

### Original Research

# The Pyroptosis-Related Signature Composed of GSDMC Predicts Prognosis and Contributes to Growth and Metastasis of Hepatocellular Carcinoma

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#### Abstract

**Background**: Pyroptosis-related genes (PRG) are closely associated with the progression and metastasis of hepatocellular carcinoma (HCC). The predictive power of PRGs could be used to assess the clinical outcomes of HCC. **Methods**: The Cancer Genome Atlas (TCGA) RNA-seq data and clinical information from patients with liver hepatocellular carcinoma (LIHC) were used to identify PRG with differentially expressed between HCC and normal samples. Univariate Cox regression, least absolute shrinkage and selection operator (LASSO) Cox method, and multivariate Cox regression analysis were used to develop a prognostic model that included three PRGs. Gene set enrichment analysis (GSEA) was performed to identify differential immune cells and their associated pathways. The expression of Gasdermin C (*GSDMC*) in the HCC samples was detected by western blotting, and the function of GSDMC in HCC proliferation and metastasis was detected by the Cell Counting Kit-8 (CCK-8), colony formation, cell invasion, and wound healing assays. **Results**: Of 52 PRGs, *GSDMC*, Bel-2 homologusantagonist/ killer 1 (*BAK1*), and NOD-like receptor thermal protein domain associated protein 6 (*NLRP6*) were selected to establish a prognostic model. The model successfully differentiated HCC patients with varied survival in the TCGA training and test cohorts, as well as the International Cancer Genome Consortium (ICGC) validation cohorts. The risk score was proven to be an independent prognostic factor. In addition, we also reported a marked upregulation of *GSDMC* in HCC tissues, which could be induced by *CD274 (PD-L1)*. Overexpression of *GSDMC* contributes to HCC cells invasion, proliferation, and migration. **Conclusions**: The three PRGs signatures containing *GSDMC* independently predicted HCC prognosis. As a new driver molecule, *GSDMC* could play a tumor-promoting role by facilitating HCC growth and metastasis.

Keywords: hepatocellular carcinoma; pyroptosis; GSDMC; prognosis; immune checkpoint

# 1. Introduction

Hepatocellular carcinoma (HCC) has a poor prognosis throughout world [1]. Patients with Hepatocellular carcinoma (HCC) often lose the opportunity for surgery because of the advanced-stage disease diagnosis. Even in patients undergoing radical surgery, metastasis and relapse still pose a threat to survival. Therefore, useful biomarkers are indispensable for predicting the prognosis and guiding therapy [2].

Immune checkpoint inhibitors (ICI) offers a new perspective for the treatment of HCC. Since HCC cells can evade immune surveillance and induce immune tolerance to facilitate the development of cancer, immunotherapy such as ICI can reprogram the immunosuppressive tumor microenvironment (TME) to enhance anti-tumor immunity [3]. The response to immunotherapy is highly variable among individuals. Therefore, there is an urgent need for new combination therapeutic strategies to activate tumor immunity. Pyroptosis has gradually entered the public eye as one of many targets.

Pyroptosis was initially found to participate in the defense against infection. It is a unique form of cytolytic cell death that is triggered by the activation of inflammatory caspases. The GSDM family consists of Gasdermin A-E (GSDMA-GSDME), and Pejvakin (PJVK) in humans [4-8]. All GSDMs contain two arrangements: a N-terminal domain and a C-terminal domain. The GSDM full-length protein does not generally induce cell death because the existing inhibitory C-terminal domain is connected to the Nterminal, which protects the N-terminal domain from being overactivated. GSDM can be cleaved by activated caspases to release the GSDM-N fragment that associates with the plasma membrane. The cells then swell and form vesicles originating from the plasma membrane [9]. Consequently, cytolysis leads to the release of cellular contents, triggering strong inflammation [10]. The hallmark of pyroptosis, distinct from apoptosis, is the activation and secretion of a variety of signaling molecules and cytokines associated with danger, leading to robust inflammation and immune system remodeling [10–14]. Given its proinflamma-

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tory effect, it is reasonable that pyroptosis can modulate the TME, thereby contributing to tumor growth and progression. Numerous studies have revealed that key components of pyroptosis, including inflammatory vesicles, gasdermin proteins, and cytokines, are involved in tumorigenesis, invasion, and metastasis. Zhang et al. [15] revealed that GSDME may serve as a tumor suppressor through activation of pyroptosis. Enhancing the function and increasing the frequency of tumor-infiltrating NK and CD8<sup>+</sup> T cells may promote the phagocytic function of tumor-associated macrophages [15]. Indeed, the pyroptosis-related pathway may be a promising target in the management of HCC, which is supported by the finding that the antitumor activity of sorafenib was partly attributed to pyroptosis [16]. It has been demonstrated that pyroptosis of macrophages triggered by sorafenib could contribute to NK cell-mediated cytotoxicity, which may be beneficial in combating HCC [16]. However, the relationship between HCC prognosis and pyroptosis has not been fully understood.

This study systematically analyzed 42 PRGs with differential expression between HCC and normal samples. A PRG-related model was established based on mRNA expression and clinical information obtained from patients with HCC. We validated the model in the The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) test cohorts. The risk score in the model was an independent prognostic factor in univariate and multivariate Cox regression analyses. Kaplan-Meier survival curves revealed a notably different Overall Survival (OS) between the two groups. In addition, a new driver molecule *GSDMC* was proposed for the first time, which could be a promising target in HCC therapy.

# 2. Materials and Methods

## 2.1 Data Acquisition

The Cancer Genome Atlas (TCGA) website (https: //portal.gdc.cancer.gov/repository) was used to gather RNA sequencing data and associated clinical information for 374 patients with HCC and 50 normal samples. We excluded 4 patients who did not have complete follow-up information. In addition, transcriptomic counts and FPKM data with clinical features from 231 patients with HCC were obtained from the ICGC database (https://dcc.icgc.org/proje cts/LIRI-JP). Standardization for gene expression was performed using the "limma" tool.

