

Immune Subtyping for Pancreatic Cancer with Implication in Clinical Outcomes and Improving Immunotherapy

Jingkai Liu

Peking Union Medical College Hospital <https://orcid.org/0000-0003-0523-8213>

Qiaofei Liu

Peking Union Medical College Hospital

Xiang Zhang

Peking Union Medical College Hospital

Ming Cui

Peking Union Medical College Hospital

Tong Li

Peking Union Medical College Hospital

Yalu Zhang

Peking Union Medical College Hospital

Quan Liao (✉ lqpumc@126.com)

Peking Union Medical College Hospital <https://orcid.org/0000-0003-1324-6213>

Primary research

Keywords: pancreatic cancer, heterogeneity, immune cell, immune checkpoints, immunotherapy, transglutaminase 2

Posted Date: November 5th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-101309/v1>

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Version of Record: A version of this preprint was published on February 26th, 2021. See the published version at <https://doi.org/10.1186/s12935-021-01824-z>.

Abstract

Background: Emerging evidence has shown that intra-tumor immune features is associated with response to immune checkpoint blockade (ICB) therapy. Accordingly, patient stratification is needed for identifying target patients and designing strategy to improve the efficacy of ICB therapy. We aimed to depict the specific immune features of patients with pancreatic cancer, and explore the implication of immune diversity in prognostic prediction and individualized immunotherapy.

Methods: From transcriptional profiles of 383 tumor samples in TCGA, ICGC and GEO database, robust immune subtypes which had different response immunotherapy, including ICB therapy, were identified by consensus clustering with five gene modules. DEGs analysis and tumor microarray were used to screen and demonstrate potential target for improving ICB therapy.

Results: Three subtypes of pancreatic cancer, namely cluster 1-3 (C1-C3), characterized with distinct immune features and prognosis were generated. Of that, subtype C1 was an immune-cold type in lack of immune regulators, subtype C2, with an immunosuppression-dominated phenotype characterized by robust TGF β signaling and stromal reaction, showed the worst prognosis, subtype C3 was an immune-hot type, with massive immune cell infiltration and in abundance of immune regulators . The disparity of immune features uncovered the discrepant applicability of anti-PD-1/PD-L1 therapy and potential sensitivity to other alternative immunotherapy for each subtypes. Patients in C3 were more suitable for anti-PD-1/PD-L1 therapy while patients in other two clusters may need combined strategies targeted on other immune checkpoints or oncogenic pathways. A promising target for improving anti-PD-1/PD-L1 treatment, TGM2, was screened out and its role in regulation of PD-L1 was investigated for the first time.

Conclusion: Collectively, immune features of pancreatic cancer contribute to distinct immunosuppressive mechanisms that are responsible for the individualized immunotherapy. Despite pancreatic cancer being considered as a poor immunogenic cancer type, the derived immune subtypes may have implication in tailored designing of immunotherapy for the patients. TGM2 has potential synergistic roles with ICB therapy.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has been striking a heavy burden on human health by increasing worldwide incidence and less than 9% survival rate [1]. Advances in chemotherapy regimens over the last two decades has only modestly prolonged the overall survival of the patients [2]. More effective treatment are still needed for PDAC patients. A promising ICB therapy, programmed cell death protein 1 (PD-1)/ programmed death 1 ligand 1(PD-L1) antibodies, has yielded significant clinical efficacy in some tumor types [3]. However, low response rate and limited patients benefited from single-agent ICB were observed in PDAC, which can be attributed to the low immunogenicity and diverse immunosuppression mechanisms [4]. To overcome the drug resistance, combination with other ICB targets or other therapeutic modalities has been regarded as a hopeful solution [5]. While the precondition

for making appropriate combination treatment strategy is a reasonable method for patient stratification based on similar characteristics of immune response.

Over the last decade, substantial progression in molecular subtyping for PDAC has facilitated the understanding of the molecular pathogenesis and provided clues for advanced therapy designing [6]. However, to date, there is still lacking an immune feature based molecular subtyping for better understanding the heterogeneity of immune response and reasons for the inefficiency of ICB therapy.

A recent pan-cancer study revealed a widely suitable immune-subtyping method based on five immune signatures which provided a potential roadmap for PDAC [7]. Herein three distinct immune subtypes of PDAC were presented based on these five immune signature modules. Each subtype showed distinct immune cell composition and expression patterns of immunomodulators, which provided a reasonable explanation for their survival discrepancy and inefficacy of single-agent ICB therapy. Furthermore, we screened out a potential immunosuppression-related gene, transglutaminase 2 (TGM2) and investigated its potentially synergistic roles with ICB therapy. TGM2 is a multifunctional protein with both signaling transduction and protein cross-linking roles. Previous studies revealed that TGM2 could promote multiple invasive phenotypes including tumor growth, metastasis, epithelial-mesenchymal transition and drug resistance in PDAC [8–10]. Overall, these findings provide a conceptual framework to understand the immune response diversity in the tumor microenvironment of PDAC, implicate PDAC patient stratification, and design combination therapeutic strategies based on ICB therapy.

Materials And Methods

Transcriptional data resources and preprocessing

Human pancreatic cancer transcriptional profiles were downloaded from public database, including The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>), International Cancer Genome Consortium (ICGC, <https://icgc.org/>) and Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>). Read count data from TCGA (PAAD) and ICGC (PACA-AU) were normalized in TMM method by edgeR (R package). Then TPM value was calculated respectively and batch effect was eliminated by Combat. There were 182 TCGA data samples, of which 4 para-cancer samples and 1 non-primary sample were excluded, and finally a total of 177 tumor samples were collected. GSE28735 and GSE62452, which are two datasets downloaded from GEO database with entire clinical following information, were integrated together. The batch effect was eliminated by Combat. The combined datasets of GSE28735 and GSE62452 contained a total of 220 samples, including 114 tumor samples and 106 para-cancer samples. Together with 92 tumor samples from ICGC and 177 tumor samples from TCGA, 383 tumor samples were taken into analysis. To accomplish combined analysis of tumor statistics, RNA-Seq and gene array transcriptional data were normalized in the manner of z-score. The flow chart of data processing was shown as Fig. 1.

Patient cohort

The use of clinical samples and clinical information for this study was approved by the institutional review board of Peking Union Medical Hospital (NO. JS-987). Totally, 97 pairs of tumor and para-tumor normal samples of patients diagnosed as PDAC were collected as the description of our previous study [11]. All of the cases were pathologically confirmed to be PDAC, and R0 radical resection was achieved and at least three cycles of adjuvant chemotherapy was performed for each patient. Histological grading was made according to the 8th edition of the TNM system established by the American Joint Committee on Cancer (AJCC) [12]. None of the patients undergone neoadjuvant chemotherapy.

Immunohistochemistry (IHC) and digital analysis

Immunohistochemistry staining was implemented in accordance with previous study [11]. Microarray chip was stained with anti-TGM2 (15100-1-AP, Proteintech, 1:200). Quant Center identified and analyzed the areas of strong positive, moderate positive, weak positive and negative pixels, as well as the percentage of positive pixels, and finally conducted an H-score.

Cell culture

Human pancreatic cancer cell lines, PANC-1 and Mia PaCa-2, were maintained in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin and cultured in the incubator with 5% CO₂ at 37°C.

