

A Novel Pyroptosis-related Gene Signature for Predicting Prognosis and Immune Infiltration in Osteosarcoma

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Abstract

Background: Osteosarcoma is the most prevalent primary malignant bone malignancy with a dismal prognosis in children and adolescents. Pyroptosis, a proinflammatory form of programmed cell death, plays a pivotal role in oncogenesis and tumor progression across various malignancies. However, the prognostic relevance of pyroptosis-related genes (PRGs) in osteosarcoma remains poorly understood. In this study, we aimed to establish and validate a novel PRG-based prognostic signature to enhance survival prediction for osteosarcoma patients. **Methods:** Transcriptomic data and corresponding clinical profiles of osteosarcoma were retrieved from the UCSC Xena database. PRGs were identified from established literature, and bioinformatics analyses were performed to isolate differentially expressed genes (DEGs). Non-negative matrix factorization (NMF) clustering was applied to identify molecular subgroups based on PRG expression patterns. LASSO-Cox regression analysis was utilized to construct the novel model. Subsequently, we constructed a nomogram and calibration curves incorporating the risk score and clinical characteristics. Finally, Gene Set Enrichment Analysis (GSEA) and immune microenvironment characterization were performed to investigate the functional pathways and immunological landscapes associated with the signature. **Results:** A novel prognostic signature comprising four key PRGs was established. Kaplan–Meier survival analysis demonstrated that patients in the high-risk group had a significantly poorer overall survival. Independent Cox analysis illustrated that this risk model serves as an independent prognostic indicator. In addition, we validated the accuracy of our signature in the validation dataset. Furthermore, the nomogram and calibration curves confirmed the robust prediction efficiency of the novel model. Notably, GSEA and immune microenvironment analysis showed that the low-risk group was significantly enriched in immune-related pathways and possessed a superior immune status, indicating that the divergent clinical outcomes between the two risk groups mainly result from the regulation of the immune microenvironment. **Conclusion:** The identified four-gene pyroptosis-related signature is a promising prognostic biomarker for osteosarcoma. These findings provide novel insights that may improve survival outcomes and guide personalized therapeutic strategies for osteosarcoma patients.

Keywords: Osteosarcoma- Pyroptosis- Prognosis- Immune microenvironment- Signature

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Introduction

Osteosarcoma (OS) is the most prevalent malignant bone tumor affecting children, adolescents, and young adults, with a median age of 16 years. This malignancy exhibits a high propensity for local invasion and early metastasis, predominantly affecting the lungs (90%) [1, 2]. To date, traditional treatment for OS has primarily relied on surgical resection, systemic chemotherapy,

and radiation therapy [3]. Despite advancements in neoadjuvant chemotherapy and surgical techniques, the five-year survival rate for patients with non-metastatic disease has reached approximately 70% [4]. However, the prognosis for patients with metastatic or recurrent OS remains dismal, with five-year survival rates stagnating below 20%, as most cases progress to fatality at advanced

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stages[5, 6]. Given these limitations in conventional treatment regimens, early and accurate diagnosis, alongside reliable prognosis prediction, are critical for the timely initiation of treatment and improving patient outcomes. Although various biomarkers and gene signatures have been identified to predict OS survival status, they largely remain in the preclinical phase and have not yet been translated into clinical application. Therefore, there is an urgent need to develop a robust and efficient prognostic gene signature for OS.

A growing body of literature has established that programmed cell death (PCD) is intricately linked to tumorigenesis, progression, and metastasis [7-9]. Pyroptosis, a proinflammatory type of PCD discovered recently, is characterized by continuous cell swelling until the cell membrane ruptures, leading to the release of intracellular contents and triggering a potent inflammatory and immune response [10, 11]. This distinguishes pyroptosis fundamentally from apoptosis, which is generally immunologically silent and non-inflammatory. By releasing danger-associated molecular patterns and inflammatory cytokines (e.g., IL-1 β and IL-18), pyroptosis serves as a crucial bridge between tumor cell death and the activation of antitumor immunity. Given that osteosarcoma is characterized by a highly heterogeneous immune microenvironment where immune evasion facilitates metastasis, the immunogenic potential of pyroptosis suggests it may possess superior prognostic relevance compared to other non-immunogenic cell death mechanisms. The induction of pyroptosis depends on the activation of inflammasomes, specifically caspase-1/4/5/11 and Gasdermin D (GSDMD) [12, 13]. Once activated, caspases cleave GSDMD to release its Gasdermin-N domain, which binds to membrane lipids and forms pores in the membrane, resulting in changes in osmotic pressure, cell swelling, and eventual lysis [14].

Recently, pyroptosis has gained significant attention in the context of various pathologies, including cardiovascular diseases, liver diseases, and malignancies [12, 15, 16]. Accumulating evidence suggests that pyroptosis plays a pivotal role in ovarian cancer, gastric cancer, glioma, hepatocarcinoma, breast cancer, skin cutaneous melanoma, and malignant mesenchymal tumors [17-23]. For instance, previous studies revealed that GSDMD expression is downregulated in gastric cancer, thereby promoting tumor proliferation by regulating cell cycle-related proteins [24]. In addition, the pro-inflammatory effects of pyroptosis have been linked to the modulation of the tumor immune microenvironment [25]. Despite the established importance of pyroptosis across various cancers, its specific role in OS remains unexplored, and a prognostic signature based on pyroptosis-related genes for OS is currently lacking.

In the present study, we retrieved gene expression data and corresponding clinical features of patients with OS from the Genotype-Tissue Expression (GTEx) database and the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) data portal. We then identified pyroptosis-related differentially expressed genes (DEGs) in OS and constructed a prognostic multigene

signature based on these candidates. Functional enrichment analysis was performed to elucidate potential underlying mechanisms. Finally, we systematically investigated the correlations between this novel pyroptosis-related signature and the tumor immune microenvironment.