#### 2.2 Mutation Analysis

Mutation data corresponding to 370 patients with HCC were from TCGA, and four incomplete information patients were excluded. The remaining patients were divided into two risk groups and the mutational spectrum was analyzed using the MAftools package, with measurement of tumor mutational burden (TMB) [17].

#### 2.3 Copy Number Variation (CNV) Analysis

Data for CNV corresponding to 370 HCC patients also were from TCGA. The lolipop plot was generated to show the gain and loss function of genes in the HCC database.

### 2.4 PPI Analysis of PRGs

The STRING database (http://string-db.org) provides the integration of protein interactions, including direct and indirect associations. The STRING database was used to show the correlation between the PRGs.

#### 2.5 Differential PRG Analysis

There were 52 PRGs identified in the earlier literature. 42 PRGs showed differential expression between tumors and normal tissues. The "limma" R package was used to analyze the differentially expressed genes (DEGs) in the TCGA cohort and a p value of 0.05 was used to construct a heatmap or volcano plot paragraph. In addition, a network of protein-protein interactions was also created by the tool GENEMANIA (http://genemania.org/) for the PRGs.

### 2.6 Consensus Clustering

TCGA-LIHC were divided into two groups of the 42 DEGs by using the "ConsensusClusterPlus" R program [18].

#### 2.7 Developing and Validating a Prognostic Model

A Univariate Cox analysis was performed to eliminate nine survival related PRGs for further investigation. The prognostic model was constructed using the LASSO-Cox method and multivariate Cox analysis to reduce overfitting. Three genes were ultimately retained with their coefficients, and the penalty parameter was calculated using the minimum criteria. The Equation 1: risk score =  $(0.311 \times BAK1 \text{ exp.}) + (1.143 \times GSDMC \text{ exp.}) + (-0.402 \times NLRP6 \text{ exp.})$  was used to calculate the risk score.

The prognostic model was based on dividing patients with HCC into two risk clusters according to their median risk score. We then used the "limma" and "scatterplot3d" packages in R to generate a principal component analysis (PCA) for both risk groups on the basis of gene expressions. The R package "survminer" was used for survival analysis comparing the prognosis between the two risk groups, and the R packages "survival" and "timeROC" were used to construct the ROC curve. A signature of three genes was constructed using the LASSO-Cox method and the multivariate Cox regression analysis, and then to assess efficacy in the patients from the TCGA test or ICGC external validation cohort. The pyroptosis-related gene (PRG) mRNA level and the risk score was normalized using the "scale" function. Using the risk score, patients in the TCGA test or ICGC cohorts could be divided into two risk groups.

#### 2.8 Analysis of Function Enrichment

The GSEA 4.0.1 (Broad institute, Boston, MA, USA) was utilized to carry out GSEA analysis between two risk groups to determine the differential and enrichment studies performed in the "clusterProfiler" R package [19].

### 2.9 Comprehensive Analysis of Tumor Immune Cell Infiltration Characteristics in Different PRG Subgroups

To examine the correlation between immune cell infiltration and PRG scores, 28 infiltrating immune cells in each case of HCC were calculated using the ssGSEA algorithm [20]. In addition, the "GSVA" R package was also used to perform a ssGSEA using gene-specific markers from the TCGA cohort in order to determine immune function between the PRG subsets. The Spearman correlation test was conducted to determine whether the PRG score was significantly correlated with immunosuppressive molecules or immune scores.

#### 2.10 Clinical HCC Tissues

The tissue samples were approved by the Zhongshan Hospital. The patients signed an informed consent form before undergoing surgical resection. Protein was extracted from cancer and adjacent tissues from 12 patients with HCC for validation by western blotting.

#### 2.11 Cell Lines

Huh7 and Hep3B cells were cultured in complete DMEM medium (D5796, sigma) containing 10% FBS (16140071, Gibco) and 1% penicillin/streptomycin (3810-74, Sigma) and used in the present study. IFN- $\gamma$  (CST, 13684S) was used for PD-L1stimulation. All the cell lines used in our study is negative for mycoplasma (detected by Betotime, C0296). The Hep3B and Huh7 cell lines were purchased from American type culture collection (ATCC, Manzas, Virginia,US), which was already authenticated by Short Tandem Repeat (STR) profiling for excluding cell cross contamination. And these cell lines have not been reported to be contaminated or misidentified in ExPASy Cellosaurus databases.

#### 2.12 Western Blotting

Cells were lysed for extract total protein extraction and pelleted at 95 °C for 8 min. Electrophoretic separation was performed by 10% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) at a 100 V constant voltage, and at 330 mA, protein was transferred to polyvinylidene difluoride (PVDF) membrane and then incubated with 1:2000 diluted GSDMC (ab225635, Abcam, Cambridge, UK), PD-L1 (CST, 13684S, Boston, MA, USA), caspase-8 (ab119809, Abcam, Cambridge, UK) and Actin (AA128, Beyotime, Shanghai, China) antibodies for 12 h at 4 °C. The PVDF membrane was incubated with a 1:10000 diluted secondary antibody. Finally, chemiluminescence was performed.

#### 2.13 Stable Cell Line Construction

The cDNA or shRNA (Genepharma, Shanghai, China) targeting *CD274* or *GSDMC* was recombined into lentiviral vectors to overexpress or knockdown *GSDMC*. The recombinant plasmid was transfected into 293T cells, and the mature infectious lentivirus was collected after three days. Stable *CD274* or *GSDMC*-overexpressing Hep3B and *CD274* or *GSDMC*-knockdown Huh7 cell lines were constructed and verified by western blotting.

### 2.14 Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in 96-well plates ( $10^3$  cells/well). After 8 h, CCK-8 (C0037, Beyotime, Shanghai, China) was added to the wells on day 1. Plates were placed in an incubator at 37 °C for 30 min, and cell absorbance was measured using a microplate readout reader, measurements were performed for 6 consecutive days.

#### 2.15 Colony Formation Assay

The 6-well plates were used to seed the HCC cells (800 cells/well), and then were placed at 37 °C incubator for 2 weeks. After the culture medium was discarded, the cells were fixed with 4% paraformaldehyde, then the cells were stained using crystal violet (C0121, Beyotime, Shanghai, China), washed with phosphate buffered saline (PBS) (P1020, Solarbio, Beijing, China), and dried.