Western blotting

Total protein were extracted using 2% SDS lysis buffer and heated for 10 minutes. Protein samples were separated by 8% (v/v) SDS-PAGE gels and transferred onto nitrocellulose membranes (Millipore, Ireland). After blocked in 5% (w/v) Albumin Bovine-V (BSA-V, Solarbio, China) for 1 h at room temperature. The protein bands were incubated with primary antibodies at a dilution ratio of 1:1000 overnight at 4 °C. The primary antibodies contains anti-GAPDH (FL-335; Santa Cruz Bio technology), anti-TGM2 (Proteintech), anti-PD-L1 (Proteintech), anti-STAT3 (CST) and anti-Phospho-STAT3 (Ser705, CST), anti-Akt (CST) and anti-Phospho-Akt (Ser473, CST), anti-P65 (Abcam) and anti-Phospho-P65 (Ser536, Abcam). The protein bands were incubated in HRP-conjugated secondary antibodies (Zsbio, China, 1:5000) at room temperature for 1 h.

Transfection assay

Lentiviral particles for TGM2 knockdown assay were purchased from Syngen Tech (Beijing, China). Lentiviral particles were added into PANC-1 cells and Mia PaCa-2 cells supplemented with 5 µg/ml polybrene. After 48 hours transfection, target cells were selected with 1 µg/ml of puromycin for two weeks. The TGM2 knockdown in cells were validated by Western blotting. The sequence of shTGM2 is acquired from previous study and showed as below: 5'-AAGGGCGAACCACCTGAACAA-3' [13].

Statistical analysis

Identification of immune subtypes

Five immune signature sets, including CSF1_response (Macrophages), Llexpression_score (Lymphocyte), Module3_IFN_score (IFN- γ), TGFB_score_21050467 (TGF- β) and CHANG_CORE_SERUM_RESPONSE_UP (Wound healing) modules, were selected to run clustering analysis. They respectively represented the activation of macrophage/monocytes, overall lymphocyte infiltration, TGF- β response, IFN- γ response and wound healing activity in tumor immune microenvironment. GSVA enrichment analysis was conducted and ssGSEA values were calculated. Unsupervised clustering with ssGSEA values of samples was conducted by McLust R package, and K values corresponding to maximized Bayesian Information criterion (BIC) were selected to obtain immune subtypes.

Depiction of molecular and cellular signatures in immune subtypes

To further understand the cellular and molecular characteristics of immune microenvironment, we assessed the enrichment degree of 75 immunomodulator genes and 64 immune cell types. The enrichment of immune cell types and immune related genes were analyzed by xCell software and ImmuneCellAI (<http://bioinfo.life.hust.edu.cn/ImmuneCellAI>). Kruskal-wallis test or Wilcox test were used to analyze the differences in enrichment scores of immune cells among groups. Boxplot was drawn for distribution of immune cells. The accuracy of immune cells in predicting survival was analyzed by C-index analysis.

Prognostic analysis

To evaluate the impact of immune signatures on patients' survival, we performed Kaplan-Meier analysis, univariate and multivariate COX analysis. The results were presented as the mean \pm standard deviation. C-index was used to analyze the accuracy of gene sets model in predicting survival. $p < 0.05$ was considered statistically significant.

Identification of immune related gene target

Based on the gene expression profiles of GSE28735 and GSE62452 differential expressed genes (DEGs) were collected by limma (R package) with filtration of p -value < 0.05 and Fold Change (FC) > 1 . To identify target gene among the DEGs, further filtration was set as negative relation with prognosis, positive relation with the infiltration of macrophages and the expression of PD-L1, PD-1, TGF- β 1 and GM-CSF and analyzed by online tool of GEPIA (<http://gepia.cancer-pku.cn/>) and TIMER (<https://cistrome.shinyapps.io/timer/>). Set median expression value of target gene as cutoff and we divided the whole samples into high and low expression groups. The distribution of high and low groups in three immune subtypes then was described in a percentage and count manner. The prognostic analysis of target gene was conducted by online tool of ImmuneCellAI. The influence of immune modulator genes on patients' survival in the above two high and low expression groups was also analyzed.

Results

Identification of immune subtypes in PDAC

PDAC harbored highly heterogeneous tumor microenvironment. By performing clustering analysis with five immune signature gene sets, we classified three immune subtypes, namely C1-C3, in the 383 pancreatic tumor samples. The five immune signatures represented the activation of macrophage/monocytes (Macrophages), overall lymphocyte infiltration (Lymphocyte), TGF- β response (TGF- β), IFN- γ response (IFN- γ) and wound healing activity (Wound healing), respectively.

Five immune signatures showed disparate enrichment patterns in three immune subtypes (Fig. 2a). Subtype C3 had the highest Lymphocyte and Macrophages as well as a favorable enrichment in IFN- γ , indicating an immune-hot phenotype (Fig. 2b). Subtype C1 had generally poor enrichment scores, especially the lowest enrichment in IFN- γ , TGF- β and Wound healing, indicating an immune-cold phenotype (Fig. 2b). In comparison, subtype C2 had the highest TGF- β enrichment scores and the lowest Lymphocytes enrichment scores, indicating an immune suppressive phenotype (Fig. 2b). Meanwhile, C2 also had the highest Wound healing enrichment scores indicating an active tissue remodeling (Fig. 2b).

Cellular composition and immunomodulators diversity of immune subtypes

Immune cell composition varied across three subtypes. Subtype C3, the immune-hot phenotype, had dominant cellular enrichment scores with the highest T lymphocytes (both CD8⁺ and CD4⁺), NKT cells, dendritic cells (DCs), macrophages and B cells as well as granulocytes (Fig. 3a,b). Meanwhile, three subtypes displayed distinct expression patterns of immunomodulators (Fig. 3c). Compared with subtype C1 and C2, subtype C3 had higher CD27 and inducible synergistic co-stimulation molecules (ICOS) expression, indicating a favorable lymphocytes activation. In addition, higher IL2, IFN- γ and TNF families (e.g. CD40) represents a favorable cellular immunity and anti-tumor ability of C3 (Fig. 3d). The expression pattern of inhibitory immune regulators varied from three subtypes. In addition to a higher TGF- β 1, subtype C3 had higher immune checkpoint expression of CD274 (PD-L1, also as B7-H1), PDCD1 (PD-1), CTLA4, TIGIT, LAG3, HAVCR2 (TIM3) but lower expression of CD276 (B7-H3) and VTCN1 (B7-H4), compared with C1 and C2 (Fig. 3d, e).

Opposite enrichment patterns of immune-infiltrating cells were found between subtype C1 and C2. Subtype C1 had higher enrichment scores in T cells, NKT cells, DCs and B cells but lower in macrophages, while C2 had higher scores in macrophages but lower scores in T cells, NKT cells, DCs and B cells (Fig. 3a,b), indicating a stronger immunosuppressive microenvironment of subtype C2. On the other hand, both C1 and C2 showed a poor expression of stimulatory immunomodulators. As for the expression discrepancy of immune checkpoints, subtype C1 was mainly enriched in VTCN1, while C2 was dominated by CD274, CD276 and VTCN1 (Fig. 3d,e), which may have clinical implication for further designing tailored immunotherapy strategy.

The stromal and immune score of tumor samples were calculated by ESTIMATE software. Different from the discrete distribution of C1 and C2, subtype C3 mostly enriched in the zones with high immune scores,

which indicated a robust immune activity in C3 (Fig. 3f).