Materials and Methods

Datasets

The Therapeutically Applicable Research to Generate Effective Treatments database (TARGET; <https://ocg.cancer.gov/programs/target>) is an open database of childhood tumors, which aims to use comprehensive genomics methods to determine the molecular changes in the occurrence and development of childhood cancers to help researchers develop effective treatment methods [26]. The Genotype-Tissue Expression database (GTEx, <https://www.gtexportal.org/home/>) provides various normal human tissue transcriptome data, including 1641 samples across 43 tissues from 175 individuals [27]. The University Of California Santa Cruz (UCSC) Xena Hub datasets(<https://xenabrowser.net/>) is one of the most common gateways to access genomic sequence and annotation data of humans and other organisms [28]. The mRNA expression profile and clinical characteristics of patients with OS in the TARGET database were extracted from the UCSC database. The 88 OS cases (84 cases with survival information and 4 without survival information) were obtained and transformed by $\log_2(x+1)$. In addition, the gene expression profile data of 396 normal individuals' muscle tissue were obtained as a corresponding control group. To compare with the TARGET data, the gene expression data from the GTEx database were also transformed by $\log_2(x+1)$. Before the subsequent difference analysis, the conversion of Ensembl identifiers and the merging of expression data between the TARGET and GTEx data sets were also carried out.

Identification of pyroptosis-related DEGs

The corresponding pyroptosis-related genes were extracted from prior reviews as the candidate gene set and are presented in Table S1 [13, 23, 29-31]. The "limma" package in R was used to find the pyroptosis-related DEGs between OS tissue and normal tissues with a P-value <0.05. Then, we constructed a protein-protein interaction network (PPI) for pyroptosis-related DEGs using the Search Tool for the Retrieval of Interacting Genes (STRING, <https://string-db.org/>). Hub genes in pyroptosis-related DEGs were selected using the Maximal Clique Centrality (MCC) method of Cytoscape [32].

Classification of molecular subgroups

The nonnegative matrix factorization (NMF) clustering was used to identify distinct differential subgroups relating to pyroptosis-related DEGs, which was conducted by the "NMF" R package. Then, the immune score, stromal score, ESTIMA score, and tumor purity of different molecular subgroups were calculated with the Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression (ESTIMATE) algorithm [33].

Functional analysis

The “ggplot2” package was used to conduct Gene Ontology (GO) analysis, which includes the cellular component (CC), biological process (BP), and molecular function (MF) categories of genes in biological processes. Meanwhile, the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was also performed by this package. KEGG is a data resource for exploring advanced functions and the practicality of unique biological systems at the molecular level. $P < 0.05$ were considered as statistical significance.

Pyroptosis-related prognostic signature development

After removing the patients without complete survival information, 84 patients with complete survival information were finally extracted and randomly divided into a training group ($n = 42$), a testing group ($n = 42$), and an entire group ($n = 84$) for further investigation. Firstly, we performed a univariate cox regression analysis to assess the predictive efficiency of the pyroptosis-related DEGs in the training group, and the critical P -value was set to 0.2 to avoid omissions [34]. Then, the least absolute shrinkage and selection operator (LASSO) cox regression analysis and multivariate cox regression analysis were used to narrow down the candidate genes and develop the optimal prognostic model. The signature was established from the independent prognostic genes according to their corresponding coefficients calculated by multivariate cox regression analysis. After the model was established, we obtained the risk score according to the following formula: $\text{Risk score} = h_0(t) * e^{(\sum \text{Expression level of mRNA} * \text{Corresponding regression coefficient})}$ ($h_0(t)$ is the benchmark risk value, which is automatically calculated by the predict() function in R software). Subsequently, the patients with OS were categorized into low-risk and high-risk groups based on the median cutoff of the risk score. Moreover, the Kaplan-Meier survival curve analysis, time-dependent receiver operating characteristic curve (ROC) analysis, and risk score analysis were used to compare the survival status between the differential risk groups and evaluate the predictive efficiency of the models. Finally, we further used the data from the testing and the entire group to validate the prediction performance of the constructed prognostic model.

Independent prognostic analysis

To evaluate whether the novel prognostic model was an independent prognostic factor in predicting the prognosis of patients with OS, the clinical information of OS in the TARGET databases were extracted. Then, univariate and multivariate Cox regression analyses were performed to evaluate the relationship between the risk score and clinical characteristics in the training group and further verification in the test group and the entire group.

The predictive nomogram

The nomogram is used to diagnose or predict the onset and progression of the disease, which is widely used to predict the specific prognosis of cancer by quantifying each prognostic factor [35]. We constructed a composite

nomogram further to inspect the probability of overall survival of OS. According to the degree of influence of the risk factors on the clinical outcome, they were graded to assess their level, and the total score is obtained by the sum of the scores of each factor. Then, the predicted survival time of OS patients is calculated through the inverse relationship between the total score and the outcome event. Moreover, a calibration curve depicting overall survival was applied to verify the accuracy of the nomogram-predicted probabilities.

Gene Set Enrichment Analysis (GSEA)

GSEA (<http://software.broadinstitute.org/gsea/index.jsp>) is a scientifically sound and effective tool for gene function enrichment analysis in gene sets between two states [36]. We used GSEA to compare the enrichment levels of biological processes in the low-risk group and the high-risk group, revealing the underlying biological mechanisms of key genes. The c2.cp.kegg.v7.4.symbols.gmt in Molecular Signatures Database (MSigDB, <http://software.broadinstitute.org/gsea/msigdb/index.jsp>) was chosen as the reference gene collections in GSEA software. Gene sets with P -value < 0.05 were considered significantly enriched.

