Pyroptosis regulators exert crucial functions in prognosis, progression and immune microenvironment of pancreatic adenocarcinoma: a bioinformatic and in vitro research

Zhenghai Bai, Fangshi Xu, Xiaodan Feng, Yuan Wu, Junhua Lv, Yu Shi, and Honghong Pei

ABSTRACT

Pyroptosis is an inflammatory programmed cell death, showing potentials to be a novel anti-cancer approach. However, the roles of pyroptosis-related (PR) genes (PRGs) in pancreatic adenocarcinoma (PAAD) remain elusive. In the present study, we constructed a novel PR risk signature through the lasso regression analysis. The risk signature was greatly conducive to PAAD prognostic assessment. PR risk score was identified as an independent prognostic factor and could distinguish the prognostic differences of most clinical subgroups. Meanwhile, it could improve the traditional prognostic models based on TNM-staging. Next, its prognostic value was also tested in five validation cohorts. Using CIBERSORT, ESTIMATE, and ssGSEA algorithms, the effects of PR risk signature on tumor immune microenvironment (TIM) were explored. High PR risk suppressed antitumor immune through decreasing the infiltrating levels of CD8 T and NK cells. The genomic information and histological expression of risk PRGs were uncovered by USCA and HPA databases. Somatic mutation, methylation alteration, and homozygous CNV of eight PRGs barely occurred in PAAD samples. As for therapeutic correlation, PR risk score may not predict the efficacy of PD-1/L1 inhibitors and was weekly associated with multiple drug susceptibilities. Finally, the biofunctions of toll like receptor 3 (TLR3) in pancreatic cancer (PC) cells were investigated through qPCR, MTT, colony formation, and Transwell assays. Overexpression of TLR3 could promote the proliferation, migration, and invasion of PC cells. In conclusion, PRGs play crucial roles in prognosis, progression, and immune microenvironment of PAAD. TLR3 is expected to be a promising therapeutic target.

1. Introduction

Pancreatic adenocarcinoma (PAAD) is a highly malignant tumor, resulting in about 43,000 deaths annually worldwide that account for 4.5% in all cancer-related deaths [1]. Despite continuous improvements in its diagnostic approaches, more than half of patients develop metastatic disease at the time of diagnosis [2]. Surgical resection is the best option for PAAD treatment; however, the 5-year overall survival rate (OSR) of patients who receiving radical treatment is commonly less than 20% [3]. Concerning chemotherapy, FOLFIRINOX (Fluorouracil, Leucovorin, Irinotecan, and Oxaliplatin) and Gemcitabine regimen that act as the mainstream adjunct intervention has limited improvements in patients’ prognosis. The median overall survival of patients in FOLFIRINOX and Gemcitabine groups was only 54.4 and 35.0 months, respectively [4]. The highly anticipated programmed cell death protein 1/ligand 1 (PD-1/L1) inhibitors, such as pembrolizumab, have been proven to prolong median progression-free survival (PFS) [5]. Nonetheless, the overall response rate of pembrolizumab is only 13.2%, suggesting that only a small fraction cases could benefit from PD-1/L1 blockers [6]. Therefore, it is urgent and meaningful to develop the novel therapeutic strategies and improve prognostic evaluation system.

Pyroptosis is a proinflammatory form of programmed cell death (PCD), which was first proposed by Cookson et al. in 2001 [7]. Morphologically, pyroptosis and apoptosis share some similar characteristics such as DNA fragmentation and nuclear condensation.
Nonetheless, pyroptosis is characteristically accompanied by pore formation on cell membrane and inflammatory mediators release from cell lysis, which are not possessed by apoptosis [8]. Pyroptotic death relies on inflammasome induction, the activation of caspase (CASP) members, and the cleavage of gasdermin (GSDM) proteins, which comprises canonical and non-canonical regulatory pathways [9]. In canonical pathway, inflammasomes bind to the adaptor protein pyroptosis-related speck-like protein (ASC) and activate caspase 1 (CASP1). Activated CASP1 cleaves gasdermin D (GSDMD) to expose its N-terminal domain with pore-formation abilities [10]. Subsequently, cells are targeted by N-terminal domain and lysis, while releasing cell contents containing a large number of inflammatory mediators, especially IL-1β and IL-18 [9]. In non-canonical pathway, CASP4, 5, and 11 can be activated by bacterial lipopolysaccharide (LPS) and then directly cleave GSDMD to induce cell pyroptosis [11]. As it can be seen, pyroptosis is regulated by multiple genes and produces proinflammatory effects while causing cell lysis.