Prognosis analysis of immune subtypes

The association between immune subtypes and patients' survival was also analyzed. The immune suppressive subtype C2 had the worst prognosis, while C1 and C3 had relatively favorable prognosis (C1:C2:C3, $p = 0.0019$, Fig. 4a; C1:C2, $p = 0.00094$; C1:C3, $P = 0.24$; C2:C3, $p = 0.038$, Additional file 1: Figure S1). The results of K-M analysis revealed that IFN- γ ($p = 0.001$) and Wound healing ($p = 5.645e-04$) modules were inversely correlated with patients overall survival (Fig. 4b). The univariate and multivariate cox analyses also showed the same results (Fig. 4c and Table 1). Furthermore, with concordance index (CI) analysis, we evaluated the validity of the five immune signatures in survival prediction. The results showed that IFN- γ , Wound healing and TGF- β modules had favorable prediction accuracy on patient survival among the three subtypes (Fig. 4d). Especially, Wound healing module in C2, Macrophages module in C3, TGF- β and Lymphocyte modules in C1 seemed to be the best predictor for each subtype, respectively (Fig. 4d).

Table 1
Univariate and multivariate cox analyses of five immune signatures.

Parameter	Univariate analysis			Multivariate analysis		
	HR	95%CI	P	HR	95%CI	P
Wound healing	9	2.38–34.01	0.001	4.28	1.03–17.67	0.045
Macrophages	1.29	0.67–2.44	0.438	-	-	-
Lymphocyte	1.06	0.60–1.86	0.846	-	-	-
IFN- γ	2.22	1.32–3.76	0.003	1.77	1.03–3.06	0.04
TGF- β	5.42	1.80–16.34	0.003	3.21	0.99–10.34	0.051

Prognostic analysis showed that NKT cells correlated with a favorable prognosis ($p = 0.014$) (Fig. 5a) while macrophages were associated with a poor prognosis ($p = 0.0031$) (Fig. 5b). The CI analysis showed that both NKT cells and macrophages could serve as favorable predictors for patients' survival in C2 (Fig. 5c). Although other cell types showed no significant correlation with patients' survival in our study (Additional file 1: Figure S2), immune cells especially CD8⁺ T cells, NKT cells and B cells showed greater impact on patients survival based on CI scores (Fig. 5d).

TGM2 was involved in the immune-suppressive microenvironment in PDAC and high TGM2 expression predicted poorer survival

To find underlying target for anti-PD-1/PD-L1 treatment, we performed DEGs analysis with the two GEO datasets (GSE62452 and GSE28735). Among all the up-regulated DEGs, following the set filters, the result

led to gene TGM2 (Additional file 1: Figure S3). With IHC analysis in our tissue microarray, we also verified that TGM2 was elevated in tumor tissue compared with adjacent normal tissue (Fig. 6a) and had a negative impact on prognosis (Fig. 6b). In addition, survival analysis within TCGA-ICGC-GEO cohort verified the prognostic impact of TGM2 (Additional file 1: Figure S4).

Besides, the expression patterns of TGM2 across the three subtypes were different. Most patients comprising subtype C2 had high TGM2 expression, while subtype C1 and C3 were dominated by low TGM2 expression (Fig. 6c,d). Meanwhile, in the analysis of the immune cells composition between high- and low-TGM2 groups, we found that macrophages mostly enriched in high-TGM2 group, while NKT cells mostly enriched in low-TGM2 group (Fig. 6e). Furthermore, compared with low-TGM2 group, high-TGM2 group seemed to have higher M2 macrophages ($p = 0.014$) and Treg cells ($p = 0.068$) but lower pro-B cells ($p = 0.00052$) and memory B cells ($p = 3.7e-6$) (Fig. 6f, g). And high-TGM2 group harbored a higher expression pattern of inhibitory immunomodulators (CD274, CD276, CTLA4, EDNRB, HAVCR2, LAG3, PDCD1, TGFB1, TIGIT and VTCN1) in the whole cohort (Fig. 6h). In the prognostic analysis with IMs between the two groups, higher CD276 was associated with worsen prognosis in high-TGM2 group and higher VTCN1 was related to poorer overall survival in low-TGM2 group (Fig. 6i). Together, these findings suggested that TGM2 may be involved in the immunosuppression in PDAC.

TGM2 regulated PD-L1 expression in PDAC via STAT3 and NF- κ B

To further explore the role of TGM2 in the immunosuppression, we analyzed the relation between TGM2 and the suppressive factors (Fig. 7a). The relative coefficient between TGM2 and PD-L1 is the most robust (Fig. 7b). We verified the correlation between TGM2 and PD-L1 in human PDAC cell lines (PANC-1 and Mia PaCa-2). After TGM2 knocking-down in PANC-1, we found a decreased expression level of PD-L1 (Fig. 7c). In cell line of Mia PaCa-2, we observed the same expression variation (Fig. 7d). NF- κ B and STAT3 are two important transcriptional factors in tumor evolution and reported to be capable to regulate the expression of PD-L1 [14, 15]. We hypothesized that TGM2 may regulate the expression of PD-L1 via STAT3 and NF- κ B signaling. Then, we knocked down TGM2 in PANC-1 and Mia PaCa-2 cells, a decreasing level of p-STAT3 was observed (Fig. 7c,d). Previous study revealed that TGM2 regulated the activation of NF- κ B by modulating phosphorylation of AKT [10]. In our study, we proved that TGM2 knocking down resulted in a decrease of p-Akt (Ser473) and p-P65 (Ser536) (Fig. 7e).

Discussion

ICB has become a promising immunotherapeutic modality for several refractory carcinomas, however its roles in PDAC is limited. The challenges majored in the identification of target patients, and finding effective combination therapeutic targets to amplify its clinical efficacy. In this study, we utilized published immune signature gene sets to depict the distinct immune features of PDAC and three immune subtypes are identified. The prognostic impact of immune gene sets, immune cell composition and

immunomodulators are evaluated. The diversity of immune response reflects the inner cause of prognostic discrepancy and immunotherapy failure in PDAC. Meanwhile, our study firstly put forward that the high expression of TGM2 tracked with an immunosuppression-promoting phenotype and TGM2 is involved in the modulation of PD-L1 expression by regulating downstream transcription factor STAT3/NF- κ B in pancreatic cancer cells. In addition, the enrichment disparity of IMs in each subtypes may suggest multiple potential combination immunotherapy strategies.

The prognostic impact of tumor immune feature is emerging to be concerned. Among all subtypes in our study, patients comprising C2 have the worst overall survival. The reasons for the prognostic discrepancy can be attributed to the following points. Firstly, the immune signatures in C2 are dominated by TGF- β and Wound healing modules which were associated with an immunosuppressive and pro-tumor phenotype [16, 17], and inversely correlated with patients prognosis based on our data. Meanwhile, the lowest enrichment degree in Lymphocyte module is another feature of C2. Consistent with that, the infiltration of T-, B-lymphocytes and NKT cells in C2 is less than the other subtypes, indicating a poor preexisting anti-tumor immunity [18]. Moreover, the highest enrichment degree of IFN- γ module is found in C2. IFN- γ is a major mediator inducing the death of tumor cells. However, continuous exposure to IFN- γ also stimulates tumor cells to express multiple inhibitory modulators including PD-L1 which are majorly enriched in C2 and C3 in our study, thereby suppress the secretion of IFN- γ by effector T cells and result in T cell exhaustion [19, 20]. Together, a more prominent feature of immunosuppression may be promoted by these features of C2 and therefore the prognosis of C2 is worse. In addition to these molecular immune features, our results show that the overall burden of NKT cells is associated with a better prognosis in PDAC, while that is rarely reported before. Similar with CD8⁺ T cells, NKT cells is also regarded as one of the front-line anti-tumor forces [21]. Apart from directly targeting on the tumor cells with CD1d positive, recent literature on mice model demonstrated that NKT cells could restrict the tumor evolution of PDAC indirectly by suppressing the pro-immunosuppression role of macrophages through prostaglandin E synthase-1 (mPGES-1) and 5-lipoxygenase (5-LOX) [22]. Consistent with that, subtype C2, with high fraction of macrophage and low fraction of NKT, shows more prominent immunosuppression phenotype than C1 and C3. These distinct molecular and cellular features across three subtypes show a diverse immune response of PDAC, which would be condition for prognostic evaluation and immunotherapy strategy designing.

