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Effects of Hypoxia in Pancreatic Cancer on Immune Cell Behavior in the Tumor Microenvironment

Xiaojun Wen[1], Zhaoqiang Fan[1], and Hua Yu[2]

[1] *Department of General Surgery, Ningbo Hangzhou Bay Hospital, Ningbo, China*
[2] *Hepatobiliary and Pancreatic Surgery Unit 1, Ningbo No.2 Hospital, Ningbo, China*

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ABSTRACT

Hypoxia serves as a fundamental component of the tumor microenvironment, exerting a crucial influence on tumor advancement. Nonetheless, a comprehensive examination of a prognostic signature linked to hypoxia in pancreatic cancer is notably absent, presenting an urgent necessity. Therefore, our objective was to create and authenticate a robust prognostic signature capable of predicting outcomes for pancreatic cancer.

Initially, the Gene Set Enrichment Analysis (GSEA) database was used to obtain hypoxia-related genes, and prognostic genes were analyzed. Following this, we utilized the Lasso Cox regression model to construct the hypoxia risk score model. Pancreatic cancer patients were subsequently categorized into high- and low-risk groups according to the median risk score. Finally, the CIBERSORT technique was used to assess immune cell infiltration while examining the relationship between hypoxia and immune-related genes.

Applying the Lasso Cox regression model, we pinpointed 2 significant genes, *GYS1* and *ALDOB*. Following this, patients were categorized into hypoxia high-risk and low-risk groups. Notably, the low-risk cohort demonstrated a substantially heightened survival rate relative to the high-risk group. Further investigation into the immune microenvironment unveiled a greater prevalence of resting mast cells, monocytes, plasma cells, and naive $CD4^+$ T cells in the low-risk category. In addition, we detected differences in the expression of 39 immune-related genes between the 2 groups.

In summary, our study has established a predictive signature comprising molecular markers for forecasting the prognosis of pancreatic cancer patients.

Keywords: Bioinformatics; Hypoxia; Immune cell infiltration; Pancreatic cancer; Tumor microenvironment

INTRODUCTION

Pancreatic cancer stands as one of the most formidable malignant neoplasms globally, particularly prevalent in developed nations.[1] According to GLOBOCAN data from 2012, this malignancy claims over 331,000 lives annually, constituting 4% of all deaths.[2] Recent studies, as per

Corresponding Author: Hua Yu, MM;
Hepatobiliary and Pancreatic Surgery Unit 1, Ningbo No.2 Hospital, Haishu District, Ningbo, China. Tel/Fax: (+86 0574) 8387 0999,
Email: fanzhaoqiang0607@163.com

The first and second authors contributed equally to this study.

data from the American Cancer Society indicate an overall survival rate for pancreatic cancer of merely 9%.[3] The incidence and mortality rates of pancreatic tumors show a steady increase, as evidenced by 5-year overall survival data.[1,4,5] Surgical resection remains the sole potential curative intervention for pancreatic cancer; however, a significant majority (80% to 85%) of patients are ineligible for surgery due to advanced disease stage upon diagnosis.[1] Furthermore, pancreatic cancer exhibits poor responsiveness to the majority of chemotherapeutic agents.[6] Hence, it is imperative to develop a predictive signature comprising molecular markers to prognosticate the outcomes of pancreatic cancer patients.

The hypoxic microenvironment is a hallmark of many solid tumors, including pancreatic cancer, and plays a critical role in tumor progression and resistance to therapy.[7,8] Hypoxia-induced stress triggers a range of adaptive responses that contribute to tumor cell survival and immune evasion.[9-11] The hypoxic tumor microenvironment (TME) is known to induce the expression of key genes such as hypoxia-inducible factors (HIFs), which regulate various pathways involved in immune suppression and tumor progression. In particular, hypoxia has been shown to promote tumor resistance and subsequent immunosuppression, creating a shield that allows tumors to evade immune surveillance and resist treatments like chemotherapy and immunotherapy. Studies have consistently demonstrated the upregulation of PD-L1 expression under hypoxic conditions, a well-established immune checkpoint molecule that inhibits T-cell activity and facilitates tumor immune escape. Recent years have seen a growing body of research focused on the TME and the role of immune cells within it. Immune cells within the TME can have either anti-tumor or tumor-promoting effects, depending on the complex signaling and interactions between tumor cells and the immune system. These interactions are influenced by hypoxic stress, which drives the polarization of immune cells towards an immunosuppressive phenotype. For example, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) are often recruited to the hypoxic areas of tumors, where they suppress effective immune responses. Hypoxia also leads to the recruitment of protumorigenic macrophages and the activation of various immune checkpoint pathways, such as the PD-1/PD-L1 axis, which further suppresses anti-tumor immunity.[12-15]

The impact of hypoxia on immune suppression has been well-documented in pancreatic cancer, where it not only aids tumor growth but also creates an environment that limits the effectiveness of chemotherapy and immunotherapy.[16] However, the influence of hypoxia on the immune system is not limited to pancreatic cancer. In lung cancer, breast cancer, and colorectal cancer, similar mechanisms of hypoxia-induced immune suppression have been observed.[17-19] For instance, in lung cancer, hypoxia has been shown to promote immune checkpoint upregulation, leading to immune evasion by inhibiting cytotoxic T lymphocyte function. In breast cancer, hypoxia not only affects immune cell infiltration but also influences the metabolic reprogramming of immune cells, making them more likely to support tumor growth rather than mount an effective anti-tumor response. Colorectal cancer similarly exhibits hypoxia-induced immune suppression, with evidence showing that Tregs and MDSCs accumulate in hypoxic regions, contributing to a pro-tumor environment.