Recently, accumulating evidence has witnessed the closely relationships between pyroptosis and human diseases, including atherosclerosis, Parkinson's disease, cancer, and even COVID-19 [12–14]. For instance, blocking aquaporin 4 (AQP4) expression could alleviate myocardial ischemia-reperfusion injury by restraining cardiomyocyte pyroptosis [15]. Zinc finger E-Box binding homeobox 2 (ZEB2)-induced astrogliosis is competent to protect neuron through retarding pyroptosis in cerebral ischemia [16]. In colorectal cancer (CRC), PR lncRNA XIST promotes proliferation and epithelial-mesenchymal transition (EMT) of cancer cells by targeting miR-486-5p [17]. Analogously, XIST also facilitates CRC progression by directly binding with miR-200b-3p [18]. It is worth noting that pyroptosis plays a dual role in carcinogenesis and progression [19]. On one hand, it can inhibit tumor progression by pyroptosis-mediated PCD, and some chemotherapy drugs can exert antitumor functions through inducing pyroptosis [19,20]. On the other hand, the inflammatory cytokines released by pyroptotic cells are conducive to form a suitable microenvironment for tumor cell onset and growth [19,21]. In a word, pyroptosis is proposed to be a novel anti-cancer strategy.

Several studies have explored the roles of pyroptosis-related genes (PRGs) in lung adenocarcinoma (LUAD) [22], ovarian cancer [23], hepatocellular carcinoma (HCC) [24], and cervical cancer (CC) [25]. For example, mirtitone can induce HCC cell death through GSDM-dependent pyroptosis [24]. Zhou C et al. have identified a pyroptosis-related (PR) signature for prognostic assessment of CC [25]. Song J et al. constructed a PR lncRNA signature to predict patients' survival outcomes in LUAD [26]. Regrettably, their functions in PAAD remain elusive. In the present study, we comprehensive investigated the functions of PRGs in PAAD from multi-perspectives, including expression, prognostic value, immune effect, prediction for therapeutic efficacy, and transcriptome information. The novel PR risk signature was proven to profoundly impact prognosis and immune microenvironment of PAAD. More importantly, we first confirmed the oncogenic competency of TLR3, a core regulator in pyroptosis, in pancreatic cancer (PC) development through experiments in vitro. It is conceivable that our findings could bring new insights into the treatment and prognosis assessment of PAAD after further experimental and clinical trials.

2. Materials and methods

2.1 Data source

Clinical information and transcriptome data obtained from multiple public databases, including TCGA (https://portal.gdc.cancer.gov/), ICGC (https://dcc.icgc.org/releases), and GEO (https://www.ncbi.nlm.nih.gov/geo/) databases. Six TCGA samples were excluded due to their too short follow-up (less than 30 days). Because of the insufficient normal samples in TCGA database (n = 4), we supplemented 167 normal pancreatic samples from GTEX database (https://xenabrowser.net/data pages/). PACA-AU project in ICGC database and four GEO datasets (GSE62452, GSE21501, GSE28735, and GSE57495) were implemented as validation cohorts. Besides, GSE67501, GSE11636, and GSE93157 datasets were applied to probe into
the potential linkage between PR risk score and the therapeutic response of PD-1/L1 inhibitors. To ensure the comparability between different datasets, gene expression data was standardized by log2 (FPKM+1) transformation. The clinical characteristics of TCGA, ICGC, and GEO cohorts are presented in Supplementary table 1 and 2.

2.2 Establishing an improved pyroptosis-related gene set

A reasonable and comprehensive gene set is the foundation for constructing prognostic model. In the present study, the PR gene set mainly consisted of three parts. First, previous establishing strategies of PR gene set. Ye Y et al. [22] and Lin W et al. [23] adopted a same PR gene set consisting of 33 PRGs to investigate their roles in lung adenocarcinoma and ovarian cancer, respectively. We included these 33 PRGs in our gene set. Second, the Molecular Signatures Database (MSigDB) is a collection of annotated gene sets for GSEA analysis (https://www.gsea-msigdb.org/gsea/msigdb/) [27], which provides a pyroptosis gene set containing 27 members. Through consulting the literature [28,29], GZMB, CYCS, HMGβ1, IRF1, and IRF2 were selected into our PR gene set. Third, pyroptosis bypass pathway. Recent studies have reported that toll-like receptor 3 and 4 (TLR3/TLR4) could activate receptor interacting threonine kinase 1 (RIPK1)-dependent caspase 8, which in turn cleaves GSDMD to induce pyroptosis [9]. Therefore, TLR3, TLR4, RIPK1, and DIABLO (Smac) were included. Finally, we established an improved PR gene set consisting of 45 members (Supplementary table 3).

2.3 Construction of pyroptosis-related risk signature

The ‘Limma’ package in R software (Ver 3.6.3) was used to screen out PR differentially expressed genes (DEGs). Adjusted p-value < 0.05 and the absolute value of Log2FC ≥ 0.58 were considered statistically significant (1.5 fold difference in gene expression). Next, PR prognostic genes were identified by cox univariate regression analysis. The intersection part between DEGs and prognostic genes was obtained by Venn diagram. Finally, intersection genes were entered into Lasso regression analysis to construct a novel pyroptosis-related risk signature in PAAD.