#### 2.16 Wound-Healing Assay

After 10 h, HCC cells were seeded in 6-well plates to form a dense monolayer. Lines were drawn with the tip of a pipette size 20  $\mu$ L pipette perpendicular to the cell layer to form straight cell wounds. The width of the cell wound width was then washed with PBS and recorded under a microscope (Olympus, Japan) by taking photograph. Plates were placed at 37 °C incubator for 48 h, then the wound width was recorded again.

#### 2.17 Cell-Invasion Assay

About 100  $\mu$ L BD Matrigel mixture (diluted 1:5 with DMEM) was pre-coated in a chamber (3513, Corning, Corning, NY, USA) at 37 °C incubator for 5 h. DMEM without serum was used to dilute the HCC cells and 4 × 10<sup>4</sup> cells were plated in the upper chamber. Then, 600  $\mu$ L complete DMEM was added to the bottom chamber. Cells from the lower chamber were then incubated for 48 h at 37 °C, fixed, stained with crystal violet, washed with PBS, and photographed.

#### 2.18 Analytical Statistics

The DEGs between HCC and normal liver tissue were analyzed by a one-way ANOVA. The Mann-Whitney test was used to calculate the ssGSEA scores using the adjusted *p*-value from the BH method. The whole statistical investigations were performed using R 4.0.1 (Auckland, New Zealand). A p < 0.05 was used to define statistical significance.

## 3. Results

# 3.1 The Genetic Diversity of Pyroptosis-Related Genes in HCC.

Fig. 1 shows the detailed protocol. A total of 52 PRGs selected from the KEGG database were included in this study. To find gene alterations of PRGs in patients with HCC, we downloaded data from the TCGA-LIHC databases. At the genetic level, 157 of the 364 samples (43.13%) showed mutations in regulatory genes linked to pyroptosis. Of these, *TP53* had the highest mutation frequency (30%) followed by *NLRP2* (2%). No mutations were found in other PRGs such as *BAK1*, *CASP1*, *CASP5*, *CHMP2A*, *CHMP3*, *CHMP4A*, *CHMP4B*, *CYCS*, *GSDME*, *IL1A*, *CASP6*, *CASP9*, *GPX4*, *GSDMA*, *PJVK* as well as *SCAF11* (Fig. 2a). Our results indicated that most PRGs, except *TP53*, have no significant changes in gene variation.



**Fig. 1.** Flow chart of the whole study. LIHC, liver hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; ICGC, International Cancer Genome Consortium; GSEA, gene set enrichment analysis; GSDMC, Gasdermin C.

We also found copy number variation (CNV) alteration in 7 of the 52 PRGs, which showed that *CASP9*, *HMGB1*, and *TP53* had a significant frequency of CNV deletions (Fig. 2b), whereas *GSDMC*, *AIM2*, *GSDMD*, and *CHMP6* had a significant frequency of CNV amplifications (Fig. 2b). Fig. 2c showed the CNV alterations location on the chromosomes.

# 3.2 Identification of DEGs and the Interactions between Them

The expression levels of 52 PRGs were compared based on 50 normal samples and 374 HCC samples in TCGA data. Forty-two DEGs were identified (all p < 0.05, Table 1), among which 39 genes were enriched in the tumor cluster, while three genes (*IL-6*, *IL-1B* and *NLRP3*) were down-regulated. Fig. 2d showed the heatmaps of RNA levels (blue: low expression; red: high expression) and the histograms in Fig. 2e.

#### 3.3 The Interactions among PRGs

We then utilized the STRING platform to analyze the network of protein-protein interactions of 52 PRGs' regulators (Fig. 2f). The PRGs correlation network is shown in Fig. 2g (blue: negative correlations; red: positive correlations).

# 3.4 Tumor Classification Based on Pyroptosis Regulators of Prognosis

Our next step was to use consensus cluster analysis to explore the association of all 370 HCC subtypes in the TCGA cohort with these prognostic genes (**Supplementary Fig. 1a**). When  $\kappa = 2$ , the intragroup correlation was the smallest (**Supplementary Fig. 1b,c**), indicating that it was appropriate to split 370 patients into two clusters on the basis of 42 DEGs obtained previously (Cluster 1 = 160, Cluster 2 = 210). Then we found that patients in cluster 1 survived for a shorter period than those in cluster 2 (p <0.05, **Supplementary Fig. 1d**), indicating that these DEGs may indicate a prognosis. The baseline clinical features and gene expression profile of the two clusters are shown as a heatmap. However, no notable baseline differences were observed between the two groups (**Supplementary Fig. 1e**).

#### 3.5 Establishment and Validation of the Prognostic Risk Model

Through univariate Cox regression analysis, we identified nine genes (BAK1, BAX, CHMP4B, CASP8, GSDMC, NLRP6, NOD1, NOD2, and PLCG1). The curves in Fig. 3b represent the trajectories of each independent variable coefficient. The two dashed lines in Fig. 3c indicate two special  $\lambda$  values: lambda. min and lambda. lse. Based on LAS-SON Cox analysis (Fig. 3b,c), lambda. min was chosen to construct a nine genes PRGs model. Finally, we obtained three key prognosis-related PRGs (BAK1, GSDMC, and NLRP6) by multivariate regression analysis with a cutoff of 0.1, and the other six genes were narrowed with a p-value > 0.1. A total of 370 HCC specimens were randomly separated into two cohorts (training cohort, n = 186; test cohort, n = 184). In the training set, we found that three genes (BAK1, GSDMC, and NLRP6) were closely associated with HCC occurrence after performing a multivariate Cox regression analysis of PRGs (Table 2). We classified





**Fig. 2. Pyroptosis-related genes (PRGs) Screening of patients with hepatocellular carcinoma (HCC).** (a) The mutation frequency of PRGs in 370 HCC patients. Each column represented a patient. The proportion of each variant type was showed by right-hand bar plots, while stacked bar plots show the conversion fractions in each sample. (b) TCGA-LIHC cohort's CNV variation frequency for pyroptosis-related genes. A column's height represents the frequency of alteration. Green dot: deletion frequency; red dot: amplification frequency. (c) Based on the LIHC cohort, the Copy Number Variation (CNV) alteration location of PRGs is shown. (d) The differential expression of PRGs were showed by heatmap. (e) The differential PRGs expressed showed by box plot. (f) Protein-protein interaction network. (g) Gene association network (Blueline: negative correlations; Redline: positive correlations. The depth of color reflects the relevance's strength).