The heterogeneity of the tumor microenvironment across different cancers highlights the diversity of immunophenotypes within tumors, even from the same tissue origin. This variability presents both a challenge and an opportunity for identifying predictive biomarkers that could guide the selection of immunotherapies tailored to individual patients.[20] The identification of such biomarkers, particularly those involved in the hypoxia-induced immune suppressive microenvironment, has spurred the development of novel immunotherapeutic strategies. Combined immunotherapies that target both the tumor and the immune system, such as immune checkpoint inhibitors and hypoxia-targeting therapies, are rapidly advancing through clinical trials and are showing promise in improving patient outcomes across a variety of cancers. These therapies are poised to mature further in clinical practice as the understanding of the tumor microenvironment and its interactions with the immune system deepens.

The research commenced with the extraction of genes linked to hypoxia to formulate a hypoxia risk model, subsequently utilized for outcome prediction in pancreatic cancer patients. Furthermore, we explored the intricate interplay between hypoxia and the tumor immune microenvironment, uncovering unique patterns of immune gene expression among groups stratified by varying degrees of hypoxia risk. These methodologies

are pivotal for enhancing comprehension of the tumor microenvironment and informing immunotherapeutic approaches in pancreatic cancer investigation.

MATERIALS AND METHODS

Extraction of Hypoxia-Related Genes and Gene Expression Data

The process of extracting hypoxia-related genes and gene expression data involved several steps. Initially, RNA-seq data for pancreatic cancer were acquired from the Gene Expression Omnibus (GEO) database (GSE78229), encompassing 59 samples, which were then merged into a gene expression matrix using Strawberry Perl. Following this, hypoxia-related gene sets were acquired from the HALLMARK gene sets through the Gene Set Enrichment Analysis (GSEA) website. Utilizing the STRING database, we established a PPI model for hypoxia-related genes, identifying core genes based on the count of adjacent nodes.

Establishment of the Prognostic Signature

To formulate prognostic characteristics, we performed univariate Cox proportional hazards regression analysis to pinpoint hypoxia-related genes exhibiting differential expression linked to overall survival. A significance threshold of $p<0.05$ was applied. Subsequently, multivariate Cox regression was employed to construct the hypoxia-related risk signature, incorporating weights derived from the Cox regression coefficients (β). The risk score of each patient was then calculated according to the following formula: Risk score = β gene(1) × expression gene(1) + β gene(2) × expression gene(2) + ⋯ + β gene(n) × expression gene(n). Subsequently, patients were categorized into 2 groups based on the median hypoxia-related signature risk score. The Kaplan-Meier method was employed to evaluate discrepancies in survival, while the prognostic utility of the genetic risk score model was further assessed through multivariate Cox regression and stratified analysis. Additionally, receiver operating characteristic (ROC) curve analysis was utilized to gauge the predictive precision of the model.

Correlations between Prognostic Related Genes and Immune Cell Type Fraction

To find correlations between prognostic genes and immune cell types, we analyzed expression data from the GEO database and used the CIBERSORT algorithm to predict the relative proportions of 22 immune cell subtypes. Significant differences in immune cell infiltration between the 2 groups were determined when $p<0.05$. For each sample, the sum of all estimates of the immune cell type score equals 1.

Relationship between Gene Expression and Hypoxia Risk

After identifying key genes involved in immune cell regulation, we analyzed the relationship between gene expression and hypoxia risk using R packages `ggplot2`, `ggpubr`, and `ggExtra`. In addition, we assessed differences in expression levels between the groups.

RESULTS

Identification and Establishment of Hypoxia-Related Signature in Pancreatic Cancer

Initially, data pertaining to gene expression in pancreatic cancer, coupled with pertinent clinical details, were acquired from the GEO database. This dataset comprised information for 49 patients diagnosed with pancreatic cancer. Subsequent to this acquisition, annotations were performed for all genes encompassed within this dataset. Concurrently, hypoxia-related genes were extracted from the GSEA website, and their expression levels within the GEO dataset were extracted. Utilizing the STRING database, we constructed a protein-protein interaction (PPI) network for the hypoxia-related genes (Figure 1A), subsequently identifying the top 50 core genes characterized by the highest number of adjacent nodes (Figure 1B). Subsequently, we conducted univariate Cox regression analysis for these core genes to investigate their association with overall survival (OS) in the GEO dataset. Notably, 15 genes exhibited significant prognostic value for pancreatic cancer patients ($P<0.05$). These genes comprise *ENO1, SLC2A1, PFKP, HK1, LDHA, GAPDH, TPI1, ALDOA, GPI, GYS1, HK2, PDK1, SDC4, ALDOB*, and *HSPA5* (Figure 1C). From the multivariate Cox regression analysis (Figure 1D), we identified two central genes, namely *GYS1* and *ALDOB*. Subsequently, we established the characteristics of the genetic risk score, calculated using the formula: genetic risk score = −0.002 × ALDOB + 0.015 × GYS1. Notably, GYS1 was found to be associated with a higher risk of malignancy, whereas ALDOB exhibited potential as a protective factor in tumorigenesis.

