Pyroptosis-Related IncRNA Signatures Predict Prognosis and Indicate Immune Microenvironment Infiltration in Hepatocellular Carcinoma

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Abstract

Background. Hepatocellular carcinoma (HCC) remains a major cause of cancer-related deaths worldwide, with limited treatment options. Long noncoding RNAs (IncRNAs) are essential regulators of HCC progression and are closely related to the pyroptotic cell death. However, the influence of pyroptosis-related IncRNAs on HCC remains indefinite.

Methods. We systematically explored the expression profile of pyroptosis-related IncRNAs to establish a novel signature for predicting the outcome of HCC patients based on The Cancer Genome Atlas (TCGA) database by multiple analysis methods. Four IncRNAs with the highest hazard ratio in the above model were selected for external verification in the Gene Expression Omnibus (GEO) database. Finally, the expression of these IncRNAs was verified by real-time quantitative polymerase chain reaction (RT-qPCR) in different cell lines.

Results. The results showed that 25 pyroptosis-related IncRNAs were evidently correlated with the prognosis of HCC patients. Cox regression analyses demonstrated the prognostic ability of the risk model established by the pyroptosis-related IncRNAs. And the high and low-risk groups were linked to different types of infiltrating immune cells and affected the physiological state of the tumor microenvironment in HCC.

Conclusion. We constructed and validated an independent pyroptosis-related IncRNAs prognostic model and preliminarily described the potential immune correlations, providing novel prognostic factors and directions of immunotherapeutic strategies for HCC.

Introduction

As the major cause of tumor-related deaths worldwide, liver cancer has contributed to 700,000 deaths recorded globally every year [1]. In all cases of liver cancer, 75–85% of them are hepatocellular carcinoma (HCC) patients. There are many risk factors that lead to HCC, from long-term obesity, alcoholism, hepatitis virus infection to various metabolic diseases [1–3]. The incipient clinical features of HCC are insidious and lack specificity. Meanwhile, owing to the high rate of metastasis, the overall prognosis of HCC remains limited, and early monitoring methods need to be improved. Although tumor removal, liver transplantation, interventional therapy, and other therapies can effectively improve the prognosis of HCC, a considerable number of patients would miss the best period of treatment [4]. Therefore, it is critical to identify promising novel biomarkers for better prognosis evaluation and effective therapy guidance for HCC.

Pyroptosis has been gradually emerging as a novel form of programmed cell death, which could trigger a momentous natural immune response in the human body [5, 6]. Pyroptosis leads to cell and organelle swelling, membrane lysis and DNA cleavage, followed by the release of pro-inflammatory compounds, inducing a local or systemic inflammatory response [5]. Many studies have confirmed that pyroptosis is
intimately related to the occurrence and development of multiple cancers like liver cancer, gastric cancer and breast cancer\textsuperscript{[7–9]}. Xia \textit{et al.} pointed out that abnormal expression of pyroptosis-related genes is related to metastasis, invasion, drug resistance and mediates cell death in cutaneous melanoma\textsuperscript{[10]}. Wang \textit{et al.} suggested that some lncRNAs would be able to inhibit gastric cancer progression via pyroptosis\textsuperscript{[11]}. Studies on the relationship between pyroptosis and cancer have provided new directions for cancer prevention and treatment. However, whether pyroptosis is correlated with the prognosis of HCC patients remains unclear.

LncRNAs are approximately 200 nucleotides in length and are capable of transcriptional regulation, mRNA processing and mRNA post-transcriptional control, without protein-coding\textsuperscript{[12–14]}. In addition to gene regulation, lncRNAs are also inextricably linked with a variety of biological processes like proliferation, transformation and metastasis of tumor cells\textsuperscript{[15]}. In recent years, some studies have proved that pyroptosis-related lncRNAs can become potential prognostic markers or even therapeutic targets in cancer treatment strategies. Deng \textit{et al.} found that lncRNA MEG3 could accelerate the process of renal tubular epithelial pyroptosis through miR-18a-3p/GSDMD pathway in acute kidney injury (AKI)\textsuperscript{[16]}. Moreover, Lin \textit{et al.} discovered that several lncRNAs fundamentally affect the development and changes of papillary thyroid carcinoma cells by adsorbing microRNAs and regulating the PI3K/Akt and Wnt signaling pathways\textsuperscript{[17]}. However, even though the dysfunction of pyroptosis-related lncRNAs is closely related to the tumor phenotype, few studies have paid attention to them and their relationships with the prognosis of HCC patients.