To improve the clinical efficacy of anti-PD1/PDL1 therapy in PDAC, reasonable patient stratification and combination strategy designing should be concerned. For the former, prior studies indicated that the level of preexisting anti-tumor immunity and the expression level of PD-1/PD-L1 are two vital factors for the efficacy of anti-PD-1/PD-L1 treatment [23]. In our study, tumor samples comprising C3 are rich in various anti-tumor cytokines, such as TNF and IFN- γ and have abundant infiltration of immune cells including NKT cells and T lymphocytes as well as B lymphocytes. Despite the anti-tumor effect of B cells remained unclear, a recent study in melanoma indicated that B cells may contribute to the response to ICB treatment by altering T cell activation and function [24]. As well, C3 has the highest PD-1 and PD-L1 enrichment across the three subtypes. Thus, patients within C3 subtype seem to be more suitable for anti-

PD treatment. Meanwhile, based on our data, C3 is also rich in CTLA4, TIGIT and HAVCR2 expression which may provide alternative options for combined targets. Compared with single-agent ICB therapy, combination treatment of anti-CTLA4 (ipilimumab) and anti-PDL1 (nivolumab or pembrolizumab) led to better tumor response and patient survival in melanoma, sarcoma and small cell lung cancer [25–29]. Besides, preclinical studies have demonstrated that anti-TIGIT or anti-HAVCR2 can effectively control tumor evolution, suggesting a promising combination target for anti-PD-1/PD-L1 treatment [30, 31].

In contrary with subtype C3, anti-PD-1/PD-L1 treatment may be not appropriate for the patients within subtype C1 due to the relatively low infiltration of T/B lymphocytes and the poorest enrichment in PD-1/PD-L1. Of note, C1 has the highest VTCN1 expression as well as favorable enrichment scores in NKT cells. As a newly discovered immune checkpoint expressed on APC cells and tumor cells, VTCN1 is expected to become a novel target for immunotherapy in the future despite its regulatory mechanism in cancer immunity remained to be further explored [32]. Due to advantages of targeted on tumor cells and suppressive effect on graft versus host disease, NKT cells (majorly invariant NKT cells) is regarded as a viable vector for the CAR or rTCR treatments with multiple pre-clinical animal models supporting favorable anti-tumor effects in solid tumors [33–35].

Subtype C2 has favorable enrichment scores in PD-L1, CD276 and VTCN1 but is poor at lymphocytic infiltration. In addition to anti-PD-L1 treatment to restore the anti-tumor immunity, strategies to target oncogenic pathway is needed for patients in C2 to restrict tumor progression. TGM2 is a promising target for improving the response to chemotherapy in solid tumors including PDAC [36]. While its role in the immune evade process of PDAC remains unclear. In this study, we find that TGM2 is positively related with the expression of multiple inhibitory immunomodulators, and the high-TGM2 group is mainly enriched in the immunosuppressive subtype C2, suggesting that TGM2 may be involved in the regulation of immunosuppression in PDAC. Through *in vitro* experiments, we verified that TGM2 has a positive impact on PD-L1 in PANC-1 and Mia PaCa-2 cells. The underlying mechanisms may refer to two aspects. Firstly, STAT3 has been reported to be involved in the regulation of PD-L1 expression in various cancer types [37–39]. However, whether TGM2 alters the expression of PD-L1 in PDAC via STAT3 signaling remains to be unknown. Our present study reveals that down-regulating TGM2 in PANC-1 and Mia PaCa-2 cells results in a decreased phosphorylation of STAT3, which indicates a potential pathway for the regulation of PD-L1 by TGM2. Secondly, previous studies revealed that TGM2 promotes the activation of AKT by suppressing PTEN, then result in the activation of downstream substances including transcription factor NF- κ B in PDAC [10, 40]. Consistent with that, knocking down TGM2 in PANC-1 cells leads to a decrease in p-Akt and p-P65 expression. As a vital transcriptional factor, NF- κ B can regulate the transcription of PD-L1 by directly binding to the promoter region of PD-L1 [41]. Thus, TGM2 may take a positive impact on PD-L1 expression via Akt/ NF- κ B pathway. These findings provide insights into the regulation network of PD-L1 expression in PDAC (Fig. 7f) and thereby TGM2 is expected to be a promising target for anti-PD-1/PD-L1 therapy.

Some limitations in this study need to be addressed. Firstly, as a retrospective study, the clinical value of these findings need to be further validated in a larger prospective cohort. Secondly, the potential selection

bias of tumor specimens may exist. Thirdly, though transcriptional profiles in our study provided us underlying features of immune response in PDAC, with multi-omics data gathering, more solid evidence will promote our insight into tailored treatment for PDAC.

In summary, our study uncovers the specific immune features among PDAC patients. For the discrepant immune response mechanisms, more personalized ICB treatment should be considered. Moreover, TGM2 regulates the expression of PD-L1 in PDAC via STAT3 and Akt/NF- κ B signaling pathway and predicts poorer survival of PDAC patients, indicating a potential role in immunotherapy.

Abbreviations

ICB: immune checkpoint blockade; PDAC: pancreatic ductal adenocarcinoma; PD-1: programmed cell death protein 1; PD-L1: programmed death 1 ligand 1; TGM2: transglutaminase 2; TCGA: The Cancer Genome Atlas; ICGC: the International Cancer Genome Consortium; GEO: Gene Expression Omnibus; AJCC: American Joint Committee on Cancer.

Declarations

Ethics approval and consent to participate

The studies involving in human participants were reviewed and approved by the institutional review board of Peking Union Medical Hospital (NO. JS-987). All patients involved in this study signed the informed consent.

Consent for publication

Not applicable.

Availability of data and material

The public datasets of PAAD, PACA-AU, GSE28735 and GSE62452 for this study can be found in The Cancer Genome Atlas (<https://portal.gdc.cancer.gov/>), International Cancer Genome Consortium (<https://dcc.icgc.org/>) and Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>).

Competing interests

The authors declare that they have no competing interests

Funding

This work was supported by National Natural Science Foundation of China (81673023, 81872501 and 81502068).

Authors' contributions

Quan Liao and Jingkai Liu designed the research concept of this manuscript. Jingkai Liu wrote the article. Qiaofei Liu, Xiang Zhang and Ming Cui reviewed the draft. Qiaofei Liu made critical revision. Xiang Zhang, Ming Cui and Tong Li conducted the collection of clinical data and prepared pathological analysis. Jingkai Liu ran the bioinformatics analysis of public data. Jingkai Liu and Yalu Zhang conducted *in vitro* experiments. Jingkai Liu checked the data. All authors approved the final version.