Immunity analysis

We further explored the correlation between the signature and immune infiltration through the ESTIMATE, CIBERSORT, and single-sample gene set enrichment analysis (ssGSEA). The ESTIMATE algorithm was used to calculate the stromal score, immune score, ESTIMA score, and tumor purity of different risk groups. The CIBERSORT algorithm is a robust method based on gene expression data to describe the immune cell composition of complex tissues. It is used to evaluate the cellular immune response or cellular components between the two risk groups of OS patients. In addition, ssGSEA was used to quantify the tumor-infiltrating immune cell between the two risk groups and assess their immune function. Finally, potential immune checkpoints retrieved from previous studies were used to explore the connection between immune-related genes and risk scores [37].

Statistical analysis

Statistical analyses were conducted by using Bioconductor packages in R software. The Wilcoxon test was used to compare the expression differences of various types of immune cells and immune checkpoint molecules between the low-risk group and the high-risk group. The univariate and multivariate Cox proportional hazard regression analyses were performed to identify potential prognostic factors of overall survival. The nomogram and calibration curve, including risk score and clinical features, were constructed using R software’s “rms” package. The survival status of patients with OS between differential groups was assessed by the Kaplan–Meier survival analysis. For each analysis, $P < 0.05$ was considered statistically significant.

Results

Pyroptosis-related DEGs between normal and tumor tissues

The 52 pyroptosis-related gene expression levels were extracted from the TARGET (tumor tissues, n=88) and GTEx (normal tissue, n=396) RNA sequence data, and we identified 46 DEGs. Figures 1A showed all DEGs. To further explore the potential interactions of these pyroptosis-related DEGs, PPI analysis was performed, and the results were presented in Figure 1B (The highest confidence: the minimum interaction score required for PPI analysis is set to 0.9). In addition, ten hub pyroptosis-related DEGs were obtained by the MCC method in Cytoscape, namely CHMP3, CHMP7, CHMP4A, CHMP6, CHMP4B, CHMP2A, CHMP4C, CHMP2B, NLRP3, PYCARD (Figure 1C). Finally, Figure 1D shows the correlation network containing all pyroptosis-related DEGs.

Clustering of molecular subgroup associated with the pyroptosis DEGs

To further investigate the association between the 46 pyroptosis-related DEGs and OS subgroups, we classified molecular subgroups through NMF uniform clustering. By increasing the subgroup number from 2 to 10, the results revealed that the correlation within the group is the highest, and the correlation between the groups is low when divided into two subgroups, indicating two subgroups classified are reasonably (Figure 2A). Figure 2B shows the gene expression data and clinical characteristics, including primary tumor site (arm/hand, leg/foot, and pelvis), age (≤ 18 or >18), and metastasis status (Non-metastatic and Metastatic), but there were few noticeable differences in clinical characteristics between the subgroups. In addition, the overall survival

analysis results between the two clusters indicated that cluster 2 underwent better overall survival (Figure 2C). Then, we performed a functional analysis between the two subgroups, and the results were shown in Figure 2D and Figure 2E. Finally, we also used the ESTIMATE algorithm to estimate the immune status of each OS patient tissue and compared whether there was a difference between the two subgroups. As presented in Figure S1, the osteosarcoma tissues in cluster 2 showed higher stromal scores. However, there were no differences in the immune score, ESTIMATE score, and tumor purity between the two clusters.

Development of a prognostic gene model based on the TARGET dataset

The OS patients with complete survival data were divided into a training group, a testing group, and the whole group to construct and verify the prognostic genetic risk model of OS. First, the survival-related genes were initially screened from 46 pyroptosis-related DEGs by univariate Cox regression analysis. Seven genes (CASP6, CHMP4C, GSDMA, TP63, PYCARD, TIRAP, and GZMA) that met the criteria of $P < 0.2$ were extracted for the next analysis, and among them, 5 genes (CASP6, GSDMA, TP63, PYCARD, and GZMA) were protective genes with HRs <1 , while the other 2 genes associated with increased risk with HRs >1 (Figure 3A). Then, the LASSO Cox regression analysis and multivariate Cox regression were performed to define candidate genes (Figure 3B, 3D). As a result, a 4-gene signature was constructed and the risk score for each patient was calculated as follows: Risk score = $17.1698 * e^{(\text{mRNA expression level of CASP6} * (-1.010) + \text{GSDMA} * (-2.704) + \text{TP63} * (-1.536) + \text{TIRAP} * 1.072)}$. According to the median risk score, the OS patients in the training group were equally stratified into low-risk and high-risk subgroups (Figure 3C).

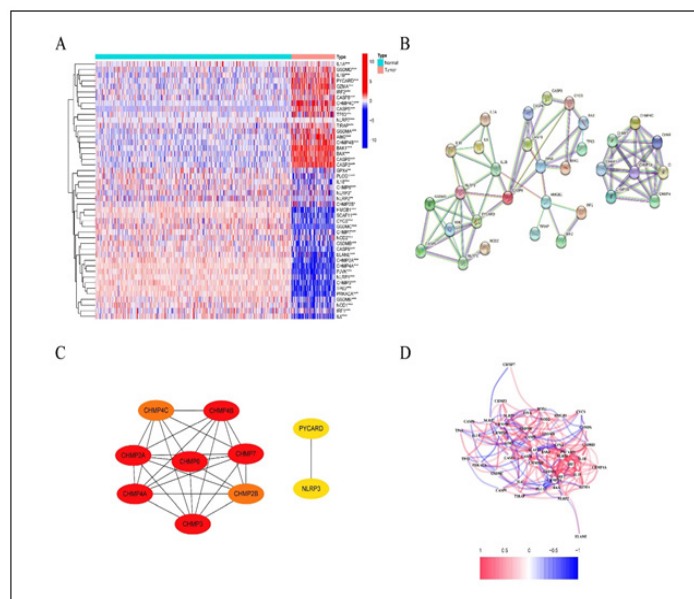


Figure 1. The Expression and Interaction of the Pyroptosis-related Gene. A. The heatmap of pyroptosis-related DEGs between OS tissue and normal tissue (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$); B. PPI network of pyroptosis-related DEGs based on the string database; C. The hub pyroptosis-related DEGs were obtained by Maximal Clique Centrality; D. The correlation network of pyroptosis-related DEGs (blue: negative correlations; red: positive correlations).

