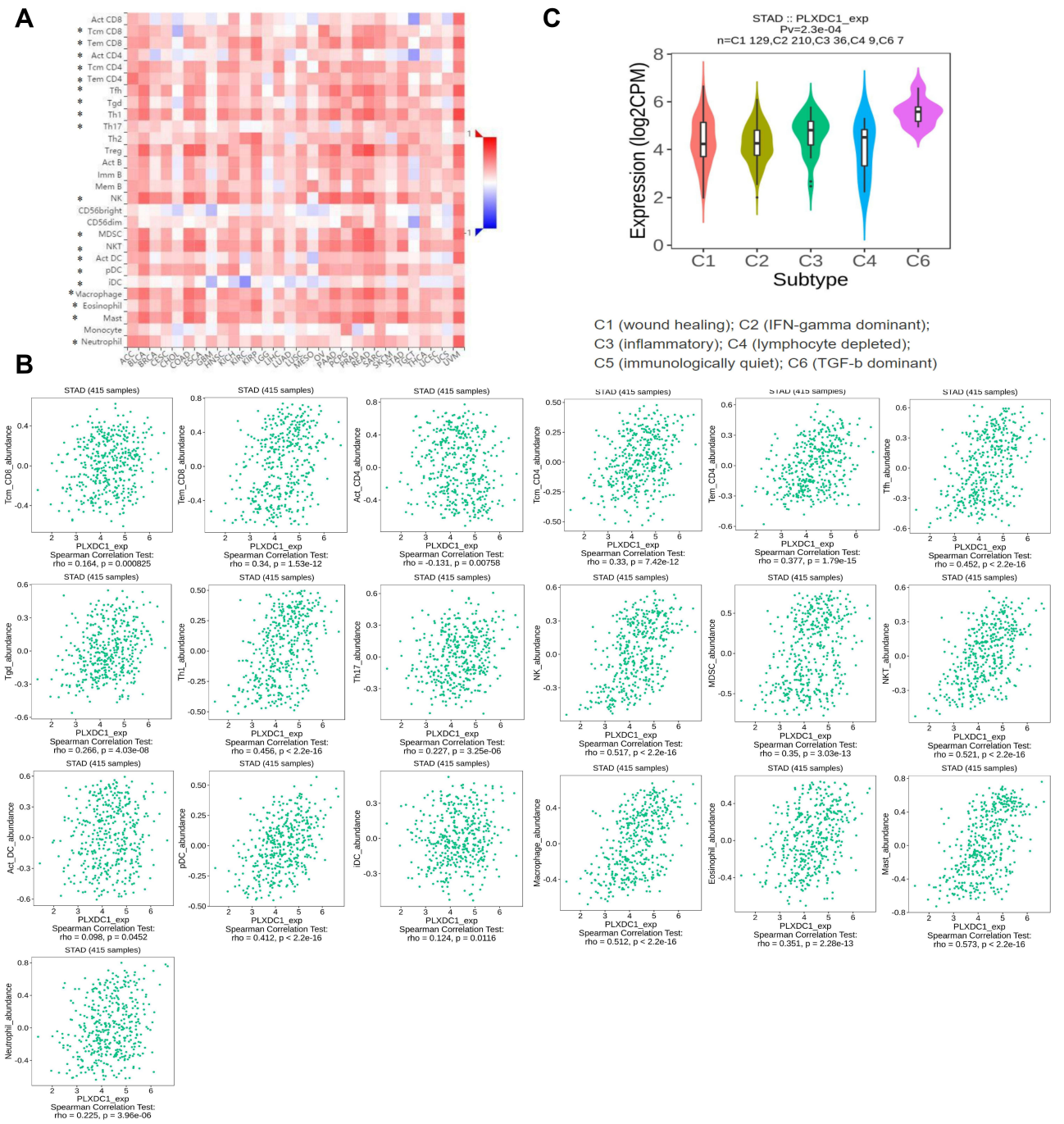


Figure 5 Correlation analysis of PLXDC1 expression and immune cell infiltration in the TME of gastric cancer. (**A**) Heatmap demonstrating the correlation of PLXDC1 expression with immune cell infiltration in pan-cancer. Red represents positive correlation and blue represents negative correlation. The deeper color indicates the stronger correlation. (**B**) Correlation analysis of PLXDC1 expression and immune cell infiltration in the TME of gastric cancer. (**C**) Correlation analysis of PLXDC1 expression and infiltration of immune subtypes in gastric cancer. *P value < 0.05.