In order to identify the mechanism of pyroptosis-related lncRNAs in HCC, we first used Cox regression analysis to set up the signature of pyroptosis-related lncRNAs and construct the corresponding prognostic model. Accordingly, HCC patients were divided into low-risk group and high-risk group based on the risk score model. Then the accuracy of the prognostic model was further verified using ROC analysis. Furthermore, we explored the relationship between pyroptosis-related lncRNAs and the HCC immune microenvironment in two groups, which guides the future immunotherapy strategy of HCC. Significantly, we used the external database and RT-qPCR to verify the expression difference between tumors and non-tumors of lncRNAs in the prediction model. In brief, our prediction model provides new prognostic information and a novel method for promoting individualized immunotherapy in patients with HCC.

Materials And Methods

2.1 Data resources

RNA sequencing and dependent clinical data in this study were downloaded from the TCGA-LIHC database (https://portal.gdc.cancer.gov/repository). Firstly, we picked up 33 pyroptosis-related genes through a systematic analysis of previous relevant reviews\textsuperscript{[10,12,18–20]}. Secondly, the “limma” software package in R was performed to distinguish the differentially expressed genes (DEGs) between ordinary
tissue and cancer tissue. Then, correlation coefficient $R > 0.4$ and $p < 0.001$ were used as the judging criteria to screen the pyroptosis-related lncRNAs. FDR $< 0.05$ and $|\log_{2}FC| \geq 1$ were employed to identify the different expression pyroptosis-related lncRNAs.

### 2.2 Construction of prognostic pyroptosis-related lncRNAs signature

We assessed the correlations between pyroptosis-related lncRNAs and overall survival status by univariate Cox regression analysis. Then a hazard ratio (HR) was utilized to categorize those lncRNAs (HR $> 1$ means “risk” and HR $< 1$ means “protective”), and 25 survival-related genes were identified for further analysis. Furthermore, the multivariate Cox regression analysis was used to set up the best prognostic model. Finally, 7 optimal pyroptosis-related lncRNAs were chosen to build the prognostic model. The risk score model was calculated by the level of lncRNA expression and the corresponding formula as follows:

$$RiskScore = \sum_{i=1}^{n} (\beta_{lncRNAi} \times Expression_{lncRNAi})$$

### 2.3 Evaluate prediction ability of the risk scoring model

Based on the previous risk scoring model, we divided HCC patients into high-risk and low-risk groups according to the median risk score and visualized the results using the “pheatmap” R software package. Then we employed the “survival and survminer” R packages to appraise the prognosis between these two groups. To evaluate the specificity and sensitivity of the risk score model, we used the analysis methods of multifactor ROC and time-dependent ROC curves. In consideration of other clinicopathological features, univariate and multivariate Cox regression analyses were utilized to assess the effectiveness and advantage of the established model.

### 2.4 Estimation of the immune cell infiltration

The evaluation of infiltrated immunocytes was mainly analyzed in this way: the immune cell subsets with tumor infiltration and immunological functioning were quantified by single sample gene enrichment analysis(ssGSEA) in two different risk groups.

### 2.5 Analysis and verification of GEO database

HCC related microarray datasets, GSE121248, GSE36376, and GSE76427, were obtained from GEO (https://www.ncbi.nlm.nih.gov/geo/) as an external validation set[21,22]. The expression of lncRNAs were visualized by using “ggplot2” R package.

### 2.6 Cell lines and culture

The human HCC cell lines HCC-LM3, Huh7, MHC-97H, SMMC-7721, and human normal liver cell line LO2 were cryopreserved and passaged in our laboratory. These cell lines were incubated in a Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine
serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/ml penicillin G and 100 µg/ml streptomycin (Beyotime, China). All of the cell lines were grown in a humidified incubator containing 5% CO₂ at 37°C.