Acknowledgments

We thank Prof. Yuande Wu and Prof. Xiaolin Yang from institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

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Figures

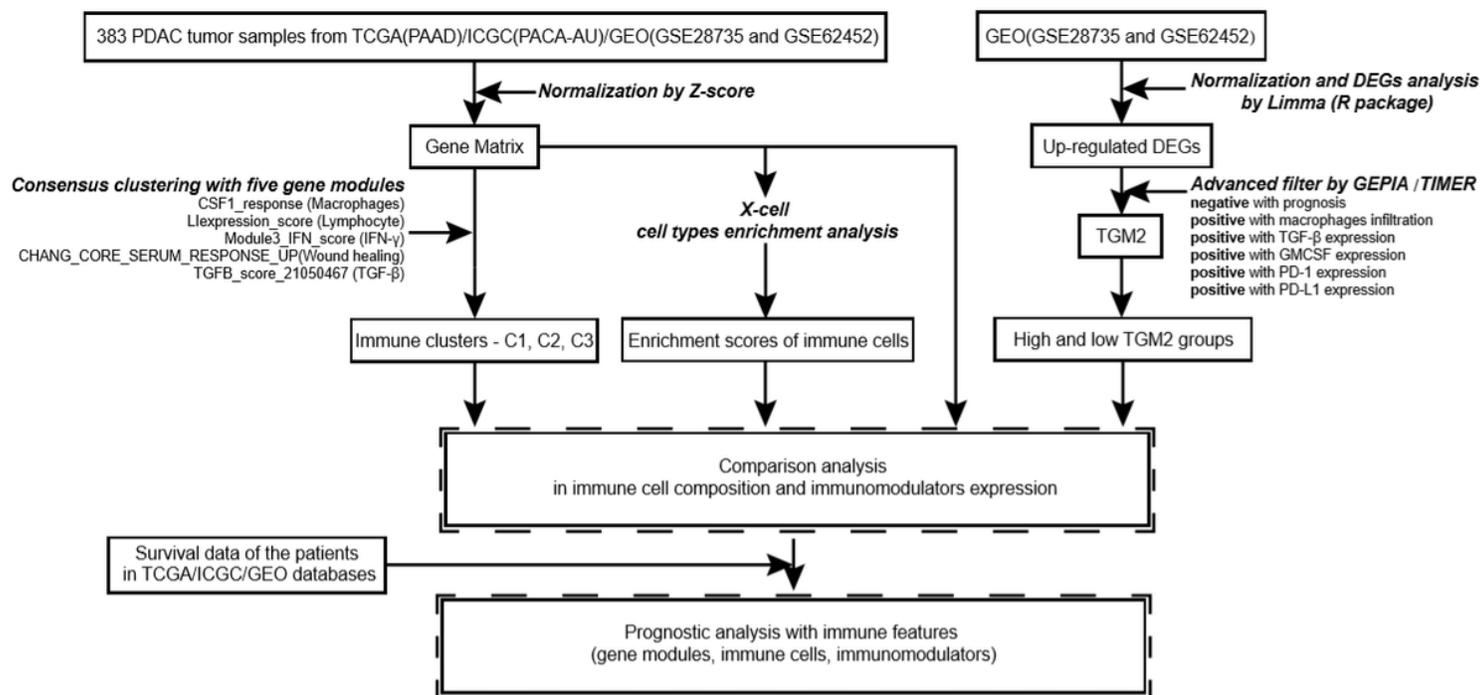


Figure 1

Flowchart of data collection and analysis in present study. Transcriptional profiles were collected from TCGA (PAAD)/ICGC (PACA-AU)/GEO (GSE28735 and GSE62452). Three immune clusters were derived by consensus clustering with five gene modules. The enrichment scores of cell types were calculated by X-

cell. Then, comparison analysis in immune cell composition and immunomodulators expression were performed in groups and whole cohort. Combined with survival data of the patients TCGA/ICGC/GEO cohort, the prognostic analysis with immune features (gene modules, immune cells, and immunomodulators) were performed. To find promising target for anti-PD-1/PD-L1 treatment, DEGs analysis and correlation analysis were performed in GEO (GSE28735 and GSE62452) database. The results led to gene TGM2, an oncogenic target for PDAC. The comparison analysis and prognostic analysis were performed in high-/low- TGM2 groups.

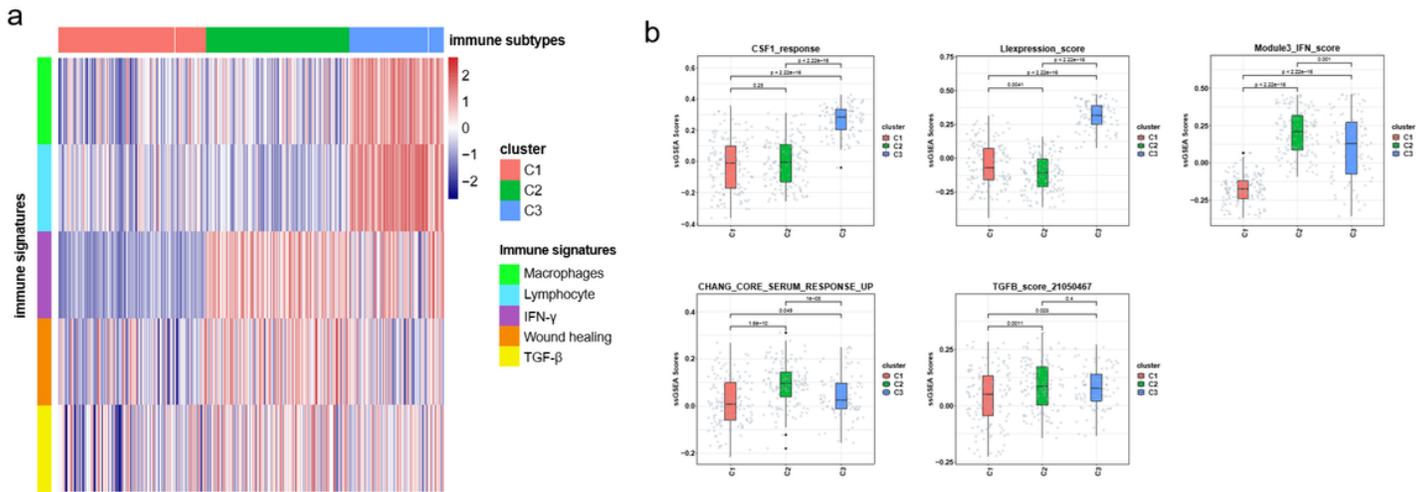


Figure 2

Immune subtypes of PDAC and immune signatures in TCGA and ICGC and GEO mix. a Immune subtypes of PDAC: column was for tumor samples and row was for five immune signatures. b Enrichment comparison of the five immune gene modules across three immune subtypes. Gene modules include CSF1_response (correspond to Macrophages), Llexpression_score (correspond to Lymphocyte), Module3_IFN_score (correspond to IFN- γ), CHANG_CORE_SERUM_RESPONSE_UP (correspond to Wound healing), and TGFB_score_21050467 (correspond to TGF- β).