that PLXDC1-related immunomodulators are mainly involved in cytokine–cytokine receptor interactions and the regulation of immunity (Figure 6B). Overall, PLXDC1-related immunomodulators may play an important role in the TME of gastric cancer.

(Figure 7A) demonstrates the differential expression of immunomodulators in the high and low PLXDC1 expression groups, and the results revealed that immunomodulators in the TME of gastric cancer were expressed at significantly higher levels in the high PLXDC1 expression group than in the low PLXDC1 expression group. Univariate Cox analysis

Table 4 Correlations Between the Expression of PLXDC1 and Immunoinhibitors

Immunoinhibitors	Rho	*P* value	Immunoinhibitors	Rho	*P* value
ADORA2A	0.363	2.98e-14*	IL10RB	0.055	0.263
BTLA	0.19	0.000104*	KDR	0.533	2.2e-16*
CD96	0.206	2.42e-05*	LAG3	0.038	0.442
CD160	0.086	0.0809	LGALS9	−0.085	0.0833
CD244	0.088	0.0735	PDCD1	0.062	0.206
CD274	0.045	0.361	PDCD1LG2	0.398	2.2e-16*
CSF1R	0.446	2.2e-16*	PVRL2	−0.173	0.000412*
CTLA4	0.107	0.0287*	TGFB1	0.444	2.2e-16*
HAVCR2	0.337	2.48e-1*	TGFBR1	0.287	3.11e-09*
ID01	0.041	0.403	TIGIT	0.157	0.00133*
IL10	0.386	1.43e-16*	VTCN1	0.008	0.873

Note: *P value < 0.05.

Table 5 Correlations Between the Expression of PLXDC1 and Immunostimulators

Immunostimulators	Rho	*P* value	Immunostimulators	Rho	*P* value
C10orf54	0.212	1.46e-05*	NT5E	0.1	0.0425*
CD27	0.214	1.11e-05*	PVR	−0.103	0.0368*
CD28	0.373	4.15e-05*	RAET1E	0.065	0.187
CD40	0.283	5.28e-09*	TMEM173	0.378	1.29e-15*
CD40LG	0.229	2.49e-06*	TMIGD2	0.044	0.368
CD48	0.277	1.07e-08*	TNFRSF4	0.304	3.31e-10*
CD70	0.08	0.103	TNFRSF8	0.238	1.02e-06*
CD80	0.243	5.83e-07*	TNFRSF9	0.21	1.62e-05*
CD86	0.35	2.81e-03*	TNFRSF13B	0.219	7.4e-06*
CD267	0.255	1.58e-07*	TNFRSF13C	0.116	0.0183*
CXCL12	0.524	2.2e-16*	TNFRSF14	−0.11	0.0246*
CXCR4	0.4	2.2e-16*	TNFRSF17	0.159	0.00121*
ENTPD1	0.69	2.2e-16*	TNFRSF18	0.06	0.226
HHLA2	−0.028	0.571	TNFRSF25	−0.136	0.00553*
ICOS	0.192	8.74e-05*	TNFSF4	0.437	2.2e-16*
ICOSLG	0.179	0.000256*	TNFSF9	−0.053	0.28
IL2RA	0.285	4.23e-09*	TNFSF13	−0.078	0.111
IL6	0.203	3.2e-05*	TNFSF13B	0.268	3.47e-08*
KLRC1	0.11	0.0257*	TNFSF14	0.287	3.03e-09*
KLRK1	0.192	8.88e-05*	TNFSF15	0.205	2.62e-05*
LTA	0.185	0.000157*	TNFSF18	0.339	1.78e-12*
MICB	−0.016	0.74	ULBP1	−0.079	0.109