Gene	Con mean	Treat mean	Log FC	<i>p</i> -value
BAK1	2.43296064	7.45717126	1.61591549	$1.55  imes 10^{-17}$
BAX	7.3216181	21.1009946	1.52707658	$7.52\times10^{-23}$
CASP3	4.1860116	8.44081184	1.01180546	$1.98\times10^{-17}$
CASP4	4.20085128	5.10681863	0.28174311	0.03286059
CHMP2A	33.2191982	65.2138897	0.97316202	$9.02\times10^{-22}$
CHMP2B	7.37121326	9.87610132	0.42203954	$1.91  imes 10^{-5}$
CHMP3	2.05397496	4.19803902	1.03129698	$4.04\times10^{-17}$
CHMP4A	0.44395801	0.76114955	0.77775671	$4.87  imes 10^{-6}$
CHMP4B	32.0528566	59.5627723	0.89395797	$6.59\times10^{-18}$
CHMP4C	2.54545884	6.03191047	1.24468928	$2.37\times10^{-14}$
CHMP6	7.38079992	13.8223234	0.90515105	$7.64\times10^{-20}$
CHMP7	4.30753654	5.45855639	0.34165642	0.00610607
CYCS	12.623064	25.3375992	1.0052177	$1.71\times10^{-15}$
ELANE	0.08227496	0.08760968	0.09063689	0.03218088
GSDMD	12.9530553	31.0751544	1.26246913	$2.96\times10^{-22}$
GSDME	0.42152436	1.12730849	1.41919444	0.00054835
HMGB1	11.3255013	15.9875838	0.49737702	$2.20  imes 10^{-9}$
IL1A	0.01072757	0.02485794	1.21238333	0.00735294
IL1B	0.87439016	0.41835073	-1.0635642	$2.07\times10^{-10}$
IRF2	7.4236917	8.64728488	0.22011042	0.02426076
TP53	4.95454942	8.22316697	0.73094026	$5.80  imes 10^{-6}$
TP63	0.03249136	0.12194168	1.90806327	0.00237048
AIM2	0.25001586	0.37839679	0.59788023	0.00972577
CASP6	5.03020808	6.86427733	0.44848976	$3.49  imes 10^{-5}$
CASP8	1.37688528	2.83142406	1.04011948	$1.52\times10^{-15}$
CASP9	1.78856694	2.52060221	0.49496435	$3.54  imes 10^{-5}$
GPX4	118.53575	188.693886	0.67072544	$1.28 \times 10^{-10}$
GSDMB	1.34402605	3.63674743	1.43608764	$1.66\times10^{-13}$
GSDMC	0.00830341	0.2340813	4.81716107	$8.32\times10^{-19}$
IL6	0.86703163	0.3207223	-1.43476	$3.10  imes 10^{-5}$
NLRP1	0.40595995	1.07178314	1.40060373	$1.31 \times 10^{-9}$
NLRP3	0.35190932	0.27927313	-0.333527	0.00231316
NLRP6	1.07212382	1.19241641	0.15341661	0.00113618
NLRP7	0.01351051	0.0204933	0.60106999	0.00202789
NOD1	0.3254706	0.66369271	1.02798821	$4.82 \times 10^{-14}$
NOD2	0.19403987	0.34029517	0.81043355	0.0077925
PJVK	0.24180884	0.4645166	0.94186313	$1.21 \times 10^{-9}$
PLCG1	1.26078037	3.99551069	1.66406294	$1.77 \times 10^{-21}$
PRKACA	14.1108017	18.2172065	0.36850179	0.00340267
PYCARD	3.24194386	9.79743175	1.59554451	$7.65  imes 10^{-6}$
SCAF11	3.499931	4.37598781	0.32228224	0.00655423

Table 1. 42 differentially expressed genes (DEGs) in 370 HCC and 50 normal tissue.

TCGA training cohort patients into two clusters (high- or low-risk clusters) according to the median risk score calculated by Equation 1 (Fig. 4a). The PCA demonstrated that patients in the different risk groups were well separated into two clusters (Fig. 4b). Patients in the high-risk clusters had a worse prognosis than those in low-risk clusters (Fig. 4c). The Kaplan–Meier curve (KM) curve also showed a notable difference in OS between the two risk groups (p = 0.002, Fig. 4d). Receiver operating characteristic (ROC) analysis was performed to evaluate the specificity and sensitivity of the prognostic model. We demonstrated that the 3-gene risk model could correctly indicate the clinical outcomes of HCC patients (AUC 0.58 for 1-year, 0.658 for 3-year, and 0.656 for 5-year survival) (Fig. 4e).

# 3.6 Validation in both the TCGA Test Cohort as well as ICGC Cohort

We then assessed the predictive power of the risk signature in both the TCGA test cohort (n = 184) and the external cohort (ICGC, n = 231). In the TCGA test co-



Table 2. Multivariate Cox regression analysis of risk genes.