2.7 RNA Isolation and RT-qPCR analysis

Total RNA was extracted from indicated normal and tumor cells by AG RNAex Pro Reagent (Accurate Biology, China) comply with the manufacturer's product manual, and was quantified titer by Nanodrop one (Thermo Fisher). Total RNA was then reverse transcribed to cDNA using Evo M-MLV RT Reaction Mix (Accurate Biology, China). The expression of LncRNA was measured by Quantitative PCR using the Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific). β-actin served as the housekeeping gene. The primers was presented in Table S1. The Fold changes in relative expression level were calculated by 2^−ΔΔCt method.

2.8 Statistical analysis

In this article, all clinical and molecular data were analyzed by the R software package. For comparisons, the data from diverse groups were evaluated via the Wilcoxon test and unpaired Student's t-test. And the threshold of statistical significance was set as P < 0.05. The Kaplan Meier curve was utilized to compare the OS of patients in the low-risk group and high-risk group. Univariate and multivariate Cox regression analyses were utilized to extract the independent elements correlated with overall survival data. RT-qPCR data were analyzed using GraphPad Prism 9.0. p < 0.05 was identified as statistically significant.

Result

3.1 Functional enrichment analysis

The DEGs of 33 pyroptosis-related genes were subjected to GO and KEGG enrichment analyses. GO analysis suggested that the DEGs were primarily in relation to the defense feedback to bacteria, positive regulation of cytokine production and IL-1β production, and pyroptosis in the biological processes (BP) section. Among the molecular functions (MF), the DEGs were concentrated in phosphatidylinositol 4,5 bisphosphate (PIP2), cytokine receptor binding and phosphatidylinositol bisphosphate binding. We also found that the DEGs were concentrated in the inflammasome complex in the cellular component (CC) category (Fig. 1A). Besides, KEGG analysis represented that the DEGs were mostly concentrated in the NOD-like receptor signal transduction pathway, lipid metabolism and atherosclerosis, salmonella infection, legionellosis and pertussis (Fig. 1B).

3.2 Screening of prognostic pyroptosis-related IncRNAs and risk score model construction

To evaluate the clinical prognosis of pyroptosis-related IncRNAs, the univariate Cox regression analysis was conducted. 25 prognostic IncRNAs related to pyroptosis were discovered that markedly correlated to the survival of HCC patients (Fig. 2A). Next, seven IncRNAs (AC099850.3, MKLN1-AS, AC091057.1, NRAV,
SNHG4, AP001453.2, and LINC00205) were screened to build the prognostic model, and the patients were separated into two different risk groups by the median risk score. Specifically, the formula was “Risk score = AC099850.3 * 0.12 + MKLN1-AS * 0.80 + AC091057.1 * −1.05 + NRAV * 0.13 + SNHG4 * 0.27 + AP001453.2* -0.52 + LINC00205 * 0.20”. Figure 2B indicates the predictive ability of our structured pyroptosis-related lncRNA prognostic model used to predict OS in HCC patients. Considerably, the low-risk group in the OS of patients was longer than that in the high-risk group (p < 0.01). The distributions and survival situations of HCC patients with distinctive risks are demonstrated in Fig. 2C-D. According to the heatmap (Fig. 2E), six pyroptosis-related lncRNAs were positively associated with the risk model, whereas one was negatively correlated.

3.3 Prognostic value of the risk score model

Then the Cox regression analyses were utilized to demonstrate the prognostic ability of the risk model. The outcomes showed the risk score model is able to be regarded as a survival predictor of HCC by univariate Cox regression (CI: 1.198–1.351; HR: 1.272, 95%) and multivariate Cox regression (CI: 1.159–1.322; HR: 1.238, 95%) (Fig. 3A). In addition, there is notable independence and predictive ability for the risk score, with an area under the ROC curve (AUC) of 0.748 (Fig. 3B). In addition, time-dependent ROCs were generated, and the AUC of 1-year, 2-year and 3-year survival prediction risk scores were all more than 0.6, indicating that the prediction accuracy of the risk score model for the prognosis of HCC was stable (Fig. 3C). And in order to predict the potentiality of clinical OS of HCC patients, a prognostic nomograph model was built by employing risk scores (Fig. 3D).