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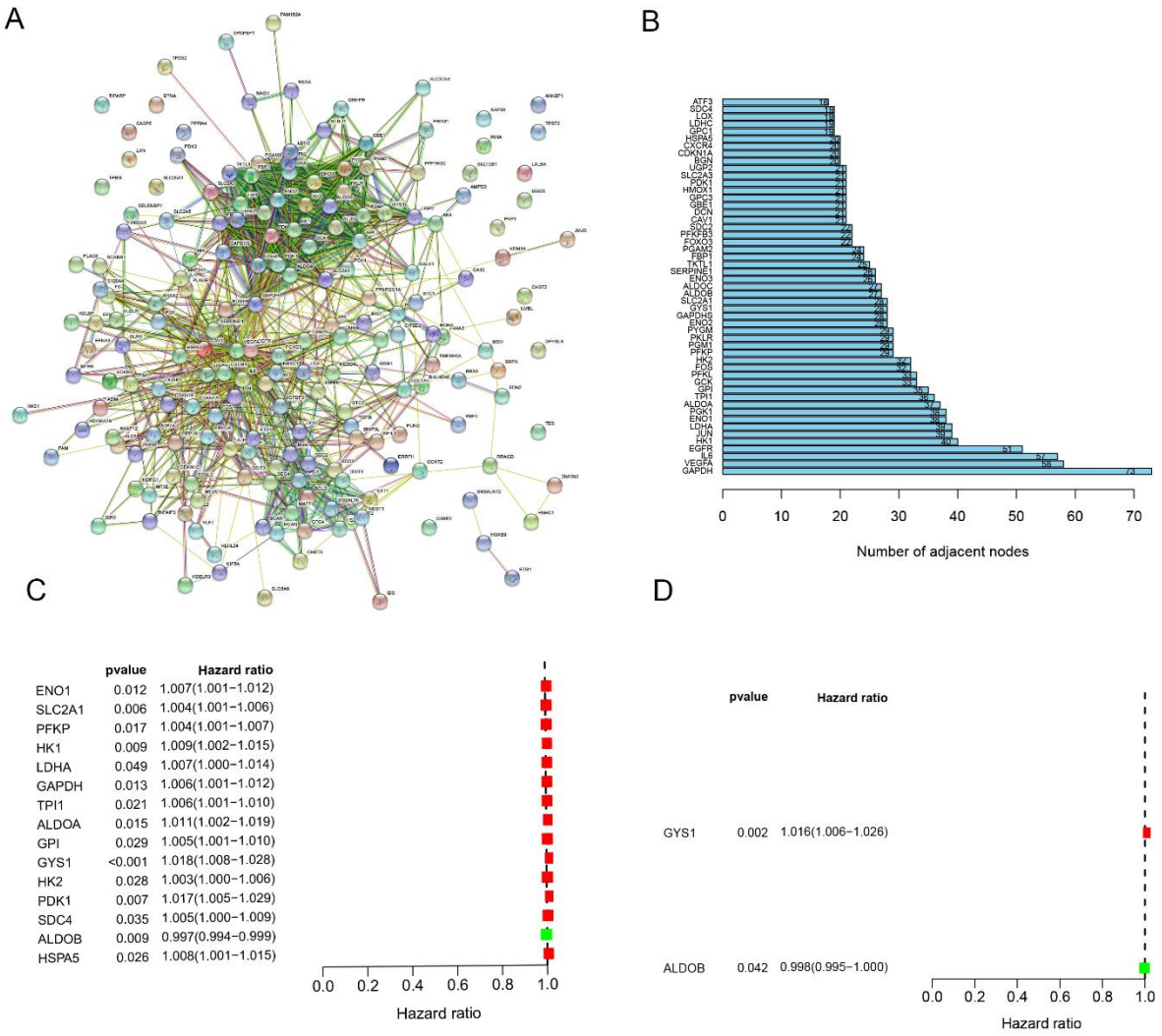

Figure 1. Identification of hypoxia-associated genes. A. The interactions between proteins encoded by hypoxia-related genes were visualized using a network model. Nodes represent individual proteins, and edges indicate interactions between these proteins. The size of the nodes reflects the degree of connectivity with other proteins. B. The top 50 genes were selected based on the number of nodes (proteins) they interact with and their adjacent sub-nodes in the protein interaction network. These genes are considered the most relevant in the context of hypoxia-related processes. C. A univariate Cox regression analysis was performed to assess the relationship between individual gene expression levels and overall survival outcomes in patients. The analysis helps identify genes that may serve as potential prognostic markers. D. A multivariate Cox regression analysis was conducted to evaluate the combined effect of multiple hypoxia-related genes on patient survival, adjusting for potential confounding variables.