Note: *P value < 0.05.

revealed that among the 48 immunomodulators, CSF1R, TGFB1, CXCR4, NT5E, and TNFSF18 were associated with poor prognosis, whereas the level of CTLA4 was associated with good prognosis (Figure 7B). Then, among these prognosis-related immunomodulators, we randomly identified four immunomodulatory genes (CTLA4, CXCR4, NT5E, and TNFSF18) to construct a PLXDC1-related prognostic risk model (Figure 7C and D). The multivariate Cox proportional hazards model had the following computational formula: risk score = (coefficient CTLA4 × expression CTLA4) + (coefficient CXCR4 × expression CXCR4) + (coefficient NT5E × expression NT5E) + (coefficient TNFSF18 × expression TNFSF18). Based on the median value of the risk score (1.126), we divided the gastric cancer patient samples in TCGA database into high- and low-risk groups. We found that patients in the low-risk group had better

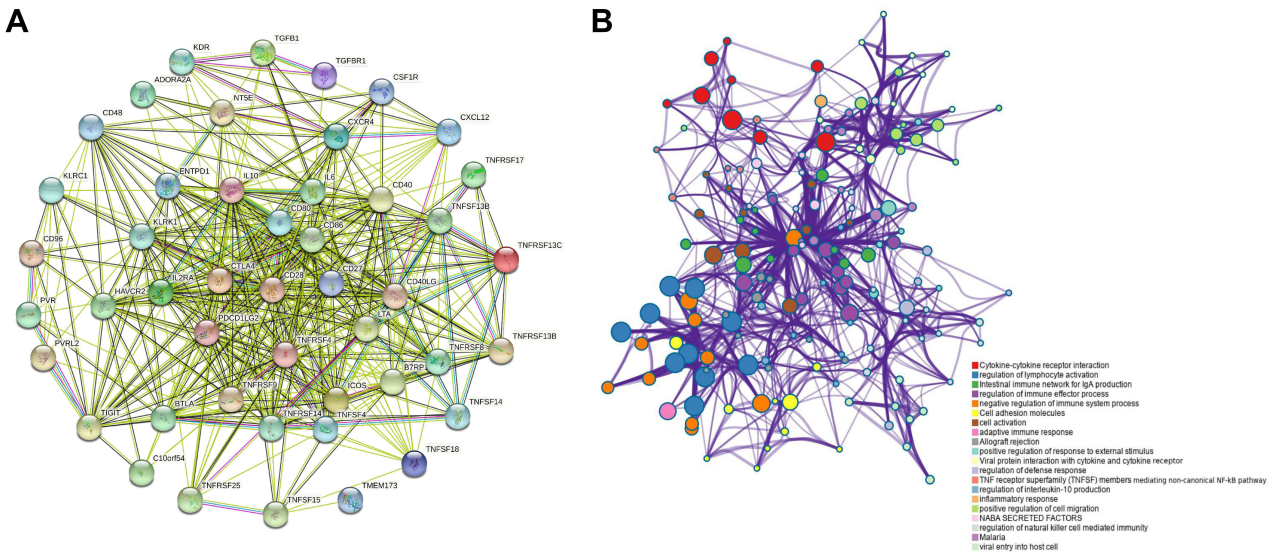


Figure 6 Analysis of immunomodulators associated with PLXDC1 expression in TME of gastric cancer. (**A**) Analysis of PLXDC1-related protein interactions of 48 immunomodulators. (**B**) Functional enrichment analysis of 48 immunomodulators whose expression is associated with PLXDC1 expression. Each color of the circles corresponds to a function and the size of the circles indicates the degree of functional enrichment.