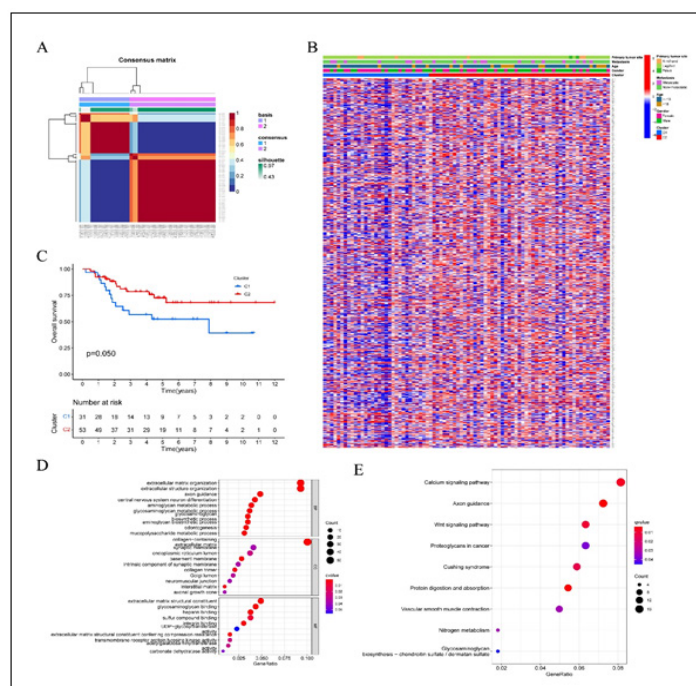


Figure 2. Clustering of a Molecular Subgroup Based on the Pyroptosis-related DEGs. A. Two molecular subgroups through NMF cluster; B. The heatmap visualized the clinicopathological characters and the expression of differential expression genes between the 2 clusters; C. The Kaplan–Meier survival curve of the 2 clusters; D. The GO enrichment analysis results; E. The KEGG analysis results.

Moreover, Figure 3E-F showed a significant difference in OS time between the low-risk group and the high-risk group ($P < 0.001$), and the patients in the high-risk group had poor survival status. Finally, we also conducted a ROC curve to evaluate the prognostic prediction efficiency of the signature. The results showed that the area under the curve (AUC) was 0.732 (1-year overall survival), 0.845 (3-years overall survival), and 0.916 (5-year overall survival), indicating that the prediction signature is well constructed (Figure 3G).

Verification of the novel signature

To further evaluate the predictive accuracy and reliability of the established signature, set the testing, and entire groups were as verified groups to assess the power of the prognostic model. The patient's risk survival status plot and Kaplan–Meier analysis indicated that patients in the low-risk group generally had better survival status than those in the high-risk group (Figures 4A-B and E-F). Figures 4C and 4G show the distribution of patients based on risk scores in the testing and entire set, respectively. In addition, the AUC for 1-year, 3-year, and 5-year were 0.641, 0.786, 0.703 in the testing group and 0.706, 0.805, 0.816 in the entire group, respectively (Figure 4D and 4H). Overall, the above results from the validation group are consistent with those from the test group, which proved that this novel prognostic signature had good predictive efficacy.

Independent prognostic value of the constructed prognostic model

To investigate whether the pyroptosis-associated DEGs signature could be used as an independent

prognostic factor for OS patients, the univariate analysis and multivariate analysis were performed to assess the independent prognostic value. The univariate analysis results indicated that the risk score was a univariate prognostic predictor for OS in both the training set and validation set (Figure 5A-C, training group: hazard ratio (HR) = 1.219, 95% confidence interval (CI) = 1.095–1.358, $p < 0.001$; testing group: HR = 1.139, 95% CI = 1.013–1.281, $p = 0.03$; entire group: HR = 1.141, 95% CI = 1.059–1.229, $p < 0.001$). After considering other confounding factors in the multivariate analysis, the risk score is still an independent prognostic indicator of OS (Figure 5D-F, training group: HR = 1.246, 95% CI = 1.102–1.409, $p < 0.001$; testing group: HR = 1.124, 95% CI = 1.002–1.261, $p = 0.046$; entire group: HR = 1.134, 95% CI = 1.050–1.224, $p = 0.001$). Additionally, Figure 5G demonstrated the pyroptosis-related signature genes and different distributions of primary tumor site, age, gender, and metastasis between the two risk subgroups.

Construction of a predictive nomogram

A predictive pyroptosis-related prognostic nomogram, including risk scores and clinical characteristics (age, gender, metastasis), was constructed to provide a stable and accurate prediction model for OS patients. As indicated in Figure 6A, each patient could calculate the total scores according to the actual situation and then predict the corresponding survival rate within 1, 2, 3 years. Moreover, the predictive values of the nomogram were verified by calibration plots, and the results showed an accordant agreement for the 2 and 3-year survival rates (Figure 6B-C). Overall, the pyroptosis-related risk signature might be a valuable tool for the clinical