The Impact of Hypoxia-Related Genes on Prognosis

Using these 2 hypoxic genes, we constructed a genetic risk score profile and plotted a survival curve. The prognostic significance of hypoxic risk scores in pancreatic cancer patients was assessed by Kaplan-Meier analysis (Figure 2A). The findings revealed a clear association between high-risk scores and poor prognosis in pancreatic cancer. Moreover, the area under the ROC curve was calculated as 0.703 (1-year), 0.736 (3-year), and 0.950 (5-year) (Figure 2B). These results underscore the predictive capability of the 2-gene risk signature in determining the survival outcomes of

patients with pancreatic cancer. Assessment of patient survival rates across the high and low-risk cohorts. It was noteworthy that a greater percentage of individuals in the low-risk category demonstrated survival compared to their counterparts in the high-risk category. More precisely, the survival rate stood at 44% among individuals classified as low-risk, a significant contrast to the mere 12% observed in those categorized as high-risk (Figure 2C). These results highlight the efficacy of our model in discriminating between patients with varying levels of risk. Furthermore, we explored the interactions among hypoxia-related genes identified as influencing patient prognosis in the model. Notably, we noted an inverse correlation between the 2 genes (Figure 2D). Considering that two genes encompass both protective and deleterious factors with diverse effects on pancreatic cancer prognosis, this negative correlation is comprehensible. Additionally, we examined the trend in mortality rates over time in the low-risk group, uncovering a decline in the number of deaths (Figure 3A). Subsequently, we illustrated the correlation between patient risk and survival through risk curves, revealing a prolonged survival duration in the low-risk group compared to the high-risk group (Figure 3B). Finally, we used thermal imaging to compare the expression levels of each gene in the 2 groups of mouse models. Notably, *GYS1* demonstrated higher expression in the high-risk group, whereas *ALDOB* exhibited elevated expression in the low-risk group (Figure 3C).

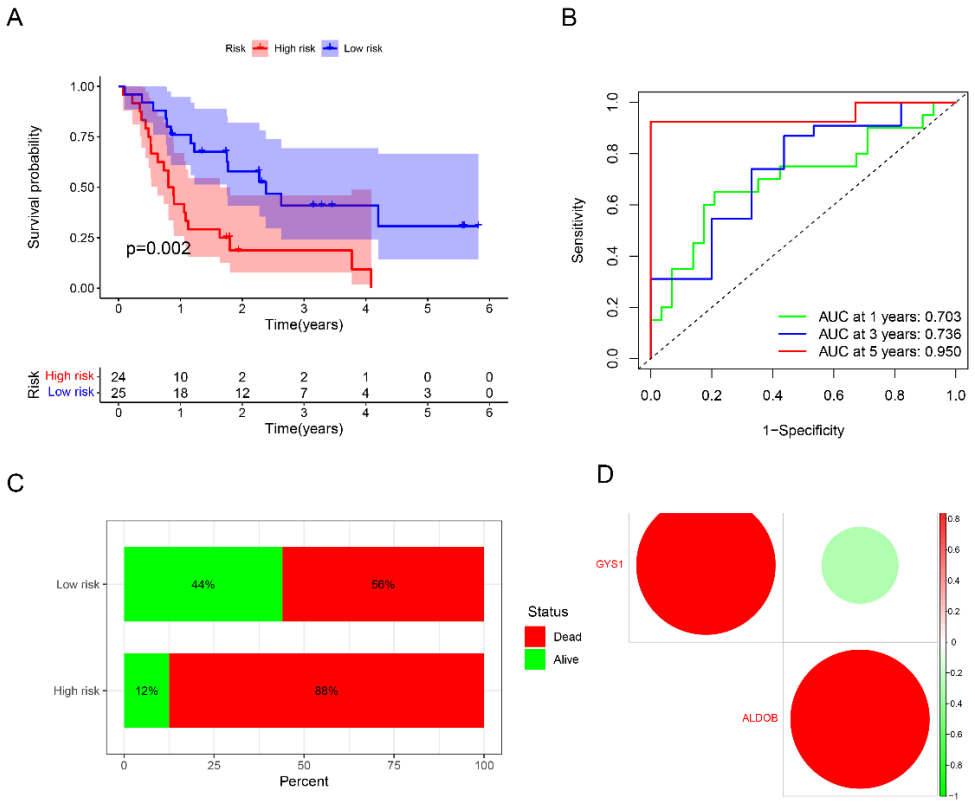

Figure 2. Evaluating the prognostic impact of the hypoxia model on patient outcomes. A. Kaplan-Meier survival curves were generated to compare the overall survival between patients in the high-risk and low-risk groups, as defined by the hypoxia-related gene model. Log-rank tests were used to assess the statistical significance of survival differences between the two groups. B. Receiver operating characteristic (ROC) curve was generated to evaluate the accuracy of the hypoxia model in predicting patient survival. The area under the curve (AUC) provides a measure of the model's discriminative ability. C. The mortality rates in the high-risk and low-risk groups were compared, showing how the hypoxia risk model stratifies patients according to their likelihood of mortality. D. The correlation between the 2 hypoxia-related genes was assessed using Spearman's rank correlation coefficient, indicating the strength and direction of the relationship between the gene expression levels.