3.4 Evaluation of the relationship between clinicopathological characteristics and pyroptosis-related lncRNAs signatures

In order to further clarify the statistical relationship between the predictive characteristics of lncRNAs and clinicopathological indications, we used the heat map to characterize the related changes between them (Fig. 4A). The high-risk group was linked to a higher grade (p < 0.001) and stage (p < 0.001) of HCC. Then the GSEA was utilized to investigate the physiological process and signal transduction pathways linked to the pyroptosis-related lncRNAs between two different risk groups. The GSEA results demonstrated that several biological pathways were concentrated in the high-risk group, including ubiquitin-mediated proteolysis, endocytosis, oocyte meiosis, RNA degradation and purine metabolism (Fig. 4B). However, primary bile acid biosynthesis, fatty acid metabolism, retinol metabolism, tryptophan metabolism, glycine and threonine metabolism, and complement and coagulation pathways were concentrated in the low-risk group (Fig. 4C).

3.5 Distinct immune landscapes between high and low-risk HCC patients
The heat map of immune responses was based on seven different algorithms: CIBERSORT, TIMER algorithms, CIBERSORT-ABS, MCP counter, XCELL, QUANTISEQ and EPIC (Fig. 5A). According to the ssGSEA analysis of the TCGA-HCC database, it can be found that the existence of multiple immune cell subsets was closely related to immune functions and related activities. ssGSEA results also showed that there were dramatic differences in cytolytic activity and MHC class I and type II IFN responses between high-risk and low-risk groups (Fig. 5B). Since the immune checkpoint inhibitors based immunotherapy is the spotlight of cancer immunotherapy, we further explored the expression level of immune checkpoints between the two risk groups. We discovered significant variations in the most of expression levels of immune checkpoints like NRP1, LGALS9, and CD276 (Fig. 5C). In addition, we inspected the expression levels of m6A-related genes between the high-risk and low-risk groups. The outcomes clarified that the expression levels of HNRNPC, YTHDC1, YTHDF2, ALKBH5, METTL3, YTHDF1, WTAP, FTO, YTHDC2, and RBM15 in the high-risk group were higher compared to the low-risk group (Fig. 5D).

3.6 External validation for expression differences of pyroptosis-related lncRNAs

Then, we further confirmed the differences of pyroptosis-related lncRNAs with higher hazard ratio in the risk model. Firstly, these lncRNAs were selected to explore their expression in the GEO microarray of HCC. The results showed that the expression levels of SNHG4 and LINC00205 were higher in tumors than in adjacent tissues (Fig. 6A-F). Furthermore, we explored the expression of higher HRlncRNAs (NRAV, SNHG4, MKLN1-AS, LINC00205) in normal and cancer cell lines by RT-qPCR analysis\cite{23–26}. As expected, the experimental results were consistent with the results of GEO microarray results. That is, pyroptosis-related lncRNAs are expressed higher in tumor cell lines (Fig. 6G-J).

Discussion

Recent studies have demonstrated that anti-tumor therapies can be improved by regulating processes of cell death, such as pyroptosis\cite{27}. As a newly recognized type of non-apoptotic cell death, pyroptosis has been described as a double-edged sword in cancer progression and effective therapy. Normal cells would turn into cancer cells due to the stimulation of an enormous number of inflammatory cytokines released by pyrolytic cells \cite{28}. In contrast, promoting pyroptosis in tumor cells is becoming popular as an innovative therapy for cancers\cite{29}. For instance, Xie et al. discovered that DNA demethylation combined with chemotherapy to initiate pyroptosis can be used as a feasible treatment for lung cancer\cite{30}. Further, Ding et al. proposed biodegradable K\(_3\)ZrF\(_7\):Yb/Er upconversion nanoparticles can act as a pyroptosis activator for tumor immunotherapy\cite{31}. As some important components controlling cell growth and differentiation, the role of lncRNAs in maintaining various cellular activities, especially in programmed cell death, has been gradually clarified. For example, Tan et al. discovered that lncRNA HOTTIP could target the miR-148a-3p/AKT2 axis to inhibit pyroptosis in ovarian cancer\cite{32}. Liu S et al. reported that lncRNA H19 alleviates pyroptosis of raw 264.7 cells by oxidized low-density lipoprotein \cite{33}. Therefore,
lncRNAs are involved in cellular pathways, such as pyroptosis, that affect tumorigenesis and cancer progression. Thus, pyroptosis and its related genes are expected to become promising biomarkers or therapeutic targets for cancer diagnosis and treatment.