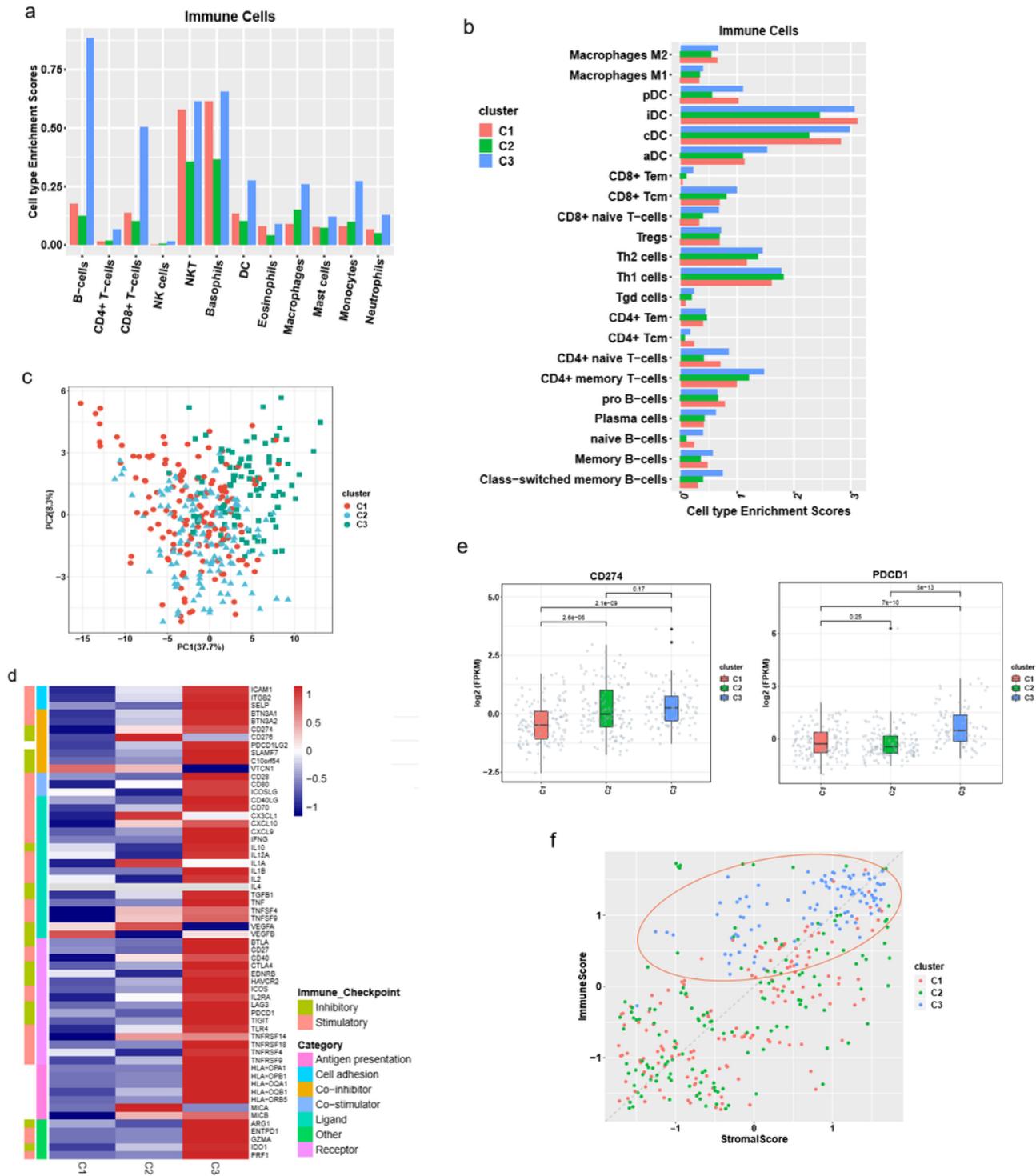


Figure 3

Immune cell composition and expression patterns of IMs in three subtypes. a,b Immune cell composition of three immune subtypes. c PCA analysis of IMs genes. d Expression patterns of IMs in three subtypes. e Enrichment disparity of CD274 and PDCD1 across the three subtypes. f Analysis of stromal and immune scores across the three subtypes: the distribution of samples in C3 was indicated by the red circle.

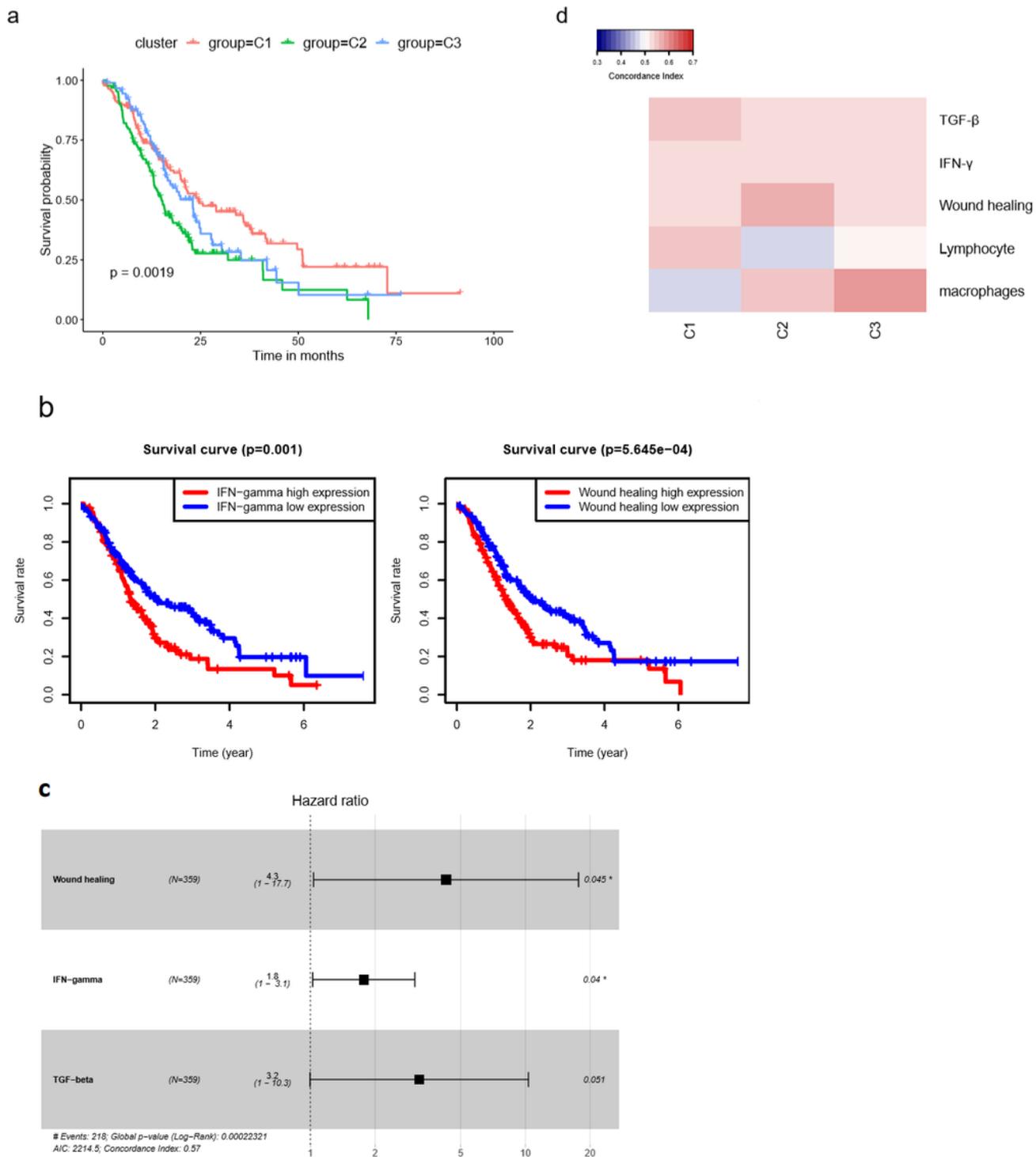


Figure 4

Prognostic impact of immune subtypes and immune signatures. a Overall survival of three immune subtypes. b Correlation of immune signatures with overall survival. The Left was for IFN- γ module. The Right was for Wound healing module. c Multivariate COX analysis of three immune signatures. Wound healing module (coef=1.45, HR=4.28, 95%CI=1.03-17.67, $p=0.045$), IFN- γ module (coef=0.57, HR=1.77, 95%CI=1.03-3.06, $p=0.04$), TGF- β module (coef=1.16, HR=3.21, 95%CI=0.99-10.34, $p=0.051$). d

Concordance index (CI) of five immune signature expression with the overall survival. Rows and columns represented immune subtypes and immune signatures respectively. Red indicated higher prediction accuracy while blue indicated lower prediction accuracy, along with the increasing expression of immune signature enrichment scores.