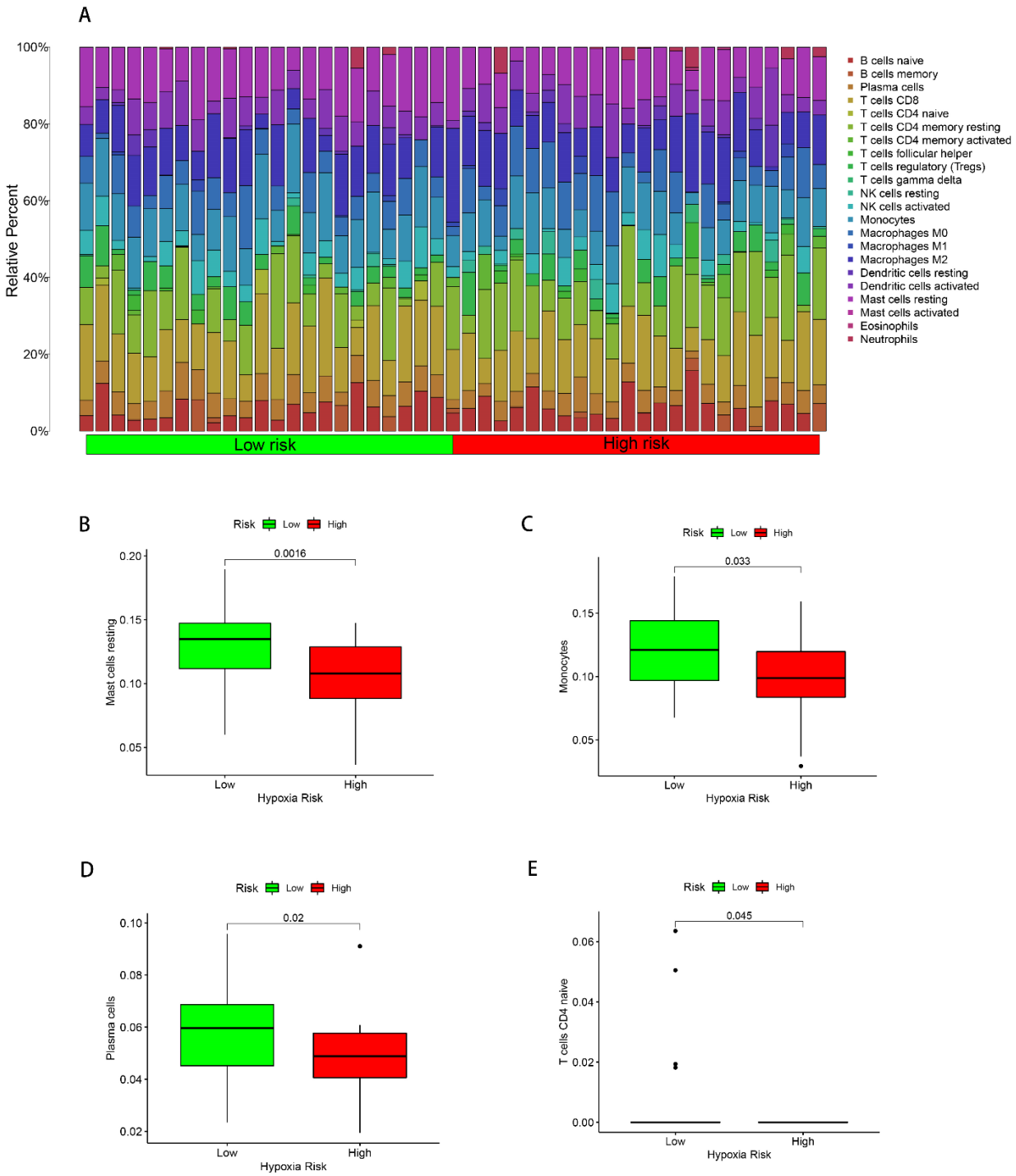

Figure 3. Evaluating patient risk using the hypoxia model. A. Survival rates were compared between patients classified into 2 cohorts based on their risk scores from the hypoxia model. The figure demonstrates the survival probability over time for high-risk and low-risk patients. B. The distribution of risk scores for patients in both high-risk and low-risk groups, as calculated using the hypoxia-related gene expression model. These scores are predictive of patient outcomes and guide treatment decisions. C. The expression of key hypoxia-related genes in the high-risk and low-risk patient groups was analyzed and compared. Significant differences in gene expression were observed between the 2 groups, contributing to their stratification.