2.4 Survival analyses

The optimal cutoff value of PR risk score was calculated by the Cutoff Finder online tool (http://molpath.charite.de/cutoff) [30], by which PAAD samples were divided into high- and low-risk groups. Survival difference analyses were conducted based on Kaplan–Meier method. Receiver operating characteristic curve (ROC) was used to evaluate the predictive performance of PR risk model. Decision curve analysis (DCA) was applied to determine whether PR risk score could improve the traditional prognostic model of PAAD. We investigated the improvements of two traditional models. Traditional prognostic model A was composed of age, histological grade, and clinical stage based on multivariate Logistic regression algorithm. Traditional prognostic model B was composed of age, histological grade, and TNM-staging. Independent prognostic factors of PAAD were identified through cox univariate and multivariate analyses. Clinical subgroup analyses were performed to estimate the applicable range of PR risk score in PAAD prognostic analysis. Moreover, we constructed a nomogram combining age, TMN staging, and PR risk level to predict the OSR of individual at 1, 3, and 5 years. Calibration plot was used to estimate the accuracy of nomogram. Due to the limited samples of M0 (n = 2) and clinical III–IV stages (n = 7) in TCGA cohort, we did not perform clinical subgroup analyses in these groups.

Five validation cohorts were employed to test the prognostic value of PR model, including ICGC-AU, GSE62452, GSE21501, GSE28735, and GSE57495 cohorts. Survival difference analysis and ROC were performed in each cohort. Meta-analysis was conducted to evaluate the combined effects of PR risk level on survival status.

2.5 Prognostic meta-analysis

The prognostic meta-analysis was conducted by Review Manager 5.2 software (The Cochrane Collaboration, Oxford, UK), based on Mantel-
Haenszel (M-H) method. Patients’ survival status is outcome measure (Dead or alive). The outcome data was extracted as follows: The number of dead patients in high- and low-PR risk groups; the total number of patients in each group. Odds ratio (OR) value was used as the evaluation indicator. I² value was applied to assess the statistical heterogeneity. If $I^2 < 50\%$, the fixed effect model was applied, inversely the random effect model was chosen. The overall effects were tested by z test.

### 2.6 Immune analyses

The immune abundances of 22 lymphocyte subtypes in each PAAD sample were obtained using CIBERSORT algorithm. Based on ssGSEA (single-sample gene set enrichment analysis) method, the activities of 13 immune-related pathways were quantified by ‘GSVA’ package. ESTIMATE algorithm can quantify the immune components in tumor parenchyma and stroma, thereby reflecting corresponding tumor immune microenvironment (TIM) [31]. Then, the stromal, immune and ESTIMATE scores, and tumor purity of different risk groups were calculated.

### 2.7 Genomic information

The mutation, copy number variation (CNV), single nucleotide variation (SNV), and methylation information of eight pivotal PRGs in four gastrointestinal cancers were analyzed using USCA database (http://bioinfo.life.hust.edu.cn/web/GSCALite/) [32]. Mutation and CNV frequencies were calculated. Meanwhile, the relationships between CNV/ methylation level and mRNA expression were also determined based on Pearson correlation analysis. A waterfall plot was generated to exhibit the number of variants and the mutation distribution of PRGs in each sample by ‘mafTools’ method. Besides, we compared the difference in methylation level of 8 PRGs between normal and tumor samples.

### 2.8 Therapeutic correlation analyses

Given that patients with high-expression of immune checkpoints (ICs) commonly have a better response to ICIs treatment, we analyzed the expressive correlations between PR risk score and six pivotal ICs (PD-1/L1, CTLA4, BTLA, HAVCR2, TIGIT, and LAG3) based on Spearman method. GSE67501, GSE111636, GSE157284, and GSE93157 datasets provide transcriptome information of tumor patients who are responsive or non-responsive to PD-1/L1 inhibitors. These datasets were used to unravel the effects of PR risk score on ICIs efficacy. Based on Genomics of Drug Sensitivity in Cancer (GDSC) data, the correlations between gene expression and drug sensitivity (IC50) were explored.

### 2.9 Cell culture and transfection

Two human pancreatic cancer cell lines (BxPC-3 and PANC1) and normal pancreatic duct epithelia cell line (HPDE6-C7) were purchased from Procell Life Science & Technology company (Wuhan, China). Three cells were all cultured in DMEM (Dulbecco’s Modified Eagle Medium) medium containing 10% FBS (Fetal bovine serum) and 1% P/S (Penicillin/ Streptomycin) (Procell, Wuhan, China). TLR3-specific shRNA and amplification plasmids were designed by HanHeng Biotechnology (Shanghai, China). Lentiviruses (HanHeng Biotechnology, Shanghai, China) were applied to transfect pancreatic cancer (PC) cells. The manipulation efficiency was tested by RT-qPCR after 72 h post-transfection.

### 2.10 RT-qPCR

Total RNA was extracted by TRIzol Reagent (TakaRa, Japan). RNA concentration was calculated by A260/A280 ratio (Nanodrop 2000 spectrophotometer). cDNA was synthesized by PrimeScript RT Reagent Kit (TaKaRa, Japan). RTqPCR reaction was marked by SYBR-Green PCR Reagent (Takara, Japan) and performed on the ABI Prism 7900 sequence detection system. GAPDH was used as an internal reference. RNA relative expression was calculated based on the $2^{-\Delta\Delta CT}$ method. Primer list is shown in Table 1.