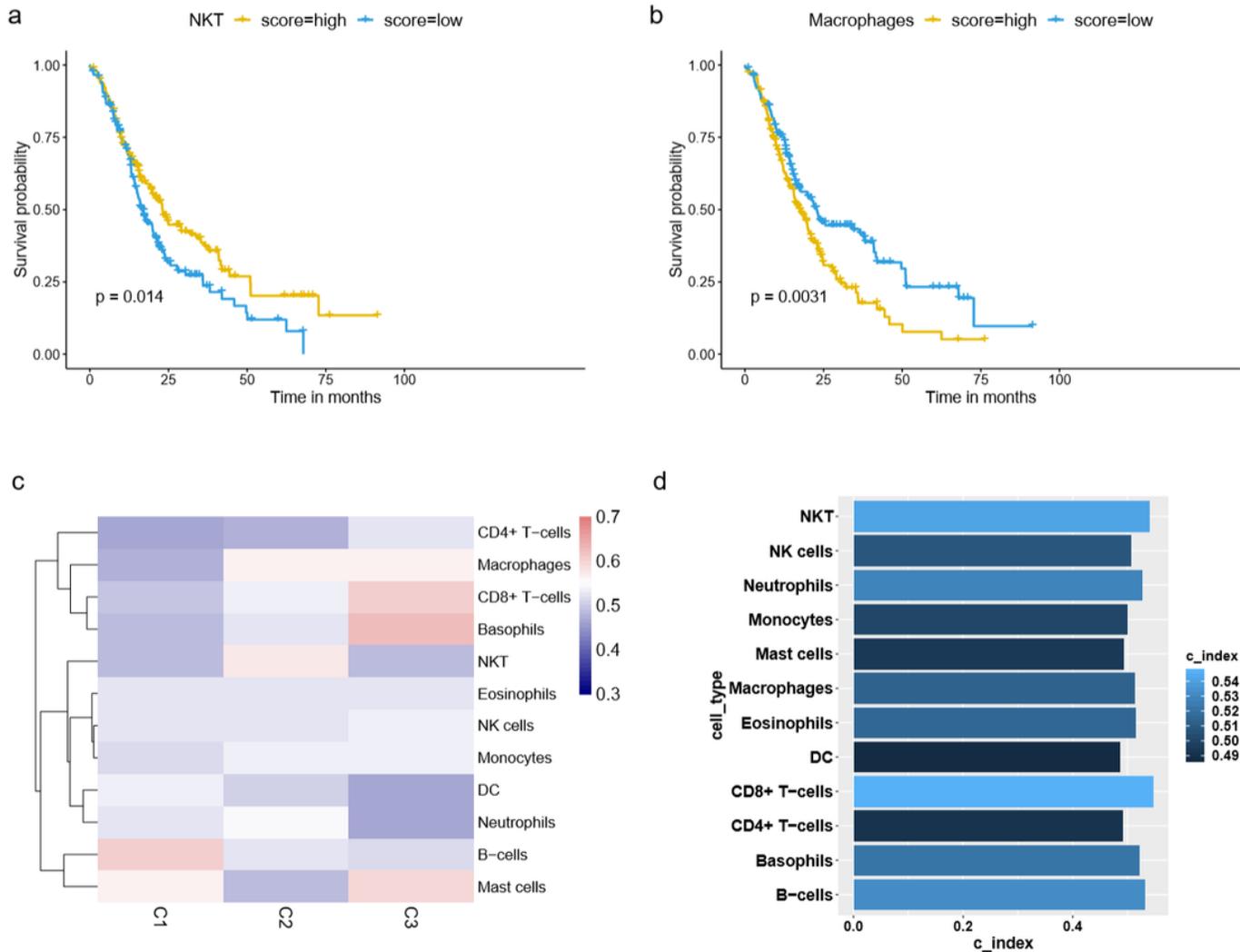


Figure 5

Prognostic impact of the immune cells. a,b Survival analysis of NKT cells ($p=0.014$) and macrophages ($p=0.0031$). c CI analysis with immune cells across the three subtypes: red indicated higher prediction accuracy while blue indicated lower prediction accuracy, along with the increasing expression of immune cells enrichment scores. d CI analysis with immune cells in the whole cohort: the lighter blue indicated greater prognostic impact while the darker blue indicated weaker prognostic impact.