In this study, we performed the correlation analysis and screened 25 pyroptosis-related lncRNAs related to patient prognosis. Then we constructed a novel independent prognostic model containing seven pyroptosis-related lncRNAs. This model included seven risk lncRNA, namely: AC099850.3, MKLN1-AS, AC091057.1, NRAV, SNHG4, AP001453.2 and LINC00205. Previous studies showed that AC099850.3 was interrelated to the prognosis of non-small cell lung cancer (NSCLC)\(^{34}\). AC091057.1 can be used to construct a prognostic method for lung adenocarcinoma\(^{35}\). Cheng et al. found that LINC00205, as a YY1-modulated lncRNA, could promote the proliferation of HCC cells by improving the level of CDK6\(^{36}\). These studies fully confirm the reliability of constructing prognostic models based on these lncRNAs.

As a method of calculating whether the set of specified genes indicates a significant difference between two biological conditions, GSEA not only tests the expression-level changes of a single gene but also includes the changes through a cluster of genes to obtain more ideal analysis results. In our study, the GSEA analysis showed that ubiquitin-mediated proteolysis and purine metabolism were active in the high-risk group, and fat metabolism was active in the low-risk group. Ubiquitin-mediated proteolysis carried out by the action of E3 ubiquitin ligases, regulates a series of cellular processes, including gene transcription, cell autophagy and apoptosis\(^{37}\). In another study, the authors proposed that targeted inhibition of purine metabolism can effectively inhibit HCC progression\(^{38}\).

The application of novel chemotherapeutic drugs has greatly improved the prognosis of patients with advanced liver cancer; however, drug resistance attenuated the therapeutic benefits. Fortunately, the emergence of tumor immunotherapy is promising for the treatment of patients with HCC. Therefore, many studies have focused on improving anti-tumor immunity to achieve anti-cancer effects. For example, a previous study demonstrated that the metastatic ATX-LPAR axis provides new therapeutic opportunities by inhibiting CD8 T cell infiltration to hinder anti-tumor immunity\(^{39}\). Zhao et al. reported that pyroptosis-associated B-type Natriuretic Peptide is a new immunotherapy strategy for solid tumors with high consistency and expansive clinical application value\(^{40}\). By combining decitabine and chemotherapy to trigger pyroptosis of tumor cells, Fan et al. evidently improve the antitumor immunity\(^{41}–^{42}\). According to the GSEA of the TCGA-LIHC dataset, the existence of various immunocyte subsets was related to immune function and related activities. Significant differences in cytolytic activity and MHC class I and type II IFN responses were shown between the two groups. We also explored the underlying connections between pyroptosis-related lncRNA signatures and immune status through various algorithms, the levels of immune cells showed similar changes. Through further analyses of immune function, as expected, we observed that the expression of cytolytic activity and type II IFN were downregulated in the high-risk group. A relatively immunosuppressive microenvironment status can expound on why the high-risk group displays a poor prognosis. In addition, genes associated with
immune checkpoints, comprising NRP1, LGALS9 and CD276, were upregulated in the high-risk group. Overall, the poor outcomes of high-risk patients with HCC may be due to limited anti-tumor immunity.