HR.95L

HR.95H

p value

HR

Coef

Fig. 3. TCGA-LIHC risk signature construction. (a) Univariate analysis showed nine DEGs (p < 0.05). (b) LASSO analyses of all nine genes. (c) LASSO regression parameter selection by cross-validation. (d) Three DEGs with p < 0.1 based on multivariate analysis.

hort, the high-risk (n = 83) and low-risk (n = 101) subgroups were well differentiated into two clusters using PCA (Supplementary Fig. 2a-c). A notably different OS was demonstrated by the KM survival curves of the two groups (p = 0.022) (Supplementary Fig. 2d). Based on the risk score, the ICGC cohort was classified into high-risk (n = 115) and low-risk (n = 116) subgroups using PCA (Supplementary Fig. 3a-c). There was a marked difference in the OS between the two groups, as represented by the KM survival curves (p < 0.01) (Supplementary Fig. 3d). The AUC values for survival at 1-year, 3-year, and 5-year survival were 0.725, 0.745, and 0.580, respectively (Supplementary Fig. 3e). Taken together, these data revealed that this risk model consisting of three genes could predict the prognosis of HCC patients well, whether in the TCGA training cohort, the test cohort, or the ICGC cohort.

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# 3.7 Independent Evaluation of the Risk Model's Prognostic Value

Cox regression analysis was used to assess whether the risk score could independently predict OS. The findings suggested that the risk score may be a promising hazard factor both in the univariate Cox regression analysis (p< 0.001, HR = 2.280, 95 percent CI: 1.572–3.307, Fig. 5a) and in the multivariate analysis (p < 0.001, HR = 2.171, 95 percent CI: 1.389–3.396, Fig. 5b).

To assess the predictive power of the risk score in HCC, we performed univariate and multivariate Cox regression analyses in the TCGA test cohort of 184 patients (Fig. 5c,d). Similar to the results in previous cohorts, the risk score was proved to be a strong prognostic predictor (p < 0.001, HR = 3.892, 95 percent CI: 2.097–7.226 for univariate analysis, Fig. 5c; p < 0.001, HR = 3.496, 95 percent CI:1.878–6.508 for multivariate, Fig. 5d). Individuals in the high-risk group tended to have higher levels of BAK1 and GSDMC but lower level of NLRP6, as shown in the



**Fig. 4. Validation in TCGA training cohort.** (a–c) Patients were classified as baseline on the basis of their risk scores in the training cohort. (d) The Kaplan–Meier curve (KM) survival curve. (e) Analysis of time-dependent Receiver operating characteristic (ROC) scores.

#### heatmap (Fig. 5e).

#### 3.8 Functional Analyses of the PRGs

GSEA was used to explore the role of PRGs in HCC. Most of the genes were enriched in the apoptosis, IL-6-JAK-STAT3, P53, and TGF- $\beta$  signaling pathways, *etc.* (**Supplementary Fig. 4a**), suggesting that genes related to pyroptosis may participate in the oncogenesis and development of HCC through these pathways.

#### 3.9 Evaluation of Tumor Immune Infiltration

On the basis of the functional analyses, GSEA analysis was performed to compare 16 enriched immune cell types between the two risk clusters in the TCGA cohorts. In the TCGA cohort, CD56 bright cells, CD4 T cells, dendritic cells (DC), NK cells, Plasmacyloid DC, CD56 dim cells, and Memory CD4 T cells showed a positive correlation, while eosinophils, neutrophils, T helper (Th)1 cells, macrophages, mast cells, memory B cells, activated CD8 T cells, Th17, activated B cells and monocytes were negatively correlated with the risk signature (**Supplementary Fig. 4b**).

### 3.10 The Validation of Independent Prognostic Genes Differential Expression

We explored changes in the protein levels of three PRGs using the Human Protein Atlas (HPA) database. The results indicated that BAK1 and GSDMC were upregulated in tumor tissues, whereas NLRP6 expression was lower level in HCC than in normal tissues (**Supplementary Fig. 5**).

#### 3.11 PD-L1 Upregulates GSDMC in HCC

PD-L1 is an immune inhibitor, that can upregulates GSDMC expression to transform apoptosis to pyroptosis in breast cancer [21]. Using the TCGA-LIHC database, we found that PD-L1 and GSDMC were positively correlated (Cor = 0.3, p < 0.001) at the mRNA level in HCC (Fig. 6a). Since PD-L1 protein is seldom expressed in Huh7 or Hep3B cells without IFN- $\gamma$  stimulation, we detected GSDMC expression in IFN- $\gamma$ -treated hepatoma cells. Immunoblotting assays showed that PD-L1 knockdown decreased GSDMC levels in Huh7 cells (Fig. 6b, left panel). In contrast, PD-L1 overexpression enhanced GSDMC levels in Hep3B cells (Fig. 6b, right panel).



**Fig. 5. Independent prognostic value assessment of risk scores.** (a,b) Univariate and multivariate analysis in training cohorts. (c,d) Test cohorts by univariate and multivariate analysis. (e) The correlation of gene heatmap with between the different risk groups.



Fig. 6. GSDMC is upregulated by CD274 (PD-L1). (a) CD274 showed positive correlation with GSDMC (Cor = 0.3, p < 0.01). (b) The full length of GSDMC, GSDMC-N and caspase-8 expression levels in PD-L1- knockdown Huh7 (sh1 and sh2) or PD-L1-overexpressing Hep3B cell (OE) lines, representative results of 3 experiments. To induce PD-L1 expression, we used IFN- $\gamma$  as a stimulator.

# 3.12 GSDMC Promotes the Invasion, Migration and Proliferation of HCC Cells

A previous study has reported that GSDMC was increased in lung adenocarcinoma, colon adenocarcinoma and breast cancer and correlated with reduced overall survival [22,23]. However, the role of GSDMC in HCC has not yet been investigated. We wondered whether GSDMC is upregulated in HCC and contributes to the malignant biological behaviors of tumors. Twelve pairs of HCC samples and adjacent tissues were collected for western blot analy-



**Fig. 7. Overexpression of GSDMC significantly promotes the proliferation and invasion of HCC cell.** (a) The full length GSDMC, GSDMC-N, and caspase-8 expression levels in HCC and adjacent hepatic tissues. n = 12. (b) Validation of GSDMC-overexpressing Hep3B cell lines, n = 3. (c) Effects of overexpression of GSDMC on the proliferation of HCC cells, n = 3. (d) Representative plot and quantitation of colony formation assay in HCC cells overexpressing GSDMC, n = 3. (e,f) Effects of GSDMC overexpression on HCC cell motility (e) and invasion (f), n = 3. \*\*p < 0.01, \*\*\*p < 0.001, unpaired two-tailed *t* test or one way ANOVA.

sis. Compared with adjacent liver tissues, GSDMC was significantly upregulated in HCC tissues (Fig. 7a). Then stable GSDMC-overexpressing Hep3B (Fig. 7b) cell line was successfully constructed. The proliferation ability was enhanced in GSDMC-overexpressing Hep3B cells, as shown by the CCK-8 proliferation assay and colony formation assay (Fig. 7c,d). Wound healing and invasion experiments demonstrated that the cell motility and invasion ability were substantially enhanced in GSDMC-overexpressing Hep3B cells (Fig. 7e,f).