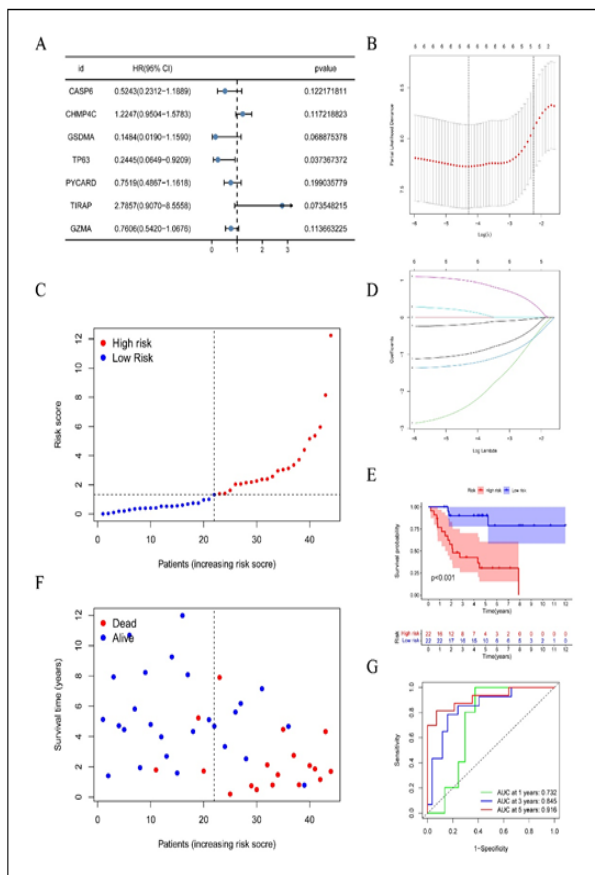


Figure 3. Construction of a Pyroptosis-related Prognostic Model. A. The forest plot results of the univariate Cox regression analyses; B. The LASSO regression result; C. The distribution and median value of the risk scores in the training set; D. Cross-validation for tuning the parameter selection in the LASSO regression; E. The ROC curves demonstrated the predictive value of the risk score; F. The distributions of survival status, survival time, and risk score in the training set; G. Kaplan–Meier survival curves for OS patients in the low-risk and high-risk group.

management of OS patients.

GSEA results

GSEA was performed to identify important functional phenotypes between high-risk and low-risk osteosarcoma patients. The pyroptosis-related risk signature was significantly enriched in 44 pathways (Table S2). Among them, “Hedgehog signaling pathway” and “Ribosome” were enriched in the high-risk group (Figure 7A). On the other hand, “Leishmania infection,” “Cytokine-cytokine receptor interaction,” “Natural killer cell mediated cytotoxicity,” “PPAR signaling pathway,” and “Systemic lupus erythematosus” were found enriched in a low-risk group in the model (Figure 7B). Beyond these pathways, we also observed a consistent enrichment of upstream immune recognition mechanisms with modest but significant scores (Table S2), such as “Antigen processing and presentation” (NES=-1.53), “Toll-like receptor signaling pathway” (NES=-1.46), and “B cell receptor signaling pathway” (NES=-1.49). Furthermore, the low-risk group was associated with metabolic and

stromal maintenance pathways, including “Glutathione metabolism” (NES=-1.44) and “Vascular smooth muscle contraction” (NES=-1.43). The collective enrichment of these diverse pathways ranging from immune surveillance to metabolic regulation – reinforces the multifaceted biological relevance of our signature beyond the primary immune response.

Assessment of the immune cell infiltration in OS

To further uncover the correlations between the pyroptosis-related risk signature and immune infiltration, we analyzed the immune score, stromal score, estimate score, and tumor purity between the two risk groups by the ESTIMATE algorithm. The results indicated there was a significant difference in terms of stromal score (Figure 8A), immune score (Figure 8B), ESTIMATE score (Figure 8C), and tumor purity (Figure 8D). In addition to calculating the relevant scores of the immune infiltration, it is also necessary to understand the proportion of the

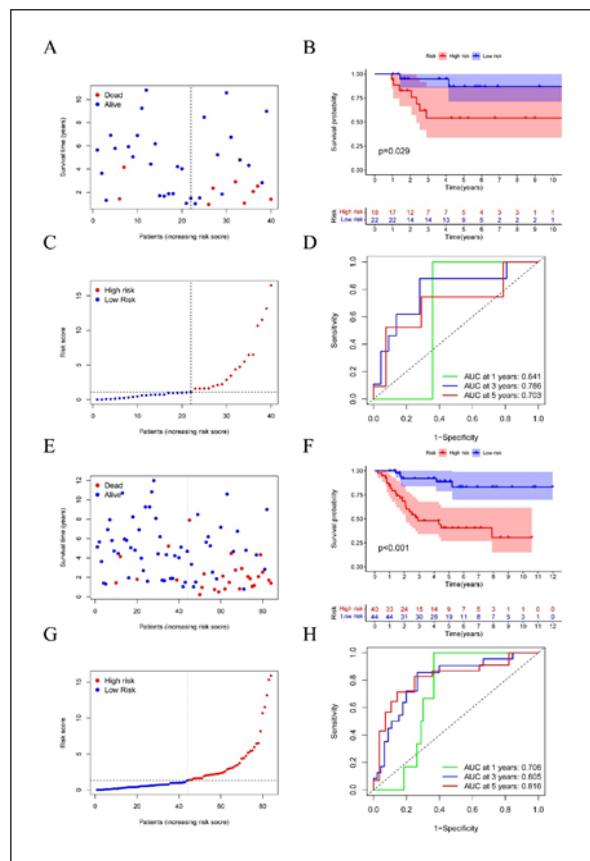


Figure 4. Validation of the Pyroptosis-related Signature in the Verification Cohort. A. The distributions of survival status, survival time, and risk score in the testing group; B. Kaplan–Meier survival curves for OS patients in the low-risk and high-risk group in the testing group; C. The distribution and median value of the risk scores in the testing group; D. The ROC curves demonstrated the predictive value of the risk score in the testing group; E. The distributions of survival status, survival time, and risk score in the entire group; F. Kaplan–Meier survival curves for OS patients in the low-risk and high-risk group in the entire group; G. The distribution and median value of the risk scores in the entire group; H. The ROC curves demonstrated the predictive value of the risk score in the entire group.