### 2.11 MTT assay

Transfected cells were seeded into 96-well plates at a density of $5 \times 10^3$ per well. At each detective time
point, MTT reagent (Solarbio, Beijing, China) was added into each well and incubated at 37°C for 4 h. After removing supernatant, DMSO was added to dissolve formazan crystals. The absorbance was measured at 490 nm.

2.12 Colony formation assay

Transfected cells were seeded into 6-well plates at a density of $5 \times 10^5$ per well. Culture medium was replaced at 2–3 days intervals. After 14 days incubation, colonies were fixed by methanol and stained by Giemsa. Colonies were counted under the microscope from five random fields.

2.13 Transwell migration and invasion assays

Transfected cells ($5 \times 10^4$ per well) were seeded in 24-well transwell chambers (Corning, NY, USA). Serum-free medium was added into upper chambers, while complete medium with 10% FBS was added into lower ones. After 24 h incubation, non-migrative cells were removed by PBS washing and cotton swab wiping. Then, cells attached to the underside of membrane were fixed by paraformaldehyde and stained by 0.1% crystal violet. The stained cells were counted in five random fields of view at 100-fold magnification. In invasion assays, the chambers were precoated with Matrigel.

2.14 Statistical analysis

All statistical analyses were conducted using R software (Version 3.6.2) and GraphPad Prism (Version 8.01). The relationships between PR risk score and the clinicopathological characteristics of PAAD were determined using the chi-square test. Survival analyses were based on the Kaplan–Meier method. The differences between groups in our experiments in vitro were compared by Student’s t-test. p-value < 0.05 was regarded as statistically significant.

3. Results

In the current study, we established an improved PR gene set (n = 45). Then, a novel PR risk signature was constructed by the Lasso regression analysis. Its prognostic value, immune effects, therapeutic effects, genomic information, and histological expressions were comprehensively analyzed. Meanwhile, we applied five validation cohorts to confirm its prognostic value. Finally, given that the crucial roles of TLR3 in pyroptosis and cancer regulation, we investigated its biofunctions in PAAD through experiments in vitro. The flowchart of our study is presented in Figure 1.

3.1 A novel PR risk signature was constructed based on eight PRGs.

Most of PRGs (35/45, 77.8%) revealed aberrant expressions in PAAD samples (Figure 2a). Among that, 33 PRGs were upregulated, while NLRP1 and PJVK was downregulated in tumor samples. According to cox univariate survival analyses, 14 PRGs possessed notable abilities to affect prognosis of PAAD patients (Figure 2b). Then, we obtained 13 intersection genes, and by which a novel PR risk signature was constructed (Figure 2c-f). The PR risk score = 0.243*(CASP4 relative expression) + (−0.085)*(GPX4 relative expression) + 0.358*(GSDMC relative expression) + 0.182*(IL18 relative expression) + (−0.285)*(NLRP1 relative expression) + (−0.379)*(PLCG1 relative expression) + 0.061*(IRF1 relative expression) + 0.155*(TLR3 relative expression). According to the optimal cutoff value of PR risk score, 171 PAAD patients were divided into high- and low-risk groups (Figure 2g). High-risk level was positive correlated with unfavorable survival status, clinical and T stages, but not with M and N stages (Figure 2h), suggesting high PR risk may promote PAAD progression.

3.2 PR risk signature is conducive to PAAD prognostic assessment.

PR risk score was closely associated with the prognosis of PAAD patients. High PR risk led a poor survival outcome (HR = 2.69, p < 0.01) (Figure 2i). The 5-year OSR of patients in high-
risk group was slightly higher than 40%, whereas that in low-risk group was just 20%. PCA analysis manifested that PR risk level could explain 55.8% of total prognostic variation, suggesting that PR model had a great predictive performance (Figure 2). Similarly, PR risk score possessed a marked advantage in predictive accuracy over other clinicopathological features (AUC = 0.702) (Figure 3a). Time-dependent ROC curve showed that predictive accuracy for 1-, 3-, 5-year OSR were all higher than 0.74 (Figure 3b). Moreover, PR risk score (HR = 3.794, p < 0.01), age, and N stage were identified as independent prognostic factors of PAAD (Figure 3c, d). PR risk score also equipped with a good applicability. Except for cases with histological grades 3–4, PR risk score could distinguish the prognostic differences of most clinical subgroups (Figure 3e-n). Besides, introducing PR risk score into traditional prognostic models would greatly increase their decision benefit (Figure 3o), which indicated that PR risk score could improve the existing prognostic evaluation system.

For providing a convenient approach to predict the overall survival rate of PAAD patients, we constructed a nomogram consisting of age, TNM-staging, and PR risk level (Figure 3p). Meanwhile, calibration plots showed that the predicted probabilities well matched with actual survival rates (Figure 3q-s). In the light of the above, PR risk signature greatly contributed to PAAD prognostic assessment.