survival outcomes than those in the high-risk group (P value < 0.05; Figure 7E). The AUC of the ROC curve for the multivariate Cox proportional hazards model was 0.706, indicating the high accuracy of our model's risk prediction capability. In addition, model risk scores combined with clinical factors had higher accuracy in prognosis prediction (AUC=0.738) (Figure 7F). Finally, the independent prognostic analysis of the multivariate Cox proportional hazards model's risk score combined with clinical factors such as age, gender, grade, stage, T stage, N stage, and M stage suggested that the risk score could be used as a prognostic risk indicator independently of these clinical factors (P value < 0.05; Figure 7G and H). In addition, we assessed the potential for immune evasion in the high- and low-risk model groups by the TIDE index, and the results showed that immune evasion was significantly higher in the high-risk group than in the low-risk group (Figure 7I). Overall, we analyzed the immunomodulators associated with PLXDC1 expression in the TME of gastric cancer and further demonstrated the correlation of PLXDC1 expression with immune evasion in gastric cancer by Cox risk modeling.

Immune Cell Infiltration in PLXDC1 High and Low Expression Groups in Gastric Cancer

To further verify that PLXDC1 mediates TIME immune evasion in gastric cancer, we performed immunohistochemical staining analysis on tissue samples with "+" and "+++" PLXDC1 expression in gastric cancer in Figure 1D. The purpose of the staining was to observe the infiltration of $CD8^+$ T cells, $CD3^+$ T cells and $CD4^+$ T cells in gastric cancer tissues and surrounding tissues to determine whether the high expression of PLXDC1 was consistent with the characteristics of immune evasion. The results showed that in gastric cancer tissues with "+++" PLXDC1 expression, $CD8^+$ T cells, $CD3^+$ T cells and $CD4^+$ T cells were mainly clustered in the tumor peripheral stroma, and immune cells were unable or rarely penetrated the stroma into the tumor parenchyma (Figure 8B). In contrast, in PLXDC1 expressing "+", $CD8^+$ T cells, $CD3^+$ T cells and $CD4^+$ T cells aggregated less in the tumor periphery, and immune cells relatively more penetrated the stroma into the tumor parenchyma (Figure 8A). Taken together, this phenomenon further confirmed that the PLXDC1 high expression group mediated immune evasion in the TIME of gastric cancer.

Discussion

In 1971, Folkman proposed a hypothesis that to promote tumor cell growth, tumors provide nutrients to their cells by creating new blood vessels.[36] Subsequently, this phenomenon was confirmed as one of the hallmarks of tumors.[37] To