Infiltration of Immune Cells and Expression of Related Genes

The CIBERSORT technique unveiled a marked rise in the percentage of quiescent mast cells, Monocytes, plasma cells, and naive $CD4^+$ T cells within the low-risk hypoxia cohort (Figure 4). Following this, we procured immune-related genes and juxtaposed their expression variations between the high-risk and low-risk factions.

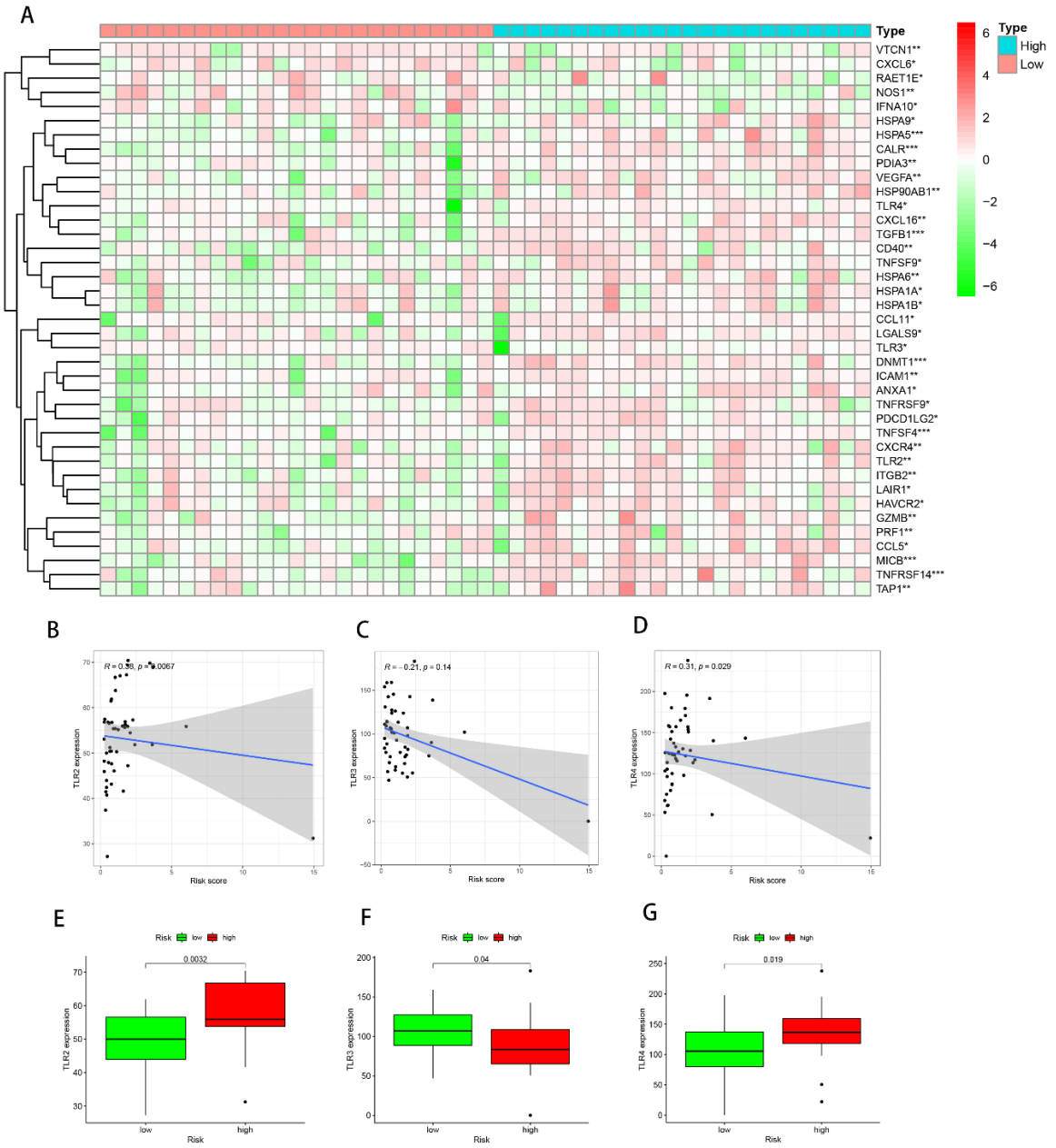

Figure 4. The composition of immune cells was examined in both high and low-risk hypoxia groups. A. The proportion of different immune cell types in the tumor microenvironment was evaluated for both high-risk and low-risk hypoxia groups. This analysis highlights how hypoxia influences the immune landscape in cancer. B. The relative levels of immune cell infiltration in the high-risk and low-risk hypoxia groups were compared. The figure illustrates differences in immune cell composition, which may have implications for immune evasion and therapeutic resistance in tumors.