3.3 PR prognostic model is also applicable to other cohorts.

To go a step further, we validated the prognostic value of PR model in other cohorts. As expected, high-risk level still conferred worse survival outcomes compared to low-risk one in ICGC-AU, GSE62452, and GSE21501 cohorts (Figure 4a, c, e). Although the predictive capacity in these validation cohorts was less than that in TCGA cohort, PR model exhibited advantages over other clinical features (Figure 4b, d, f). Regrettably, there were no obvious survival differences between high- and low-risk groups in GSE28735 and GSE57495.
Correspondingly, the AUC of these two cohorts both approached 0.5 (Figure 4h, j). To estimate the pooled effects of PR risk level on PAAD survival status, we conducted a prognostic meta-analysis. As shown in Figure 4k, high-risk level was proven to deteriorate patients’ prognosis ($Z = 3.86, p = 0.0001$) and no significant heterogeneity among these cohorts ($I^2 = 0$). The funnel plots did not show notable publication bias (Supplementary figure 1). Altogether, the prognostic value of PR risk signature was also confirmed in other cohorts and has a broad applicability.

3.4 High PR risk suppresses antitumor immune and is involved in the formation of immune-tolerant microenvironment.

The immune abundances of 22 leukocyte sub-types were variable in each PAAD sample (Supplementary figure 2). High PR risk significantly reduced the infiltrating levels of CD8 T cells and NK cells activated. On the contrary, it increased that of Tregs and macrophages M0, M1,
and M2 subtypes (Figure 5a). As acknowledged, CD8 T cells and NK cells can exert potent cytotoxic proficiency to eradicate tumor cells through perforin-granzyme and Fas-Fasl pathways [33]. The aggregation of tumor associated-macrophages (TAMs) commonly heralds onset of immune tolerance and induces therapeutic resistance [34]. Taken together, high PR risk was detrimental to antitumor immune (Table 2). Furthermore, stromal, immune, and ESTIMATE scores in high-risk group were all significantly lower than that in low-risk group, while tumor purity showed an opposite tendency (Figure 5b, c), which all supported above conclusion.

As for the activity of immune pathways, high PR risk inhibited the activities of type-II IFN (Interferon) response and cytolytic activity but promoted that of inflammation promoting and CCR (cellular chemokine receptor) (Figure 5d). Altogether, different PR risk level heralded strikingly different immune microenvironment (Figure 5e).
3.5 The genomic characteristics of PR risk signature.

The somatic mutation of eight risk PRGs (CASP4, GPX4, GSDMC, TLR3, NLRP1, PLCG1, IL18, and IRF1) hardly occurred in PAAD samples but were commonly observed in colon adenocarcinoma (COAD) and stomach adenocarcinoma (STAD) ones (Figure 6a). Only two PAAD samples (2/178, 1.1%) were accompanied with somatic mutation (Figure 6e). Besides, PLCG1 had highest mutation frequency (34%) among gastrointestinal cancers, whereas GPX4 had the lowest one (2%) (Figure 6e). By contrast, CNV was relative common in four tumors, even in PAAD samples (Figure 6b, c). Regarding CNV types, heterozygous CNV was the most predominant form, whereas homozygous pattern barely happened (Figure 6b, c). The heterozygous modes of PLCG1 and GSDMC were dominated by amplification, while those of TLR3 and NLRP1 were dominated by deletion (Figure 6b). However, genetic CNVs were weakly associated with mRNA expression level, except for PLCG1 (Figure 6d). As for methylation, although the high methylation of NLRP1, PLCG1, and IRF1 negatively affected their mRNA expressions (Figure 6g), there was no significant difference in methylation levels of 8 PRGs between normal and gastrointestinal tumor samples (Figure 6f). Briefly, somatic mutation, methylation alteration, and homozygous CNV of 8 PRGs barely occurred in PAAD samples, indicating that their ectopic expressions may be mediated by post-transcriptional regulation.

3.6 PR risk score may not predict the efficacy of PD-1/L1 inhibitors.

Given that the expression status of immune checkpoints (ICs) profoundly affected the efficacy
and therapeutic response of immune checkpoints inhibitors (ICIs), we investigated the expressive relationships between PR risk score and ICs (Figure 7a-g). The expressions of CLTA4, BTLA, and LAG3 in low-risk group were notably higher than that in high-risk group (Figure 7a). Besides, their expressions of CLTA4, BTLA, TIGIT, and LAG3 were negatively related to PR risk score.
Therefore, eight nivolumab hypothesis not observed in CD274 (PD-1) (Figure 7d). Therefore, we speculated that PR risk score may not predict the efficacy of PD-1/L1 inhibitors. Furthermore, we preliminarily validated this hypothesis through four ICIs-related GEO datasets (Table 3). Regarding pembrolizumab or nivolumab treatments for multiple cancers, there were no significant differences in PR risk level between response- and non-response patients (Fig 7h-k). However, in atezolizumab treatment for TNBC, patients with high PR risk score exhibited a better therapeutic response (Figure 7l).

In term of other drugs, the expressions of all eight PRGs were not correlated with paclitaxel sensitivity, a widely used chemotherapeutic agent (Figure 7m). Meanwhile, GSDMC and IL8 expressions were negatively related to the sensitivities of multiple molecular targeted drugs (Figure 7m).