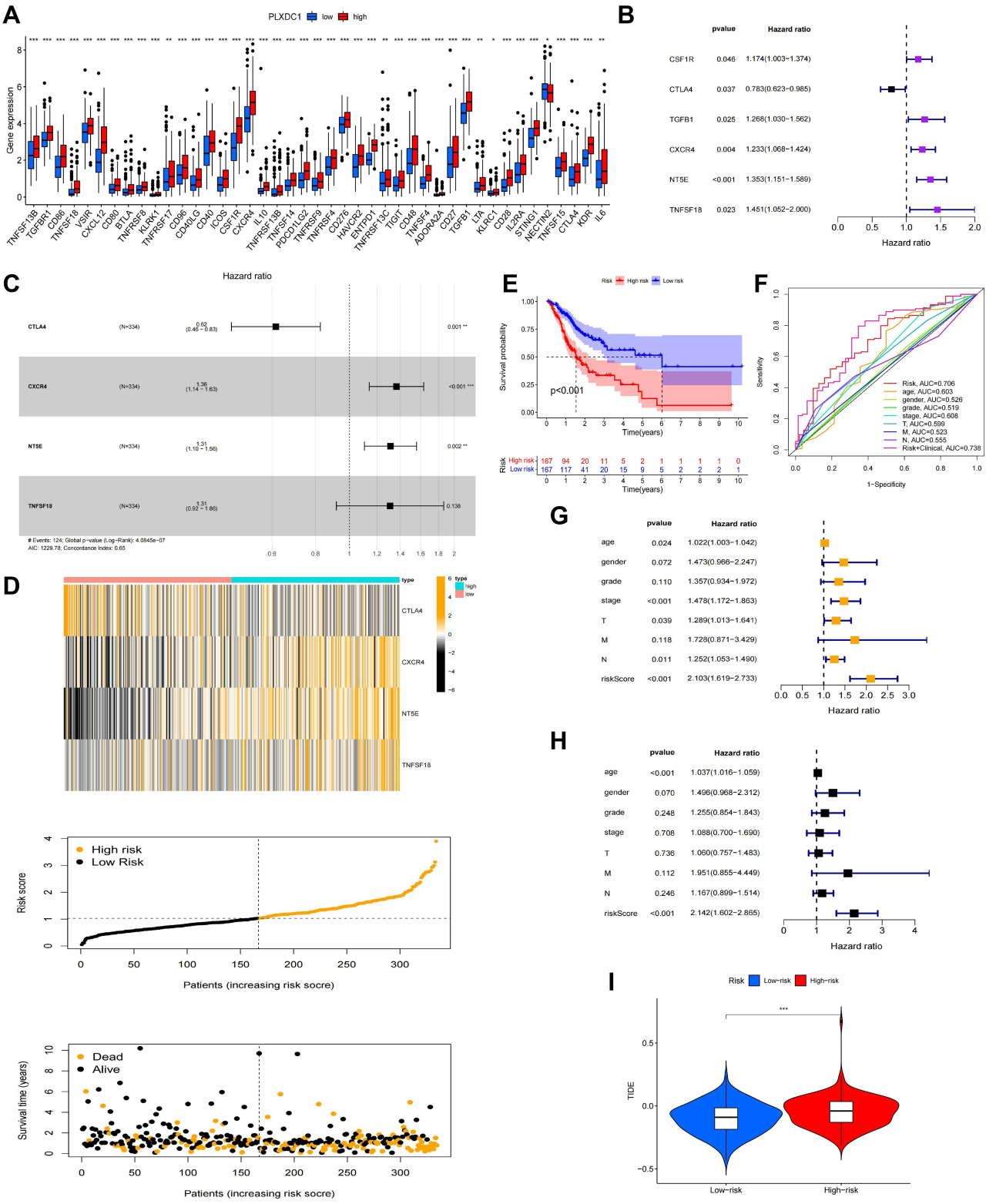


Figure 7 Construction of a PLXDC1-related immune prognostic risk model. (**A**) Differential analysis of immunomodulators in the high and low PLXDC1 expression groups. (**B**) Univariate Cox survival analysis of 48 immunomodulators associated with PLXDC1 expression in TME of gastric cancer. (**C**) Multivariate Cox analysis to screen candidate genes to construct a PLXDC1-associated Cox risk proportional regression model. (**D**) Distribution of patient samples in the high- and low-risk groups classified by the Cox risk model. (**E**) Prognostic analysis was performed by dividing patients into high and low risk groups according to the median value of the model risk scores. (**F**) Accuracy of ROC curve assessment model risk scoring and clinicopathological parameters in the prediction of prognosis. Model risk scoring combined with clinicopathological parameters for (**G**) univariate Cox and (**H**) multivariate Cox analyses. (**I**) TIDE assessment of immune evasion potential in the high- and low-risk groups of the risk model. *P value < 0.05; **P value < 0.01; ***P value < 0.001.