Next, we constructed a GSDMC knockdown Huh7 cell line (Fig. 8a). The results showed that the proliferative capacity was decreased (Fig. 8b,c), and cell motility and invasion ability were impaired in GSDMC-knockdown Huh7 cells (Fig. 8d,e).

Taken together, these findings revealed a novel tumorigenic function of GSDMC in HCC.

#### 3.13 GSDMC Expression Correlates with the Immune Cell Infiltration and Subcellular Location within the Cell in HCC

Since GSEA analysis revealed an enrichment of immune cell-related pathways, we hypothesized that infiltration of immune cells plays a critical role in the tumorigenic effect of GSDMC in HCC. GSDMC expression showed a positive correlation with immune cells and stromal cells (Fig. 9a). Furthermore, we investigated the association between GSDMC expression and 24 types of tumorinfiltrating immune cells by using the TIMER database. It is demonstrated that GSDMC was strongly correlated with macrophages and Th2 cells (Cor >0.3, p < 0.05) (Fig. 9b).

Next, we explored the correlation between GSDMC and immunosuppressive checkpoint. As shown in the heatmap, GSDMC is correlated with various immune inhibitor proteins such as CD274 (PD-L1), IL-10, and IDO1 in the TISIDB database (Fig. 9c). We further examined the expression pattern of GSDMC. It is mainly expressed



**Fig. 8.** Knockdown of GSDMC significantly inhibits the proliferative and invasive ability of HCC cell. (a) Validation of GSDMCknockdown Huh7 cell lines (sh-GSDMC), n = 3. (b) Effects of knockdown of GSDMC on the proliferation of HCC cells, n = 3. (c) Representative plot and quantification of colony formation assay in HCC cells knockdown by GSDMC, n = 3. (d,e) Effects of GSDMC knockdown on HCC cell motility (d) and invasion (e), n = 3. \*\*p < 0.01, \*\*\*p < 0.001, unpaired two-tailed *t* test or one-way ANOVA.

in keratinocytes and epithelial cells (Fig. 9d), according to the HPA dataset. GSDMC was not expressed in immune cells in HCC single-cell sequencing datasets (GSE140228 and GSE98638, Fig. 9e) using TISCH database.

## 4. Discussion

To date, the prognostic evaluation of HCC is mostly based on the pathology, staging and grading of the American Joint Committee on Cancer (AJCC), Tumor Node Metastasis (TNM), and Bandwidth Constrained Least Cost (BCLC), which are not sensitive enough. Improving the survival of patients with HCC, more accurate and less invasive prognostic models based on novel biomarkers are urgently needed.

As an embodiment of programmed cell death, pyroptosis can release cellular content and inflammatory cytokines, which occur in cells infected by pathogens. Recent studies have suggested that pyroptosis also contributes to oncogenesis and cancer development. Pyroptosis is a double-edged sword, which could be either protumorigenic or antitumorigenic. On one side, pyroptosis can promote inflammatory death of tumor cells and release chemokines that recruit immune cells to the TME, therefore suppressing tumor growth [24]. On the other side, pyroptosis-produced IL-1 $\beta$  and IL-18 can facilitate the malignant transformation of normal cells and contribute to tumor immune escape by creating a chronic inflammatory microenvironment [25,26]. However, the function of pyroptosis in HCC is not fully understood. Whether pyroptosis

can activate the TME and synergize with immunotherapy warrants further investigation. The present study provides a novel perspective for evaluating HCC prognosis. Notably, 42 DEGs were identified and divided the patients with HCC into two subtypes using the consensus cluster analysis. Interestingly, these two subtypes have different survival rates, providing a helpful classification for clinical practice. To assess the prognosis of HCC patients, we constructed an independent prognostic model in the TCGA training cohort, which was also validated in ICGC or TCGA test cohort. A prognostic model consisting of three PRGs (BAK1, GS-DMC, and NLRP6) was then derived from the LASSO-Cox method as well as the multivariate Cox analysis with a cutoff of 0.1. Among 3 PRGs, BAK1 and GSDMC were upregulated, while NLRP6 was down-regulated in tumor tissues.

Tumor cells can induce a suppressive TME to promote immune evasion and facilitate HCC progression. ICIs activate immune attack against tumor cells by specifically blocking immune checkpoints, such as PD-1/PD-L1 (CD274/CD274L). However, responses to ICIs vary among individuals. An important reason for this poor response is the scarcity of immune cells within TME. Pyroptosisinduced inflammation may increase the number of tumorinfiltrating immune cells, thereby improving therapeutic sensitivity [25]. Previous studies have suggested that pyroptosis is closely related to PD-1/PD-L1 [25]. *GSDME* and *GSDMD* can serve as biomarkers for PD-1 inhibitor [25]. Moreover, *PD-L1* was able to regulate *GSDMC* transcription and switch apoptosis to pyroptosis induced by



Fig. 9. The relationship between GSDMC expression and tumor microenvironment (TME) in HCC. (a) GSDMC was positively correlated with both immune cells and stromal cells. (b) High GSDMC expression had the strongest correlation with macrophages and Th2 cells (cor > 0.3, p < 0.05). (c) GSDMC was correlated with various immune checkpoints in the TISIDB database. (d) GSDMC was mainly expressed in keratinocytes and epithelial cells. (e) GSDMC did not express in immune cells in HCC single-cell sequencing datasets (gSE140228 and GSE98638).