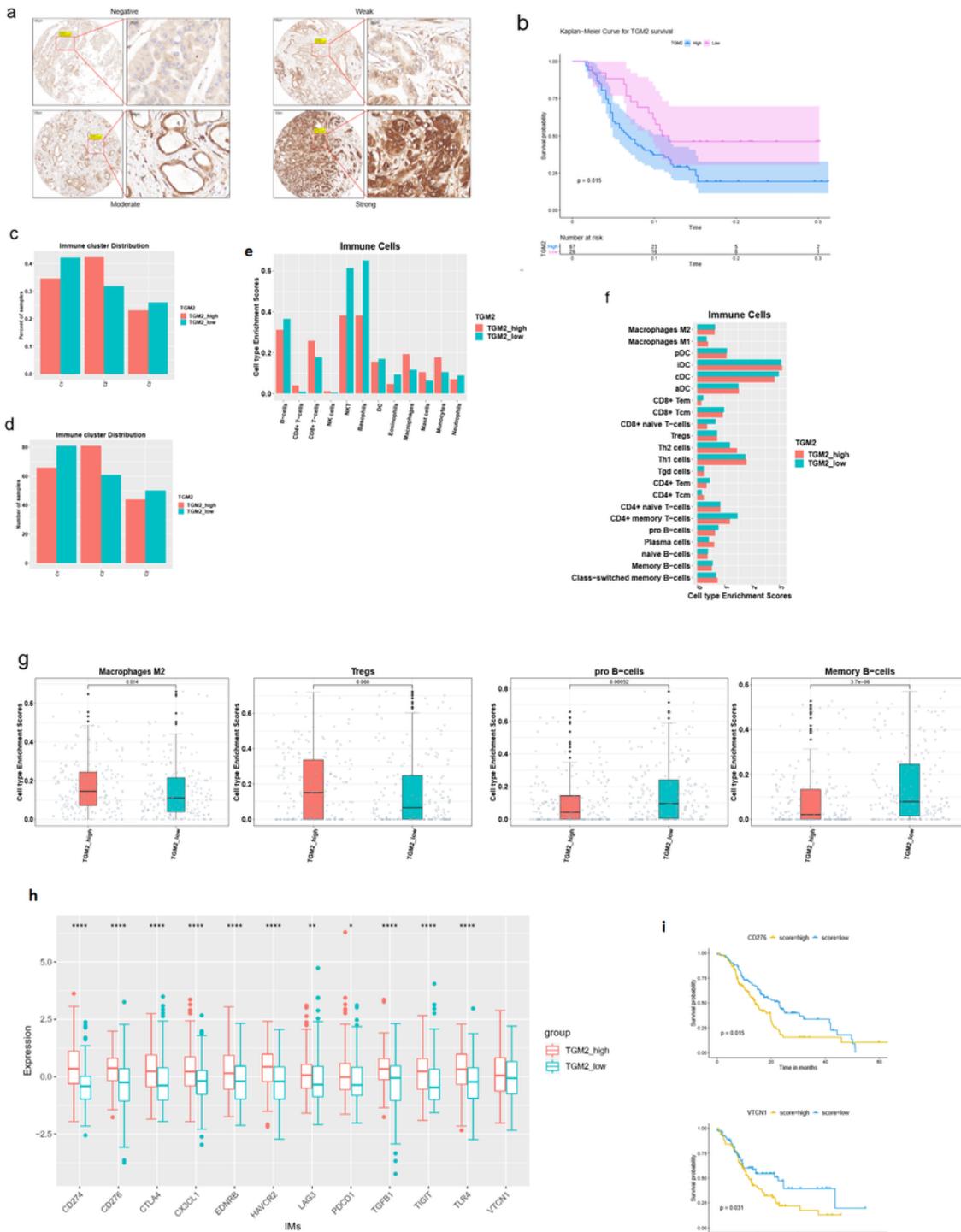


Figure 6

Relation between TGM2 and tumor immunosuppression in PDAC. a IHC analysis of TGM2 with our tissue microarray (n = 97). The scale bars were shown as indicated: 100 μ m and 20 μ m. b Survival analysis of TGM2 with tissue microarray data ($p=0.015$). c, d Distribution of high- and low-TGM2 group across the three immune subtypes showed as percentage and number. e, f Immune cell composition of high- and low-TGM2 groups. g Enrichment comparison of M2 type macrophages ($p=0.014$), Tregs ($p=0.068$), pro-B

cells ($p=0.00052$) and memory B cells ($p=3.7e-06$) between high- and low-TGM2 groups. h Comparison of IMs expression between high- and low-TGM2 groups: row is for the immunomodulators and column is for the gene expression value. (CD274, $p=1.0e-14$; CD276, $p=1.1e-10$; CTLA4, $p=5.6e-07$; CX3CL1, $p=1.6e-06$; EDNRB, $p=3.8e-06$; HAVCR2, $p=4.3e-10$; LAG3, $p=0.0046$; PDCD1, $p=0.0121$; TGFB1, $p=1.2e-12$; TIGIT, $p=6.5e-06$; TLR4, $p=3.8e-08$; VTCN1, $p=0.0585$). i Survival analysis of CD276 expression in high-TGM2 group (top) and VTCN1 expression in low-TGM2 group (bottom).

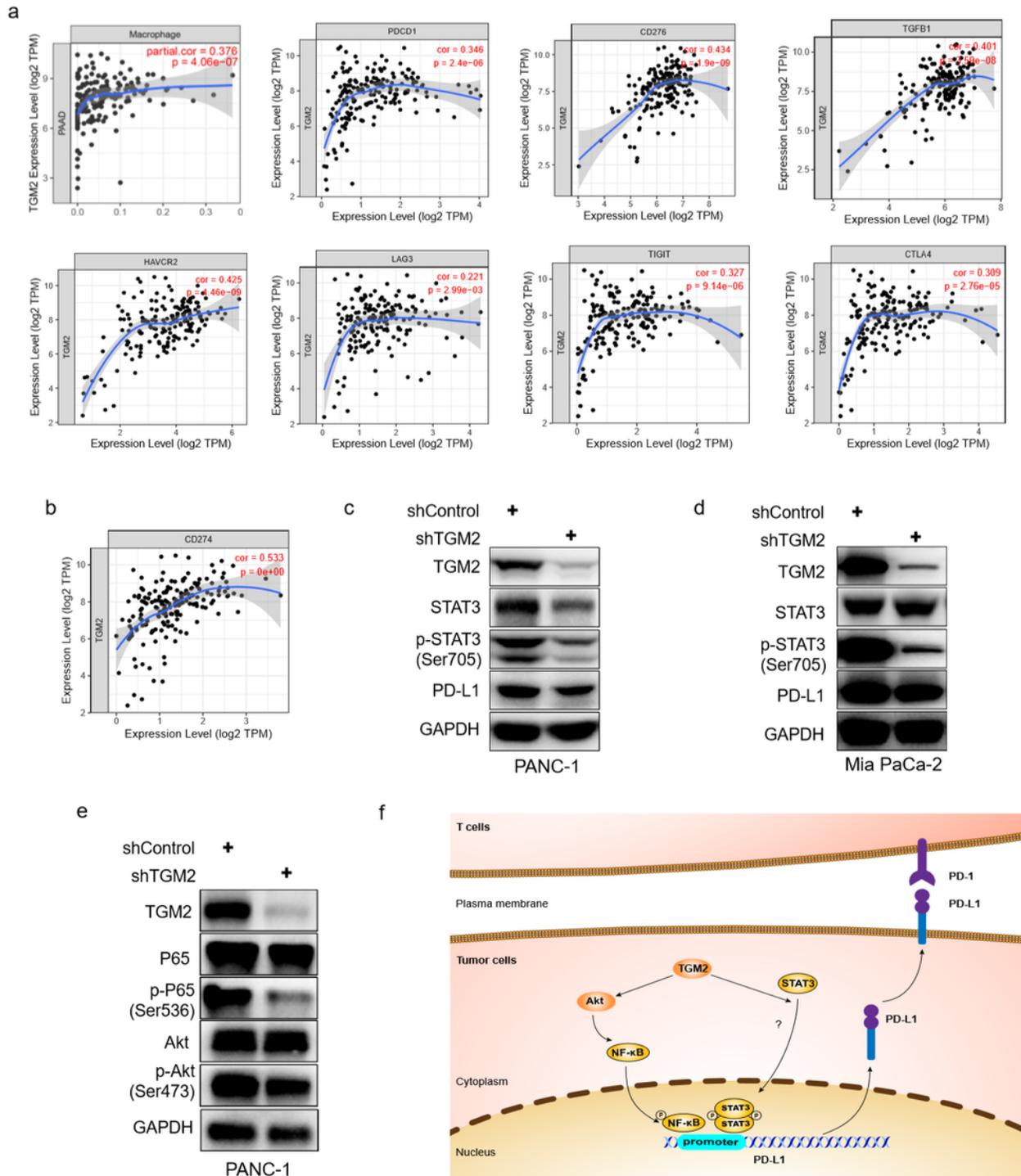


Figure 7

TGM2 regulated PD-L1 expression via STAT3 and NF- κ B in PDAC. a Correlation analysis of TGM2 and immunosuppressive factors with TIMER. b Correlation analysis of TGM2 and CD274 (PD-L1) by TIMER. c,d Western blot results showed that TGM2 knocking down resulted in a decreased expression of PD-L1 and p-STAT3 in PANC-1 and Mia PaCa-2 cells. e TGM2 knocking down in PANC-1 cells led to a decreased expression of p-Akt (Ser473) and p-P65 (Ser536) which was consistent with previous studies. f Mechanisms for TGM2 regulating PD-L1: a. TGM2 activates AKT pathway and then promotes the activation of downstream transcription factor NF- κ B which has been reported to be able to directly bind with the promoter of PD-L1 and stimulate its transcription. b, TGM2 may promote the phosphorylation of STAT3 despite the underlying pathways remains unclear, and then p-STAT3 binds to the promoter of PD-L1 and stimulate its transcription.

Supplementary Files

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