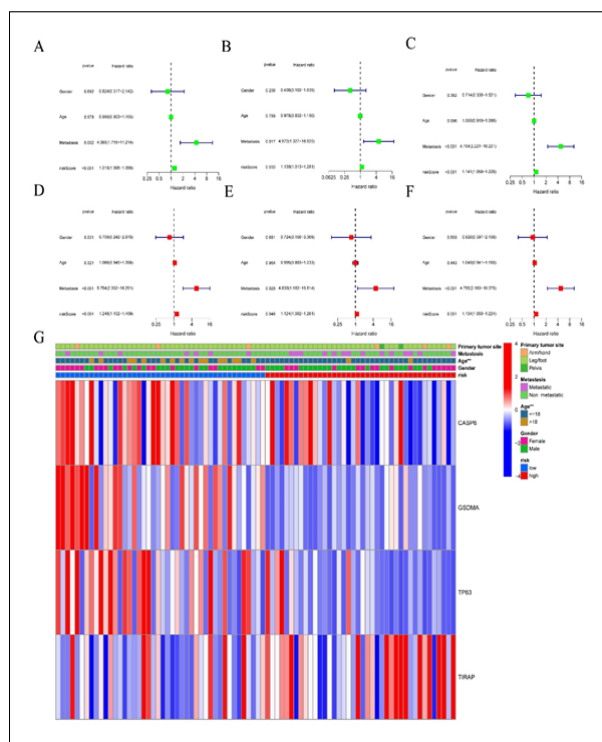


Figure 5. The Risk Score of Pyroptosis-related Signature was an Independent Prognostic Factor for OS Patients. A-C. The univariate Cox analysis results are based on the training group, testing group, and entire group, respectively; D-F. The multivariate Cox analysis results are based on the training group, testing group, and entire group, respectively; G. Heatmap for pyroptosis-related gene prognostic signature and clinicopathological manifestations (* $P < 0.05$).

immune landscape in the tumor. Thus, we employed the CIBERSORT algorithm to investigate the connection between risk score and tumor-infiltrating immune cell subsets. As presented in Figure 8E, Monocytes, Neutrophils, and Macrophages M0 were statistically significantly different between the two risk groups. Moreover, there was a significant correlation between risk scores and some immune cell subsets. Figure S2 demonstrated that the risk score was positively correlated with Macrophages M0, yet negatively correlated with T cells CD4 memory activated and Monocytes. The ssGSEA algorithm also analyzed the correlation between the risk score and the immune cell subpopulations and related functions in OS patients. The result revealed that nearly all immune cell subpopulations, related pathways, and functions were significantly reduced in the high-risk group compared with the low-risk group (Figure 8F). Only the scores for aDCs, HLA, iDCs, MHC_class_I, NK_cells, Th2_cells, and Treg did not differ significantly between the two subsets ($p > 0.05$). The heatmap of immune responses based on CIBERSORT, ESTIMATE, ssGSEA is shown in Figure 8G. Finally, we also explored the expression level of immune checkpoints in the low-risk and high-risk groups. The result showed that several immune-related genes exhibited a lower expression in the high-risk subgroup (Figure S3).

Discussion

OS is the most common primary malignant bone tumor with a high metastatic and fatal rate in children and adolescents [38]. Currently, the standard clinical therapeutic strategies for OS include surgical resection, chemotherapy, radiotherapy, and neoadjuvant chemotherapy [39]. Nevertheless, owing to the features of easy metastasis and chemotherapy resistance of OS, the overall survival rate of patients with OS remains poor, especially for the patients with metastasis, recurrence, and chemoresistance [40]. Accordingly, an increased understanding of the molecular mechanism underlying OS carcinogenesis and progression is critical for early diagnosis and improving patient outcomes. To date, this pursuit has led to the identification of various prognostic signatures based on mechanisms such as ferroptosis, aging, cuproptosis, and hypoxia [39, 41–43]. While these models provide valuable risk stratification based on metabolic or senescent pathways, they often overlook the specific inflammatory implications of cell death. In contrast, our study focuses on pyroptosis, which is distinct from the iron-dependent lipid peroxidation of ferroptosis due to its inherently pro-inflammatory nature [12]. By triggering robust antitumor immunity, this pyroptosis-related signature offers a complementary and novel perspective to the existing metabolic-focused models in the OS microenvironment.

In the present study, we investigated the expression level of pyroptosis-related genes in OS and found that most pyroptosis-related genes were different expressed

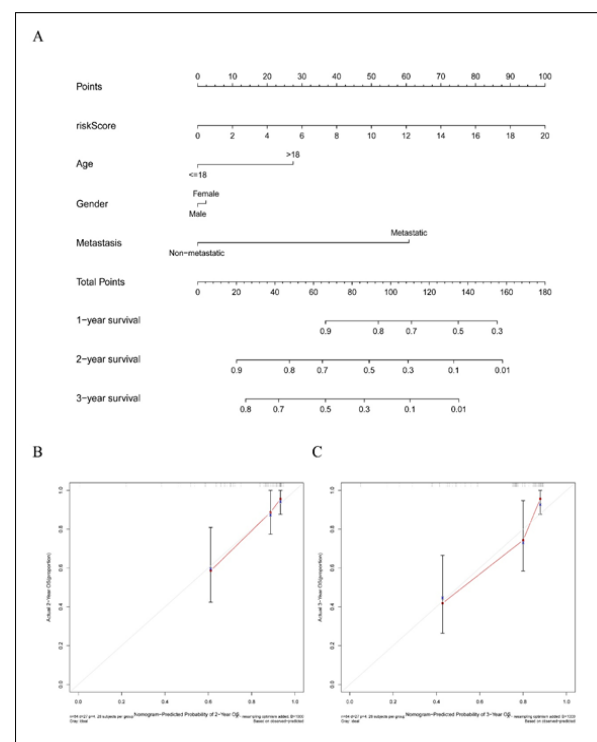


Figure 6. Construction and Calibration of a Nomogram. A. nomogram for both clinical characteristics factors and prognostic pyroptosis-related signature risk score. B. Calibration of the nomogram at 2-years and 5-years survival.