TNF $\alpha$  in breast cancer, leading to tumor regression [21]. A positive correlation between GSDMC expression and immune cells in HCC were demonstrated in the present study, as well as immune checkpoint protein including PD-L1 (CD274), which is consistent with previous findings. Pyroptosis-related pathways may be a promising targets for enhancing the sensitivity of HCC to ICIs.

The function of GSDMC in tumors is less known [27]. In a previous study, GSDMC upregulation was associated with melanoma metastatic [28], GSDMC overexpression promoted tumorigenesis and cell proliferation by inhibiting TGFBR activity in colorectal cancer. In addition, it has been shown to be an indicator of poor prognosis in lung adenocarcinoma patients [23]. In contrast, GSDMC acted as a tumor suppressor and exerted an inhibitory effect on cell growth in esophageal and gastric cancer [29]. We found that GSDMC was upregulated in HCC tissues. Furthermore, overexpression of GSDMC contributed to the proliferation, invasion, and migration of HCC cell lines, whereas silencing GSDMC showed the opposite effect. These findings revealed the pro-tumorigenic role of GSDMC in HCC. Determining the function of GSDMC in HCC will facilitate its future use as a therapeutic target in clinical practice.

Pyroptosis is a form of cell death that affects the prognosis of cancer patients by modulating tumor cell migration, proliferation and invasion. Some related studies have shown that PRGs played important roles in predicting the prognosis of hepatocellular carcinoma patient [30-38]. Li et al. [31] constructed a three-PRG prognostic model comprising CHMP4A, HMGB1 and PLK1. Further the author validate the differential expression of the three prognostic genes in HCC model and in vitro. He et al. [35] constructed five PRGs (CASP8, GSDMC, NLRP6, NOD2, and PLCG1) signatures to predict HCC prognosis. It could precisely predict survival outcomes, reveal the composition of the immune microenvironment, and strengthen the argument for more credible clinical and functional research in HCC patients. In addition, qRT-PCR analysis also demonstrated that the PRGs in HCC cell lines were differentially expressed in the prognostic signature. Zhang et al. [36] identified a 7-PRGs signatures (BAK1, CHMP4B, GSDMC, NLRP6, NOD2, PLCG1, SCAF11) for predicting the prognosis of HCC. This novel PRGs signature can predict the prognosis of patients with HCC and provide insight into new cell death-targeted therapies. The pyroptosis-related risk model developed by Xing et al. [37] can predict the prognosis of HCC, evaluate immune cell infiltration status in the tumor microenvironment and assess the efficacy of immunotherapy to guide immunotherapy. All these studies established a strong correlation between pyroptosis and the clinical risk of HCC patients, however, these studies did not focus on the genes function in HCC.

Our study aimed to identify PRGs with differential expression between HCC and normal samples in order to construct a prognostic model, that can distinguish HCC patients with varied clinical outcomes. The three PRGs signatures containing GSDMC could predict HCC prognosis. In addition, we focused on GSDMC molecules and probed the GSDMC biology in both patient samples and hepatocellular carcinoma cell lines, which could demonstrated that increased GSDMC levels contribute to cell proliferation, invasion and migration in HCC. Although we had performed verifications in multiple databases, our research still has some limitations. First of all, the validation of prognostic models was mainly based on public databases. The predictive value needs to be verified using additional clinical data. Second, this article does not have an in-depth investigation on the molecular pathway behind these pyroptosis-related biomarkers. More efforts are needed to determine how pyroptosis regulates the TME in HCC.

## 5. Conclusions

Our findings indicated that the 3-PRGs signature which contained *GSDMC* could predict the prognosis of patients with HCC. In addition, *GSDMC* exerted a protumorigenic role by promoting the growth and metastasis in HCC.

## Availability of Data and Materials

All the data included in this article are available from the corresponding author upon reasonable request.

## **Author Contributions**

RX, SC designed the study. ML and QJ performed the project. ML and XL organized the data and wrote the manuscript. QJ and LH provided help and advice on the cell function experiments. XL revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

# **Ethics Approval and Consent to Participate**

The study was carried out in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Zhongshan Hospital, Fudan University (No. B2017-134).

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# **MR Press**

## **Conflict of Interest**

The authors declare no conflict of interest.

## **Supplementary Material**

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10. 31083/j.fbl2810235.

## References

- Nia A, Dhanasekaran R. Genomic Landscape of HCC. Current Hepatology Reports. 2020; 19: 448–461.
- [2] Li H, Li T, Zhang X. Identification of a Pyroptosis-Related Prognostic Signature Combined With Experiments in Hepatocellular Carcinoma. Frontiers in Molecular Biosciences. 2022; 9: 822503.
- [3] Llovet JM, Castet F, Heikenwalder M, Maini MK, Mazzaferro V, Pinato DJ, *et al.* Immunotherapies for hepatocellular carcinoma. Nature Reviews Clinical Oncology. 2022; 19: 151–172.
- [4] Yang J, Zhao Y, Shao F. Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity. Current Opinion in Immunology. 2015; 32: 78–83.
- [5] Feng S, Fox D, Man SM. Mechanisms of Gasdermin Family Members in Inflammasome Signaling and Cell Death. Journal of Molecular Biology. 2018; 430: 3068–3080.
- [6] Shi J, Gao W, Shao F. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. Trends in Biochemical Sciences. 2017; 42: 245–254.
- [7] Wang Y, Gao W, Shi X, Ding J, Liu W, He H, et al. Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. Nature. 2017; 547: 99–103.
- [8] Yu J, Li S, Qi J, Chen Z, Wu Y, Guo J, et al. Cleavage of GSDME by caspase-3 determines lobaplatin-induced pyroptosis in colon cancer cells. Cell Death & Disease. 2019; 10: 193.
- [9] Zhao G, Xie Z. Pyroptosis and neurological diseases. Neuroimmunology and Neuroinflammation. 2014; 1: 60–65.
- [10] Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature. 2015; 526: 660–665.
- [11] Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infection and Immunity. 2005; 73: 1907–1916.
- [12] Fink SL, Cookson BT. Pyroptosis and host cell death responses during Salmonella infection. Cellular Microbiology. 2007; 9: 2562–2570.
- [13] Van Laer L, Huizing EH, Verstreken M, van Zuijlen D, Wauters JG, Bossuyt PJ, *et al.* Nonsyndromic hearing impairment is associated with a mutation in DFNA5. Nature Genetics. 1998; 20: 194–197.
- [14] Ding J, Wang K, Liu W, She Y, Sun Q, Shi J, *et al*. Pore-forming activity and structural autoinhibition of the gasdermin family. Nature. 2016; 535: 111–116.
- [15] Zhang Z, Zhang Y, Xia S, Kong Q, Li S, Liu X, *et al.* Gasdermin E suppresses tumour growth by activating anti-tumour immunity. Nature. 2020; 579: 415–420.
- [16] Hage C, Hoves S, Strauss L, Bissinger S, Prinz Y, Pöschinger T, et al. Sorafenib Induces Pyroptosis in Macrophages and Triggers Natural Killer Cell-Mediated Cytotoxicity Against Hepatocellular Carcinoma. Hepatology. 2019; 70: 1280–1297.
- [17] Mayakonda A, Lin DC, Assenov Y, Plass C, Koeffler HP. Maftools: efficient and comprehensive analysis of somatic variants in cancer. Genome Research. 2018; 28: 1747–1756.
- [18] Seiler M, Huang CC, Szalma S, Bhanot G. ConsensusCluster: a software tool for unsupervised cluster discovery in numerical