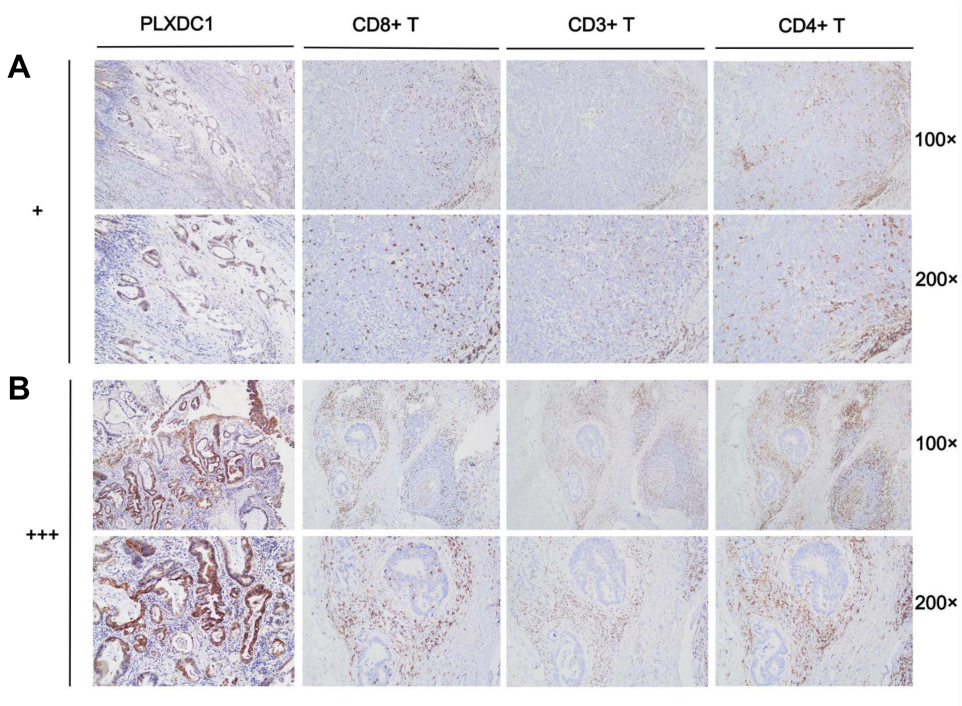


Figure 8 Immune cell infiltration in PLXDC1 high and low expression groups. (**A**) Infiltration of CD8+ T cells, CD3+ T cells and CD4+ T cells in PLXDC1 high expression group (+). (**B**) Infiltration of CD8+ T cells, CD3+ T cells and CD4+ T cells in PLXDC1 low expression group (+++).

date, antiangiogenic agents have achieved great success in treating many types of cancer, such as breast cancer,[38–40] lung cancer,[41,42] colorectal cancer[43] and gastric cancer.[44] Previous studies found that PLXDC1 expression is closely associated with angiogenesis.[19] In this study, we demonstrated that PLXDC1 was overexpressed in gastric cancer by using publicly available databases in combination with immunohistochemical staining experiments, and survival analysis revealed that high PLXDC1 expression was associated with poor prognosis in gastric cancer patients. In addition, univariate and multivariate Cox analyses indicated that PLXDC1 was an independent prognostic risk factor for gastric cancer. These findings indicated that PLXDC1 could be used as a biomarker for the prognosis of gastric cancer.

Despite the achievements of antiangiogenic drugs in the treatment of gastric cancer, some intractable problems have emerged.[45] Inhibition of angiogenesis provides only a transient survival benefit to patients because of the presence of tumor escape mechanisms, including the upregulation of compensatory pathways,[46] vasculogenic mimicry[47] and the recruitment of bone marrow-derived cells.[48] Moreover, several studies have found that angiogenesis inhibitors increase the aggressiveness and metastasis of tumors.[49,50] For example, short-term treatment with sunitinib led to accelerated tumor metastasis in a mouse model.[51] Notably, scientists have discovered that the use of antiangiogenic agents may cause tumors to develop immunosuppressive activities.[22,52] This is because the infiltration and function of immune cells depend on normal vascular channels and the oxygen and nutrients these channels provide.[53–56] Therefore, antiangiogenic agents disrupt tumor blood vessels, preventing immune cells from infiltrating and functioning. Based on the immunosuppression and multiple deficiencies of antiangiogenic agents, researchers recently proposed a program that combines antiangiogenic agents with immunotherapy,[22,57,58] which may result in a breakthrough in tumor treatment.