### Table 2. The effects of high PR risk score on TIM.

| Immune cell | Changing trend | Basic function | Final effect on antitumor immune |
|-------------|----------------|----------------|----------------------------------|
| T cells CD8 | Decreased      | CD8 + T cells clear tumor cells through perforin-granzyme and Fas-Fasl pathways. | Unfavorable |
| NK cells   | Decreased      | NK cells can kill multiple adjacent cells if these carry with oncogenic markers. | Unfavorable |
| Tregs      | Increased      | Tregs actively participate in the maintenance of immunological self-tolerance, thereby inducing cancer immune escape. | Unfavorable |
| Macrophages M0 | Increased | TAMs can enhance tumor cell invasion and metastasis through secreting VEGF, CCL2, CXCL12, and EGF cytokines. | Unfavorable |
| Macrophages M1 | Increased | M1 macrophages can mediate the differentiation of T cells and facilitate their immune functions through releasing IL-12 and IL-23. | Beneficial |
| Macrophages M2 | Increased | M2 macrophages are the majority of TAMs, possessing immunosuppressive capacity and leading poor prognosis. | Unfavorable |

PR, pyroptosis-related; TIM, tumor immune microenvironment; Tregs, regulatory T cells; TAMs, tumor-associated macrophages; VEGF, vascular endothelial growth factor; CCL2, C-C motif chemokine ligand 2; CXCL12, C-X-C motif chemokine ligand 12; EGF, epidermal growth factor.

### 3.7 Histologically, PR risk genes differentially express in pancreatic cancer samples.

Using HPA database (http://www.proteinatlas.org/) [35], the histological expressions of eight PR risk genes were exhibited in Figure 8. As previously observed in mRNA expression level (Figure 2a), CASP4, GPX4, IL18, NLRP1, and IRF1 were upregulated in tumor samples, whereas PLCG1 was downregulated. However, the protein expressions of GSDMC were hardly detected no matter in normal and tumor samples. TLR3 presented a moderate protein expression in tumor sample, while its expression was not detected in pancreatic endocrine cells but was still with medium staining in exocrine glandular cells.

### 3.8 TLR3 promotes proliferation, migration, and invasion of pancreatic cancer cells.

TLR3 was significantly upregulated in pancreatic cancer (PC) cells (BxPC-3 and PANC1) compared to normal pancreatic duct epithelia cell (HPDE6-C7) (Figure 9a). sh-TLR3 and OE-TLR3 were confirmed to effectively manipulate TLR3 expression through RT-qPCR tests (Figure 9b, c). MTT assays revealed that overexpression of TLR3 promoted, whereas silencing TLR3 inhibited the proliferation of BxPC-3 and PANC1 cells (Figure 9d, e). The same trends were observed in the results of colony formation assays (Figure 9f, g). As for migrative abilities, upregulation TLR3 enhanced, whereas blocking TLR3 expression suppressed the migration of PC cells (Figure 9h, i). Likewise, TLR3 has a stimulative effect on the invasion of PC cells (Figure 9j, k). Collectively, TLR3 possessed cancer-promoting abilities in PAAD.

### 4. Discussion

Pancreatic adenocarcinoma is a highly malignant abdominal cancer with a consistently rising incidence since 2004. Despite tough efforts on treatment and diagnosis, existing therapeutic approaches still fail to meet the patients’ intense desire for cure. Recently, pyroptosis, an
inflammatory programmed cell death, brings a new dawn for cancer treatment [8]. Here, we constructed a novel PR risk signature for scoring cancer patients. PAAD patients with different PR risk levels exhibited distinct prognostic and immune landscape, which greatly advanced clinical evaluation of PAAD. Furthermore, we focused on a core pyroptosis regulator TLR3. A series of experiments in vitro confirmed its tumorigenicity in pancreatic cancer (PC) cells and uncovered its potentials to be a therapeutic target.

Differential expression is the foundation for exerting biological function. In the present study, we found that most of PRGs (33/45, 77.8%) differentially expressed in PAAD samples compared with normal samples. Meanwhile, 31% of PRGs (14/45) were closely associated with the survival outcomes. These observations all indicated that PRGs participated in the regulation of PAAD progression. Nevertheless, the roles of PRGs in cancer present certain tumor specificity. For example, NLRP1, one member of our PR model and the
core inflammasome in pyroptosis, was widely expressed in 83% of primary breast cancer (BC) tissue [36]. Its overexpression promoted the tumorigenesis and proliferation of BC cells and facilitated EMT [36]. Conversely, we found that NLRP1 was downregulated in PAAD tumor samples (Figure 2a) and acted as an unfavorable biomarker of prognosis (Figure 2b). The reasons might lie in the dual role of pyroptosis in cancer [19]. Except for inducing cell death, some inflammatory factors that are produced by pyroptosis, such as IL-1β, IL18, and HMGB1, can activate the cancer-promoting signaling pathways including MAPK and VEGF pathways [21], which

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**Table 3.** The clinical information of four ICIs-related datasets.

| Data set | Study | Platforms | Sample size | Tumor | Treatment |
|----------|-------|-----------|-------------|-------|-----------|
| GSE67501 | PMID: 27,491,898 | GPL14951 | 11 | RCC | Nivolumab |
| GSE111636 | Homet Moreno B et al. | GPL17586 | 11 | mUC | Pembrolizumab |
| GSE93157 | PMID: 28,487,385 | GPL19965 | 65 | NSCLC/HNSCC/Mm | Pembrolizumab or Nivolumab |
| GSE157284 | PMID: 33,770,313 | GPL570 | 82 | TNBC | Atezolizumab |

ICIs, immune checkpoint inhibitors; RCC, renal Cell Carcinoma; mUC, metastatic urothelial carcinoma; NSCLC, non-small cell lung carcinoma; HNSCC, head and neck squamous cell carcinoma; Mm, Melanoma; TNBC, triple-negative breast cancer.
accelerates tumor growth. Another example is TLR3, the core subject of our study, that plays as an accomplice to PAAD.