data. OMICS. 2010; 14: 109-113.

- [19] Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012; 16: 284–287.
- [20] Hänzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. BMC Bioinformatics. 2013; 14: 7.
- [21] Hou J, Zhao R, Xia W, Chang CW, You Y, Hsu JM, et al. PD-L1-mediated gasdermin C expression switches apoptosis to pyroptosis in cancer cells and facilitates tumour necrosis. Nature Cell Biology. 2020; 22: 1264–1275.
- [22] Cui YQ, Meng F, Zhan WL, Dai ZT, Liao X. High Expression of GSDMC Is Associated with Poor Survival in Kidney Clear Cell Cancer. BioMed Research International. 2021; 2021: 5282894.
- [23] Wei J, Xu Z, Chen X, Wang X, Zeng S, Qian L, *et al*. Overexpression of GSDMC is a prognostic factor for predicting a poor outcome in lung adenocarcinoma. Molecular Medicine Reports. 2020; 21: 360–370.
- [24] Xia X, Wang X, Cheng Z, Qin W, Lei L, Jiang J, et al. The role of pyroptosis in cancer: pro-cancer or pro-"host"? Cell Death & Disease. 2019; 10: 650.
- [25] Li L, Jiang M, Qi L, Wu Y, Song D, Gan J, et al. Pyroptosis, a new bridge to tumor immunity. Cancer Science. 2021; 112: 3979–3994.
- [26] Karki R, Kanneganti TD. Diverging inflammasome signals in tumorigenesis and potential targeting. Nature Reviews Cancer. 2019; 19: 197–214.
- [27] Kovacs SB, Miao EA. Gasdermins: Effectors of Pyroptosis. Trends in Cell Biology. 2017; 27: 673–684.
- [28] Watabe K, Ito A, Asada H, Endo Y, Kobayashi T, Nakamoto K, et al. Structure, expression and chromosome mapping of MLZE, a novel gene which is preferentially expressed in metastatic melanoma cells. Japanese Journal of Cancer Research. 2001; 92: 140–151.
- [29] Saeki N, Usui T, Aoyagi K, Kim DH, Sato M, Mabuchi T, et al. Distinctive expression and function of four GSDM family genes (GSDMA-D) in normal and malignant upper gastrointestinal ep-

ithelium. Genes, Chromosomes & Cancer. 2009; 48: 261-271.

- [30] Fu XW, Song CQ. Identification and Validation of Pyroptosis-Related Gene Signature to Predict Prognosis and Reveal Immune Infiltration in Hepatocellular Carcinoma. Frontiers in Cell and Developmental Biology. 2021; 9: 748039.
- [31] Li Y, Li Y, Zhang X, Duan X, Feng H, Yu Z, *et al.* A novel association of pyroptosis-related gene signature with the prognosis of hepatocellular carcinoma. Frontiers in Oncology. 2022; 12: 986827.
- [32] Wang S, Gao S, Shan L, Qian X, Luan J, Lv X. Comprehensive genomic signature of pyroptosis-related genes and relevant characterization in hepatocellular carcinoma. PeerJ. 2023; 11: e14691.
- [33] Duan S, Gao J, Lou W, Zhang Y, Deng Y, Wang C, et al. Prognostic signature for hepatocellular carcinoma based on 4 pyroptosis-related genes. BMC Medical Genomics. 2022; 15: 166–180.
- [34] Fang G, Zhang Q, Fan J, Li H, Ding Z, Fu J, et al. Pyroptosis related genes signature predicts prognosis and immune infiltration of tumor microenvironment in hepatocellular carcinoma. BMC Cancer. 2022; 22: 999.
- [35] He J, Ran J, Li J, Chen D. Construction and validation of a pyroptosis-related gene signature in hepatocellular carcinoma based on RNA sequencing. Translational Cancer Research. 2022; 11: 1510–1522.
- [36] Zhang S, Li X, Zhang X, Zhang S, Tang C, Kuang W. The pyroptosis-related gene signature predicts the prognosis of hepatocellular carcinoma. Frontiers in Molecular Biosciences. 2022; 8: 781427.
- [37] Xing M, Li J. Diagnostic and prognostic values of pyroptosisrelated genes for the hepatocellular carcinoma. BMC Bioinformatics. 2022; 23: 177.
- [38] Deng M, Sun S, Zhao R, Guan R, Zhang Z, Li S, et al. The pyroptosis-related gene signature predicts prognosis and indicates immune activity in hepatocellular carcinoma. Molecular Medicine. 2022; 28: 16.