The examination revealed substantial discrepancies in the expression of 39 immune-related genes between the high-risk and low-risk categories, with the majority displaying significantly heightened expression levels in the high-risk cohort (Figure 5A). The TLR family acts as critical detectors of the molecular attributes of microbial pathogens and assumes a central role in orchestrating the innate immune response.[21] TLRs activate a common pathway that triggers the expression of numerous inflammatory genes. Notably, we observed significant disparities in the expression levels of *TLR2,* *TLR3,* and *TLR4* between the high and low-risk groups. Consequently, we conducted an in-depth analysis of the expression differences of *TLR2, TLR3*, and *TLR4* in these groups, along with their correlation with the risk score. Our results demonstrated elevated expression of *TLR2* and *TLR4* in the high-risk group, while *TLR3* exhibited increased expression in the low-risk group. Interestingly, there was a negative correlation observed between the expression levels of these three genes and the risk score (Figure 5B–G).

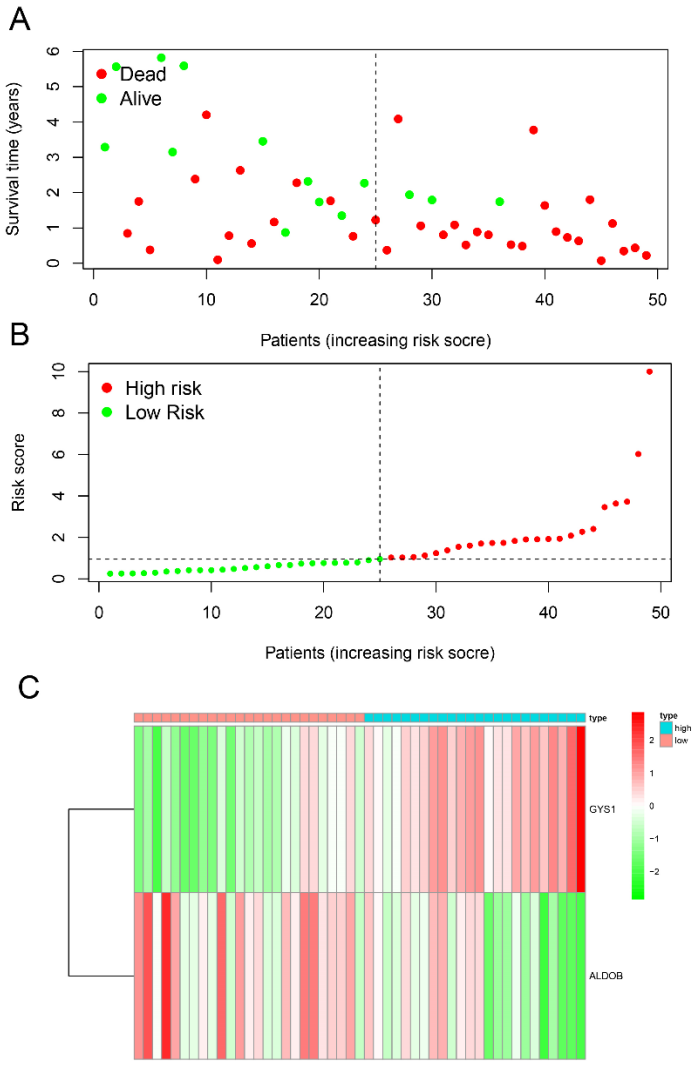

Figure 5. Immune-related gene expressions and their correlation with hypoxia risk scores. A. The expression levels of immune-related cells, including markers of various immune cell types, were compared between the high-risk and low-risk hypoxia groups. Statistical significance is denoted by *** p<0.001, **p<0.01, *p<0.05. B–G. The expression levels of TLR2, TLR3, and TLR4 were compared between the high-risk and low-risk hypoxia groups. These toll-like receptors are involved in immune responses, and their expression levels were also correlated with the hypoxia risk score to assess their potential role in the immune modulation of the tumor microenvironment.

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DISCUSSION

With one of the highest mortality rates among cancers, pancreatic cancer has garnered considerable research attention aimed at identifying effective biological targets for prediction and treatment. In recent years, there has been a growing focus on elucidating the role of hypoxia in tumor development.[22-24] Hypoxia stands as a crucial characteristic of solid tumors, intricately linked to resistance against radiotherapy and chemotherapy, heightened risks of invasion and metastasis, and dismal clinical prognoses in solid tumor patients.[7] Hypoxia exerts its influence on the tumor process through various avenues, including cell proliferation, angiogenesis, and tumor invasion.[25]

In exploring the relationship between hypoxia-related genes and prognosis, it has been established that the expression risk associated with these genes can serve as a predictor for overall survival among pancreatic cancer patients. Through multivariate analysis of these genes, two hypoxia-related genes have emerged as promising biomarkers for prognostic classification in patients with pancreatic cancer. In our study, the risk model was built using two hypoxia-related genes, *GYS1* and *ALDOB*. As tumors grow rapidly, hypoxic regions often emerge within the tumor microenvironment. Under such conditions, hypoxia enhances glucose metabolism, facilitating the adaptation of tumor cells to the hypoxic milieu and providing them with a survival and growth advantage.[26]