TLR3 belongs to toll-like receptor (TLR) family, which plays a fundamental role in pathogen recognition and activation of innate immunity. In pyroptosis, recent evidence has demonstrated that TLR3 could promote RIP-1-dependent caspase 8 activation, in turn cleave the N-terminal domain of GSDMD [9]. Likewise, TLR3 possesses multi-faceted functions in cancer regulation. For instance, triggering TLR3 could promote tumor growth and cisplatin resistance in head and neck cancer [37]. Reciprocally, TLR3 expression induces cellular programmed death in non-small-cell lung cancer (NSCLC) [38] and androgen-sensitive prostate cancer [39]. In the current study, TLR3 was both significantly upregulated in PC samples (Figures 2a and 8) and cells (Figure 9a). Meanwhile, overexpression of TLR3 led an unfavorable prognosis (Figure 2b). The experiments in vitro also revealed that overexpression of TLR3 could promote the proliferative, migrative, and invasive abilities of PC cells (Figure 9). Mechanistically, given that the activation of TLR3 relies on the signature molecules expressed on pathogens and cancer cells, namely PAMP

Figure 8. The histological expressions of 8 risk pyroptosis-related genes. The top of the figure indicates the category of tissue specimen. The name of pyroptosis regulator, the antibody type, the patient ID, and the staining intensity are listed at the bottom of each image.
(pathogen-associated molecular patterns) [9,40], PAMP may be closely associated with the tumorigenicity of TLR3. All these findings witnessed that TLR3 elicited a pro-oncogenic capacity in PAAD.

In term of prognosis, we constructed a novel PR risk signature to TNM system, greatly contributing to the prognostic assessment of PAAD. The existing survival analytical system could not fully satisfy the accurate and convenient requirements. Based on AJCC 8th edition staging system, the T stage of PAAD patients with negative node is not associated with survival outcomes; moreover, the C index of this system is only 0.57 [41]. Besides, Yin F et al. has confirmed that AJCC 8th edition TNM system could not provide effective prognostic stratification for resected distal pancreatic cancer [42]. Our novel PR model compensated for above deficiency to some extent. First, PR risk score significantly increased the clinical benefit of TNM prognostic system (Figure 3o). Second, PR risk score could distinguish the survival difference of patients with N0 stage (Figure 3n), which is TNM staging cannot achieve [41]. Furthermore, our PR risk signature was successfully validated in other cohorts (Figure 4), suggesting that this model was reliable and harbored a wide applicability.

Not surprisingly, PR risk score was closely related to immune process of PAAD. Due to inhibiting the actives of immune pathways and decreasing the enrichments of cytotoxic cells (Figure 5a-d), high PR risk might herald the suppression of antitumor immune process. Mechanistically, it has been demonstrated that...
CD8 + T cells and NK cells induce pyroptosis in tumor cells via granzyme B, thereby establishing a positive feedback loop, namely pyroptosis-activated immune microenvironment [43]. In view of this fact, high PR risk may disrupt the antitumor loop by decreasing the infiltration levels of CD8 T cells and NK cells (Figure 5a). Moreover, interferon (IFN) -γ signaling pathway is capable of enhancing the tumor clearance mediated by CD8 T cells and NK cells [44], which is also suppressed in high PR risk level (Figure 5b). It may be another reason why high PR risk imply antitumor immunosuppression.

Although PD-1/L1 inhibitors bring an improvement in the prognosis of PAAD patients, screening suitable cases for ICIs treatment is still an intractable issue due to the small portion of response patients [5]. Evidence has emerged that pyroptosis is inextricably linked to immune checkpoint and immunotherapy. For instance, many patients are nonresponsive to ICIs, in part due to a lack of tumor-infiltrating lymphocytes (TIL). Nevertheless, pyroptosis may alter the influx of TILs in tumor microenvironment through releasing the inflammatory cytokines, which may switch ICI nonresponsive state to the responsive one [45]. Regretfully, we found that PR risk score was not associated with the expression of PD-L1(CD274) (Figure 7a, d). Additionally, multiple datasets revealed that there was no statistical difference in risk score between patients who were responsive and nonresponsive to pembrolizumab or nivolumab (Figure 7h-k). These findings all pointed toward that PR risk score might not act as a biomarker for predicting the efficacy of ICIs. The possible reason for this phenomenon was that the response to ICIs was commonly determined by the states of CD8 + T cells [46], which was dramatically suppressed by high PR risk. Moreover, triggering pyroptosis alone fails to treat ICIs-resistant tumor, which needed the support of immune checkpoints actuation [43].