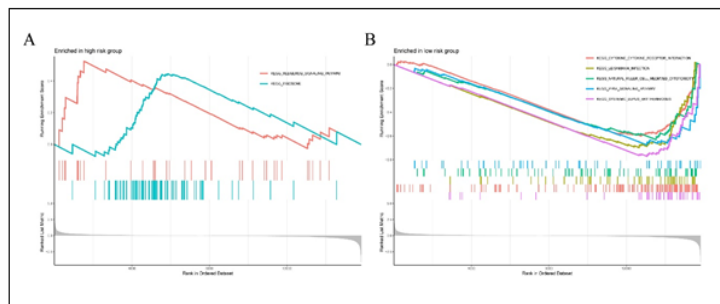


Figure 7. The GSEA Analysis Results between the High-risk Group and Low-risk Group. A. All enriched pathways in the high-risk group. B. Top 5 enriched pathways in the low-risk group.

between OS and normal tissues. Then, OS patients were divided into two clusters based on the pyroptosis-related genes. There was a significant difference between the 2 clusters in terms of survival prognosis and immunity status. Once more, to further explore the relationship between these pyroptosis-related genes and the patients with OS, the pyroptosis-related prognostic signature was constructed through univariate, LASSO, and multivariate Cox regression analysis. Its performance was also verified in different validation sets. Finally, the prognostic prediction efficiency of the novel signature was assessed by various approaches. For instance, the univariate and multivariate analyses results were confirmed that the pyroptosis signature was an independent prognostic factor of the overall survival of OS. The nomogram and calibration curves also confirmed the promising prediction efficiency of the novel model. Regarding clinical applicability, current osteosarcoma management primarily relies on staging systems where metastasis is often considered the dominant prognostic factor. However, our nomogram (Figure 6A) reveals a crucial insight: while the presence of metastasis contributes approximately 60 points to the total score, the pyroptosis-related risk score can contribute up to 100 points. This indicates that the biological heterogeneity captured by our signature can be a more potent driver of prognosis than traditional clinical staging in certain contexts. Consequently, a 'non-metastatic' patient with a high risk score could have a worse predicted survival than a 'metastatic' patient with a low risk score. This finding underscores the necessity of integrating molecular signatures into clinical practice to identify high-risk subgroups that might otherwise be undertreated based solely on their non-metastatic status.

The pyroptosis-related prognostic signature is comprised of 4 genes: CASP6, GSDMA, TP63, and TIRAP. Among them, CASP6 is an important regulator of innate immunity, host defense, and inflammasome activation[44]. Previous studies have proved that CASP6 can promote the activation of the PCD pathway, including apoptosis, pyroptosis, and necroptosis [45, 46]. Accumulating evidence indicates that CASP6 plays a vital role in carcinogenesis and progression [44]. In the present study, we found that CASP6 was one of the pyroptosis-related prognostic biomarkers in OS. Ying et al. demonstrated that CASP6 could improve the prognosis of ovarian cancer patients, which is consistent with our study findings [23]. Hence, this combined evidence

suggests CASP6 may serve as a prognostic protective factor in various cancer, but the specific role in cancer will require further exploration. GSDMA, full name gasdermin A, is one of the members of GSDM family, and it has been reported to play an important role in pyroptosis. The N-terminal domains of GSDMA exhibit pore-forming activity on cell membranes and destroy the plasma membrane of cells, which are executors for pyroptosis [47]. TP63, a paralog of TP53 and TP73, has been shown to regulate transcriptional programs related to cell differentiation, cell cycle, and apoptosis [48]. TP63 has various functionally distinct isoforms, one of which is $\Delta Np63$, and its overexpression is a clinical favorable

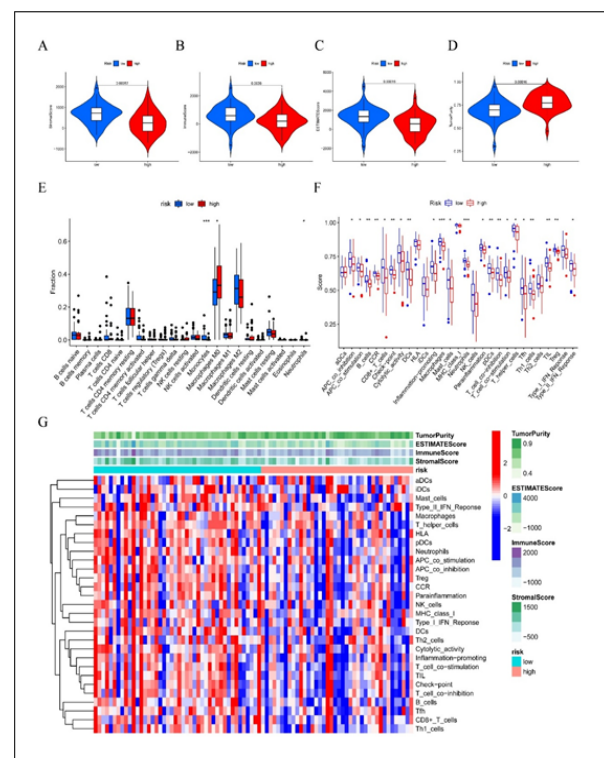


Figure 8. The Results of the Immune Infiltration. A-D. The difference ESTIMATE score between the high risk and low-risk groups in terms of Stromal score, Immune score, ESTIMATE score, Tumor purity; E. The CIBERSORT algorithm quantified 22 immune cells between the high-risk and low-risk groups; F. The ssGSEA for the enrichment scores of 16 types of immune cells and 13 immune-related functions between low-risk and high-risk groups; G. Heatmap for immune responses based ESTIMATE and ssGSEA among two risk subgroups.