Glycogen metabolism involves complex processes governed by numerous enzymes and regulatory proteins. Central to these processes are glycogen synthase (GS) and glycogen phosphorylase (GP). GS is responsible for glycogen synthesis, whereas GP catalyzes its breakdown, representing critical steps in glycogen metabolism.[27] There are two subtypes of glycogen synthase: *GYS1* and *GYS2*. *GYS1* primarily functions in muscle tissue, whereas *GYS2* is predominantly expressed in the liver.[28]

Numerous studies have demonstrated a significant increase in glycogen levels in various tumor tissues, spanning conditions such as breast cancer, bladder cancer, and kidney cancer.[29] In certain cancer cell lines, we observed the accumulation of glycogen under conditions mimicking a hypoxic environment.[26] As early as a decade ago, researchers observed that glycogen accumulation in cancer cells can improve the survival rate in response to hypoxia concentration and glucose deficiency.

After 72 hours of hypoxia, the mRNA and protein levels of GYS1 increased rapidly when the U87 cancer cell line was cultured in vitro.[30] These findings suggest a functional association between glycogen metabolism and cellular adaptation to hypoxia. HIF-1 serves as a pivotal regulator in mammalian cells' adaptation to hypoxia, playing a critical role in facilitating tumor cells' response to the hypoxic microenvironment.[31] Hypoxia induces the expression of *GYS1* in a HIF-dependent manner, which mediates glycogen accumulation after hypoxia.[32] *GYS1* expression is elevated in clear cell renal cell carcinoma and is associated with poor prognosis.[33] Analysis of the Jones renal dataset in the public Oncomine database revealed a significant upregulation of *GYS1* mRNA expression in tumor tissues compared to normal tissues. Furthermore, immunohistochemical analysis indicated that *GYS1* predominantly localizes to the cytoplasm. Through the NF-κB pathway, *GYS1* promotes glycogen accumulation and tumor progression.[33] Together, these observations suggest that glycogen can promote tumor growth in hypoxic environments.

Fructose diphosphate aldolase B (ALDOB), an enzyme involved in fructose metabolism, as one of the isoenzymes of fructose diphosphate aldolase, is mainly expressed in the liver.[34] In recent years, it has been found that the abnormal changes of ALDOB are related to many diseases, especially in various cancers.[35,36] Metabolic and transcriptomic analysis showed that *ALDOB* was up-regulated in liver metastases compared with primary tumors.[37] The findings elucidate a potential link between *ALDOB* and cancer prognosis. Recent studies have shown that in hepatocellular carcinoma, ALDOB deficiency can disrupt the stability of the ALDOB/Akt/PP2A protein complex and promote the development of liver cancer.

Depletion of *ALDOB* exacerbates hepatocellular carcinogenesis by activating insulin receptor signaling and facilitating adipogenesis.[35] Bioinformatics analysis showed that *ALDOB* was one of the most down-regulated genes in gastric cancer. The expression difference of *ALDOB* between nontumor tissues and gastric cancer tissues was more than 7 times.[38]

To date, there have been no articles suggesting the role of *ALDOB* in pancreatic cancer, highlighting the novelty of our research and the potential it holds for guiding future investigations. Our study introduces a

new direction for pancreatic cancer research. Moreover, our hypoxia risk model, composed of two genes, demonstrates versatility in analyzing pancreatic cancer. However, the absence of additional clinical data for pancreatic cancer in the GEO database precluded our ability to establish a relationship between *ALDOB* expression and the malignancy degree of pancreatic cancer. Together, our findings highlight the correlation between hypoxia and the prognosis of pancreatic cancer patients and the infiltration rate of immune cells, and these findings provide new insights into the treatment of pancreatic cancer.

The identification of *GYS1* and *ALDOB* as hypoxia-related genes with prognostic value in pancreatic cancer holds significant clinical potential. These genes could serve as diagnostic biomarkers, allowing for the stratification of patients based on their risk of poor survival and the potential need for more aggressive treatment strategies. In particular, *GYS1* and *ALDOB* may provide useful indicators for the hypoxic tumor microenvironment, which is often associated with resistance to chemotherapy and immunotherapy. Furthermore, these genes could be explored as therapeutic targets for the development of novel treatments. Targeting *GYS1* and *ALDOB* could potentially mitigate the effects of hypoxia-induced immune suppression and improve the efficacy of existing therapies. For example, inhibition of these genes might help restore immune function within the tumor, enhancing the effectiveness of immune checkpoint inhibitors or other immunotherapeutic strategies. As precision medicine continues to evolve, the inclusion of *GYS1* and *ALDOB* in personalized treatment plans could provide a more tailored approach, optimizing therapeutic outcomes for patients with pancreatic cancer and potentially other hypoxia-driven malignancies.

Despite the promising implications of these findings, there are limitations that should be considered. The study's reliance on publicly available data and the lack of validation in independent cohorts are some of the factors that should be addressed in future research. Additionally, experimental validation in clinical samples would further strengthen the potential clinical applications of these biomarkers.

STATEMENT OF ETHICS

Not applicable.

FUNDING

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Not applicable.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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