Several studies have elucidated the roles of PRGs in multiple cancers using bioinformatics methods, such as lung adenocarcinoma [22], ovarian cancer [23], HCC [26], and endometrial cancer [47]. Compared to these research, our work was equipped with some non-neglectable advantages. First, a more comprehensive PR gene set (n = 45).

In the present study, not only the participants in pyroptosis bypass, but also some crucial pyroptosis regulators from MSigDB database were added into the improved PR gene set. This greatly improved the comprehensiveness of previous gene set (n = 31). Second, we investigated the associations of PR risk score with the efficacy of PD-1/L1 inhibitors (Figure 7), which were neglected in previous studies [22,23,26,47]. In fact, pyroptosis has been proven to a promising access to treat cancer. For instance, Hou J et al. ascertained that PD-L1 could enhance the transcription of GSDMC to switch apoptosis to pyroptosis in cancer cells [48]. Third, we confirmed the tumorigenicity of TLR3 in PAAD for the first time, making it a potential therapeutic target. Of note, CASP4, IRF1, and TLR3, the members of our PR model, all belong to the non-canonical pathway [9]. Regarding mechanism, lipopolysaccharide (LPS), tumor antigens, and Gram-negative bacteria are recognized by CASP4. Then, CASP4 directly cleaves GSDMD to expose its functional N-terminal fragment, which induces pyroptosis. Besides, with the assistance of IRF1, the interactions between toll-like receptor 3,4 (TLR3, 4) and its ligand, including LPS and dsRNA, also could activate CASP8 to cleave GSDMD [49,50]. Therefore, it is conceivable that pyroptosis non-canonical pathway, not canonical pathway, may act as the predominant regulatory pathway in PAAD.

Nevertheless, there are several limitations that should be noted. First, the PR risk signature was lack of externally clinical cohort validation. Second, the detailed mechanism of PRGs in PAAD progression remains undefined. Third, the biofunctions of TLR3 were not verified by animal experiments. Here, we have two suggestions for future work. For one, pyroptosis serves both motiva-tive and inhibitory roles in cancer. What factors determine this discrepancy? It can help us to utilize pyroptosis more optimally to fight PAAD. Second, as the crucial participants of pyroptosis, which one is the best option to act as therapeutic targets among CASPs, inflammasonmes, and GSDMs? Given that GSDMs are responsible of the effector phase of pyroptosis and crosslink with PD-1/L1 [48], chemotherapy drugs [51], and anti-tumor immunity [52], GSDMs may be the
optimal solution. In fact, targeting GSDMs has been a trustworthy approach to treat cancer in clinical application. Cisplatin, Paclitaxel, and Doxorubicin all exert antitumor competency by blocking GSDM-mediated pyroptosis [43].

The PR risk score has clinical utility. On one hand, it could guide individualized treatment. Given that there was an obvious difference in 5-year OSR between high- and low-risk groups (Figure 2i), the follow-up of PAAD patients in high PR risk could be performed more densely and comprehensively. On the other hand, the nomogram that combined TNM-staging and PR risk score could help clinicians to predict patients’ prognosis more accurately. Furthermore, the oncogenic capacities of TLR3 determined its anticancer potential. In fact, there several clinical trials focusing the tumor vaccines based on TLR3 agonists have conducted in colorectal cancer, melanoma, breast cancer, and prostate cancer [53].

5. Conclusions

Given that pyroptosis presents great promises to guide new anti-cancer strategies, we elaborated the functions of PRGs in PAAD from multiple perspectives, including their expressions, prognostic value, immune effect, therapeutic correlation, genomic information, and biofunctions. The novel PR risk score provided important prognostic information for the clinical assessments of PAAD patients. Moreover, high PR score could mark the antitumor immunosuppression and immune tolerance. Regarding the therapeutic correlation, there were no clear associations of PR risk score with the efficacy of PD-1/L1 inhibitors and the sensitivities of multiple drugs. As a hub regulator in pyroptosis, TLR3 presented potent pro-oncogenic abilities in PC cells. In conclusions, our findings provide new insights into clinical assessment and development mechanism of PAAD.

Highlights:
1. We constructed a novel PR risk signature to conduct to PAAD prognostic assessment.
2. High PR risk heralds the unfavorable prognosis and the suppressive antitumor immune.
3. Somatic mutation, methylation alteration, and homozygous CNV of PRGs barely occur in PAAD samples.
4. PR risk score may not predict the efficacy of PD-1/L1 inhibitors.
5. TLR3 possesses promoting effects on proliferation, migration, and invasion of pancreatic cancer cells.

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Author Contribution

HHP conceived and designed the study. ZHB, FSX, XDF, YW, and JHL analyzed and interpreted the data. ZHB, FSX, XDF, and YW wrote the manuscript. ZHB, FSX, XDF, YW, JHL, and YS conducted the vitro experiments. ZHB, FSX, XDF, and YS made revised version. All authors have read and approved the manuscript.

Availability of Data and Materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

ORCID

Fangshi Xu http://orcid.org/0000-0002-2282-4743

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