N6-methyladenosine (m6A) is an emerging type of RNA modification discovered in recent years\footnote{43}. As one of the most significant gene modification methods in tumorigenesis, m6A can promote the self-renewal of cancer stem cells and tumor cell proliferation. An enormous amount of studies have shown that m6A plays a key role in immune responses. For example, Li et al. indicated that m6A has a regulatory role in modifying the homeostasis and differentiation of naive T cells, suggesting that m6A can be a potential target for anti-cancer immunotherapy\footnote{44}. m6A is also regulated by lncRNAs and is considered to be necessary for the metabolic function \footnote{45}. Therefore, based on the positive results for immune function in our dataset, we further analyzed the differences in m6A. Our study found that the expression of m6A-related genes including HNRNPC, ALKBH5, YTHDC1, YTHDC2, YTHDF1, YTHDF2, WTAP, METTL3, FTO and RBM15 were higher in the high-risk group. The results reveal that the risk model is closely related to the TME, which provides promising directions for HCC prognosis and immunotherapy.

In conclusion, we constructed an independent prognostic model and preliminarily described the potential immune correlations. The study indicated novel genetic markers for forecasting the prognosis of HCC and developed an essential foundation for subsequent research to elucidate the relationship between pyroptosis-related lncRNA and immunity in HCC.

However, several limitations need to be addressed. First, although the analysis of immunocyte infiltration indicated that there are divergences in the lncRNAs level between patients with different risks, the deep mechanism that pyroptosis-related lncRNAs affect the immune infiltration needs further explanation and should be verified in more detail. Moreover, considering that there are many factors affecting TME\footnote{46}, the regulatory relationship between differential pyroptosis-related lncRNAs screened in this prognostic model and HCC needs to be further verified in future experiments.

**Declarations**

**Acknowledgments**

Not applicable.

**Declaration of interest**

The authors declare that there is no conflict of interest.

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Authors’ contributions

Xiangmin Tong, Gongqiang Wu and Ying Wang conceived and designed the experiments. Jiayu Hu, Chen Yuan, Huanjuan Li performed the experiments. Chen Yang, Junyu Zhou, Lusheng Tang, Yanhua Yu, Yinghao Zhang analyzed the data. Yanchun Li and Jiayu Hu wrote the paper. Xiangmin Tong and Ying Wang revised and finalized the manuscript.

References

Figure 1

The functional enrichment analysis of differential expression pyroptosis-related genes in HCC. (A) The enrichment of the GO analysis. (B) The enrichment of the KEGG analysis. The size of bubbles circular
rings equates to the number of enriched genes. MF, molecular function; BP, biological process; PRG, pyroptosis-related gene; CC, cellular component.

Figure 2
Development of prognostic pyroptosis-related lncRNA signature. (A) the HR (95% CI) and \( p \)-value of 25 pyroptosis-related lncRNAs were shown in the forest diagram. (B) Overall survival curves for the two
pyroptosis-related clusters based on TCGA-LIHC database. (C-D) The distribution of samples based on risk scores. (E) The heatmap of 7 differently expressed pyroptosis-associated IncRNAs between the two distinct risk groups.

Figure 3
The prognostic significance of the risk score model. (A) Univariate and multivariate Cox regression analyses for OS. (B) The AUC values of the risk factors and clinicopathological features. (C) The risk score model is assessed using time-dependent ROC. (D) A nomogram including both clinic-pathological features and survival-related IncRNAs.
The Evaluation of the Risk Score Model. (A) The heatmap covered the clinicopathological features and pyroptosis-related IncRNA prognostic signature among the HCC patients. (B-C) Results of GSEA between the high-risk group and low-risk group based on the pyroptosis-related IncRNAs prognostic features.

**Figure 5**

The distinction of immune conditions between low-risk and high-risk groups.
Figure 6

External validation of pyroptosis-related lncRNA prognostic model by GEO and RT-qPCR.
(A-F) Expression levels of SNHHG4 and LINC00205 in GEO microarray between liver normal and tumor. 
(G-H) IncRNAs, NRAV and SNHG4, were differentially expressed in Huh7 and LO2 cell lines, respectively. 
(I-J) IncRNAs, MKLN1-AS and LINC00205, were differentially expressed in SMMC-7721 and LO2 cell lines, 
respectively. (*p < 0.05, **p < 0.01).

**Supplementary Files**

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