In this study, we found that PLXDC1 promotes immune activation and stromal signaling pathways in gastric cancer by GSEA and Hallmark gene set enrichment analysis. Immune cell correlation analysis in TISIDB revealed that PLXDC1 expression positively correlated with immune cell infiltration in gastric cancer, while survival analysis showed that high PLXDC1 expression in gastric cancer was associated with a poorer prognosis than low PLXDC1 expression. Immune evasion in the TME is characterized by the concentration of immune cells in the stroma surrounding the tumor tissue and the inability of these immune cells to penetrate the stroma.[32] Therefore, we suggest that high expression of PLXDC1 facilitates an immune evasive state of the TME in gastric cancer. Immunomodulators are essentially immune

cell surface markers, and the well-known marker PD-L1 is one of them. We constructed a Cox risk proportional regression model based on the immunomodulators whose expression was associated with PLXDC1 expression in the gastric cancer TME, and the TIDE assessment revealed a significantly higher immune evasion potential in the high-risk group than in the low-risk group. Finally, we performed immunohistochemical staining of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in gastric cancer tissue samples with high and low PLXDC1 expression, and the characteristics of the immune cell distribution observed further supported our previous conclusions.

However, there are some shortcomings in our study. We observed that high expression of PLXDC1 from tissue samples mediated immune evasion in gastric cancer, but the specific mechanism needs to be investigated at the cellular level subsequently. In addition, the unique angiogenic function of PLXDC1 is expected to be a promising target of tumor anti-angiogenesis treatments and immunotherapy; however, this requires additional follow-up studies.

In conclusion, we demonstrated from several biological perspectives that PLXDC1 can be a biomarker for poor prognosis and immune evasion in gastric cancer.

Abbreviations

PLXDC1, Plexin domain containing 1; TME, tumor microenvironment; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; TIDE, Tumor Immune Dysfunction and Exclusion; ICIs, Immune checkpoint inhibitors; PD-L1, programmed death-ligand 1; PD-1, programmed death-1; GSEA, Gene Set Enrichment Analysis; GSVA, gene set variation analysis; ssGSEA, single-sample GSEA; ESTIMATE, estimation of stromal and immune cells in malignant tumor tissues using expression data; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; ROC, receiver operating characteristic; AUC, area under the curve; OS, overall survival; FP, first progression; PPS, postprogression survival; LASSO, least absolute shrinkage and selection operator regression; HR, hazard ratio; MSigDB, Molecular Signatures Database; NSE, normalized enrichment score; NOM, nominal; FDR, false discovery rate.

Data Sharing Statement

The data for this study are available from the following databases: Oncomine database: https://www.oncomine.org/resource/login.html; The Cancer Genome Atlas database: https://www.cancer.gov/; Kaplan–Meier Plotter online website: https://kmplot.com/analysis/; Molecular Signatures Database online website: http://www.gsea-msigdb.org/gsea/login.jsp; Tumor Immune Dysfunction and Exclusion website: http://tide.dfci.harvard.edu/login/; TISIDB website: http://cis.hku.hk/TISIDB/index.php; Search Tool for the Retrieval of Interacting Genes/Proteins website: https://www.string-db.org/online; and Metascape website: http://metascape.org/gp/index.html/main/step1.

Ethics Approval and Informed Consent

This study was reviewed and approved by the Research Ethics Committee of The First Affiliated Hospital of Bengbu Medical College. All patients were briefed properly and voluntarily signed an informed consent form for the collection of clinical tissue samples. All specimens were processed and anonymised according to ethical and legal standards. All authors confirmed that this study complied with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors confirmed that there was no conflict of interest in the study.

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