prognostic factor for high-grade T1 bladder cancer [49]. TIRAP belongs to TLR and interleukin-1 receptor (IL-1R) families [50], which are involved in a variety of biological processes, including inflammation, cell proliferation, and apoptosis [51]. Many researchers have demonstrated that aberrant expression of TIRAP is closely related to the occurrence of multiple tumors, including lymphocytic leukemia, gastric cancer, breast cancer, and non-small cell lung cancer. Moreover, Dandan Xu et al. also reported that TIRAP was a risk factor for breast cancer prognoses, which was in line with our results [52]. Collectively, three genes (CASP6, GSDMA, TP63) in this prognostic signature were proved to be a protective factor for OS, and TIRAP as a prognostic risk factor for osteosarcoma. However, the underlying mechanism of the above genes' makeup signature impacts OS prognosis remains further investigated.

Subsequently, to further explore the relationship between the pyroptosis-related signature and OS. We conducted a GSEA analysis between low-risk and high-risk groups and found that the high-risk group mainly enriched in ribosome and hedgehog signaling pathways. A growing body of evidence has shown a vital role of the hedgehog signaling pathway in the progress of OS tumorigenesis and development [53-56]. For instance, Yi et al. indicated that MiR-212 promotes the proliferation and inhibits apoptosis of osteosarcoma cells by regulating the hedgehog signaling pathway [56]. In addition, the hedgehog signal inhibitors are expected to be used as anti-cancer drugs for osteosarcoma, which could improve the efficacy of osteosarcoma treatment and the prognosis of patients [54]. For the low-risk group, it significantly related to immune-related pathways, including cytokine-cytokine receptor interaction, natural killer cell-mediated cytotoxicity, systemic lupus erythematosus, TLR signaling pathway, B cell receptor signaling pathway, primary immunodeficiency, and antigen processing and presentation. These signal pathways played a fundamental role in the tumor immune microenvironment. It has been reported that TLRs are widely expressed in tumor cells, stromal cells and tumor-infiltrating immune cells [57]. The TLR signaling pathway participates in the regulation of tumor pathogenesis and leads to the activation of innate and adaptive immunity [57, 58]. The low-risk score group of pyroptosis-related signature in breast cancer was also significantly enriched in cytokine-cytokine receptor interaction [58]. Hence, based on the above GSEA analysis results, it is reasonable to speculate that the pyroptosis-related signature in OS might be attributed to its association with immune processes.

In the past few decades, the interaction between tumor microenvironment and immunity has attracted increasing attention [59]. As an important component of the tumor microenvironment, immune cells play a central role in tumor progression and therapeutic responses [60]. To further explore the association between the prognostic signature and infiltrated immune cells in OS. The ESTIMATE algorithms, CIBERSORT algorithm, and ssGSEA were conducted to study the immune infiltration of the tumor microenvironment in OS. Firstly,

the ESTIMATE algorithm showed that the stromal cell, ESTIMATE, and immune cell scores were lower in the high-risk group, revealing that immune cell infiltration was poor in the high-risk subgroup. These results were consistent with Lei et al., the high-risk group in Ferroptosis-related gene signature for OS also had lower immune infiltration scores and survival prognosis [39]. In addition, we found that the proportion of Monocytes in the low-risk group was higher than that in the high-risk group and was negatively correlated with the risk score through the CIBERSORT algorithm. It has also been previously reported that Monocytes could inhibit anti-tumor immunity and are associated with poor clinical outcomes [61, 62]. Similarly, ssGSEA results revealed that most immune cells showed suppressed immune function and infiltration in the high-risk subgroup. In line with the major impact of these immune cells in stimulating anti-tumor immunity, it is thus reasonable to conclude that the high-risk group has a more reduced anti-tumor immune response [63]. Finally, we also found that immune checkpoint molecules' expression was downregulated in the high-risk subgroup, confirming that immune status was poor in the high-risk subgroup. Taken together, it is entirely reasonable to believe that the poor survival status of high-risk OS patients may be caused by the reduced level of anti-tumor immunity status.

Despite the promising findings, our study has several limitations that must be acknowledged. First, this is a retrospective study relying on public databases, which inevitably introduces selection bias. The sample size of osteosarcoma patients from the TARGET database was relatively small ($n=88$), which may limit the statistical power and robustness of the analysis. Second, although we integrated normal tissue data from the GTEx database to serve as controls, potential batch effects between the TARGET and GTEx platforms could not be completely eliminated, which might influence the differential expression analysis. Third, while the signature showed good performance in internal validation, it lacks external validation in independent, multi-center clinical cohorts to confirm its generalizability. Finally, the functional roles of the four signature genes and their link to pyroptosis were inferred based on bioinformatics algorithms. We did not conduct in vitro or in vivo experiments to verify their specific biological mechanisms in osteosarcoma. Future prospective studies with larger sample sizes and experimental validation are warranted to address these issues.

In conclusion, a novel pyroptosis-related prognostic signature was constructed with high prediction efficiency. The signature can be used as an independent prognostic index for OS. Furthermore, the poor prognosis of OS with a high-risk score may be related to the tumor immune microenvironment regulation. Hence, we have adequate reason to believe that this study could provide novel insights into exploring molecular mechanisms and the prognosis prediction for OS.

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Statement of Transparency and Principles

- Author declares no conflict of interest
- Study was approved by Research Ethic Committee of author affiliated Institute.
- Study's data is available upon a reasonable request.
- All authors have contributed to implementation of this research.

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