Identification and Validation of a Necroptosis-related Gene based Prognostic Signature for Patients with Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide, nevertheless, its prognostic prediction remains obscure. Necroptosis is a tightly regulated form of cell death, which has been recently reported to be involved in HCC. However, the prognostic value of necroptosis-related genes (NRGs) in HCC has not been reported. In the present study, 159 NRGs of HCC subjects from the Cancer Genome Atlas (TCGA) cohort were downloaded and investigated. Of which, a total of thirty-six NRGs were identified to be significantly differentially expressed. Through univariate and multivariate Cox proportional regression analysis, four NRGs (HSP90AA1, PPIA, SQSTM1 and USP21) were found to have the excellent prognostic capacity for HCC, which could clearly divide patients into high-risk and low-risk groups. Survival analysis showed that the overall survival of high-risk patients was significantly shorter than that of low-risk patients. The independent prognostic value of necroptosis-related features was further confirmed by Cox regression analysis, and the area under the receiver operating characteristic curve of the panel comprising HSP90AA1, PPIA, SQSTM1 and USP21 was 0.807. Furthermore, the prognostic value of these NRGs was verified in another cohort of HCC patients from the ICGC database. Herein, this study shows that a novel necroptosis-related gene signature could be developed for prognostic prediction of HCC in future, which may also provide insights into the prevention and treatment of this disease clinically.

Introduction

Hepatocellular carcinoma (HCC) is globally one of the leading causes of cancer related death (Yang and Heimbach 2020). 90% of HCC cases originate from cirrhosis, during which hepatocytes undergo a chronic cycle of necrosis and regeneration (Kim and Viatour 2020). The treatment of HCC has greatly developed in recent years, unfortunately, the prognosis prediction seems stagnated (Siegel et al. 2018). The current prediction criteria for HCC prognosis have several disadvantages, such as aggressiveness. Therefore, it is highly necessary to establish an effective prognostic model based on the molecular heterogeneity of HCC (Forner et al. 2018; Agarwal et al. 2017).

Necroptosis is a novel form of cell death, which is mainly mediated by the RIPK1-RIPK3-MLKL signal axis (Christofferson and Yuan 2010). Accumulating evidence has suggested that necroptosis plays a key role in regulating cancer biology, including tumorigenesis, cancer metastasis, cancer immunity, and cancer subtypes (Gong et al. 2019; Seehawer et al. 2018). According to previous reports, necroptosis could serve as an alternative mode of programmed cell death to overcome apoptosis resistance, which may trigger and enhance antitumor immunity in cancer therapy (Gong et al. 2019). Increasing studies have suggested that necroptosis regulators could be used as biomarkers for the prognosis prediction of cancer (Park et al. 2020; Zhang et al. 2018). In a recent study, low-level expression of necroptosis factors was demonstrated to indicate poor prognosis in the non-small cell lung squamous cell carcinoma subtype (Lim et al. 2021). Recent studies have confirmed that necroptosis related genes can play a potential prognostic role in HCC (Nicole L et al. 2022). However, whether the necroptosis-related genes (NRGs) can be developed as biomarkers for predicting the HCC prognosis remains to be investigated.
In this study, we systematically analyzed the differentially expressed NRGs in HCC in TCGA database, and the differentially expressed NRGs that were significantly associated with OS were selected to construct a prognosis related NRGs signature. According to the cutoff value of risk score, HCC patients can be divided into high-risk and low-risk groups. Finally, we explored the prognostic role of the risk score in the TCGA database and in a validation dataset from the ICGC database. Collectively, this study may suggest a novel NRGs based biomarkers for the prognosis prediction of HCC clinically. The study design workflow is presented in Fig. 1.

Materials And Methods

Human necroptosis-related genes (NRGs) set

Based on the Kyoto Encyclopedia of genes and genomes (KEGG) database (https://www.kegg.jp/), A total of 159 genes were involved in the necroptosis signal pathway (Supplementary Table1).

Data retrieval

From the TCGA database (https://portal.gdc.cancer.gov/), RNA sequencing (RNA-seq) data and corresponding clinical information of 374 HCC patients were extracted. As a validation cohort, RNA-seq and clinical parameters of 231 HCC patients were downloaded from the ICGC database (https://dcc.icgc.org/projects/LIRI-JP). The correlation between the NRGs and the survival of patients was established by univariate cox regression analysis.

Bioinformatics analysis

A Consensus clustering analysis and a principal components analysis were carried out by the R language (version 4.1) to verify the regulatory role of necroptosis in HCC. The R package limma was used to screen the differentially expressed NRGs. Univariate Cox proportional hazard regression analysis was used to evaluate the association between total survival time (OS) and gene expression values. The relationship between independent factors and risk characteristics were determined by the multivariate Cox proportional hazards regression analyses. The regression coefficient was calculated by the Cox regression model.

Construction of prognostic model based on NRGs

Multivariate cox regression was used to construct prognostic genes. After combining the expression value of each specific gene, the risk score formula of each patient was constructed and weighted by its estimated regression coefficient in multivariate cox regression analysis. According to the risk scoring formula, taking the median risk score as the dividing point, the patients were divided into low-risk and high-risk groups. Survival differences between the two groups were assessed by Kaplan-Meier method and compared using log-rank statistical methods. Multivariate Cox regression analysis and stratified analysis were used to test the role of risk score in predicting the prognosis of patients. The area under the ROC curve was calculated for each dataset to measure the prognostic role of the model.
**Statistical analysis**

Survival curves were created by the Kaplan-Meier method and compared by log-rank test. Univariate and multivariate analysis using cox proportional hazard model. All statistical analyses were performed using the R language. All statistical tests with p<0.05 were considered significant.

**Results**

**Identification of differential NRGs**

RNA-seq and clinical data from 374 HCC and 50 healthy controls were downloaded from TCGA database. Expression values of 159 NRGs from HCC patients were extracted and compared. Of which, two genes were downregulated in the subjects with HCC such as CAMK2G and IL1B, meanwhile, thirty-four ones were upregulated, including USP21, HSP90AA1,PPIA,SQSTM1 et al (Fig. 2a, c). Heatmap revealed the expression patterns of these differentially expressed genes (Fig. 2b).

**Functional enrichment analysis**

The functions of the thirty-six differential genes were investigated by pathway enrichment analysis at the GO and KEGG levels. At the biological process of GO, these genes were involved in I-κB kinase/NF-κB and cytokine-mediated signaling pathway. At the cellular components, secretory granule lumen, cytoplasmic vesicle lumen and vesicle lumen were enriched. At the molecular function of GO, ubiquitin protein ligase binding and ubiquitin-like protein ligase binding were changed (Fig. 3a). Subsequently, in KEGG pathway enrichment analysis, these genes were demonstrated to be significantly associated with Necroptosis and NOD – like receptor signaling pathway (Fig. 3b). Most enriched pathways had z-scores greater than zero, indicating that most pathways were likely enriched (Supplementary Figure S1).

**Identification of prognosis-related NRGs and construction of prognosis prediction model**

A univariate Cox regression approach was first employed to identify prognostically relevant NRGs. Prognostic NRGs that were statistically significant in univariate analysis were selected for inclusion in subsequent multivariate analysis. The results of multivariate analysis showed that a total of four genes were significantly associated with prognosis. A prognostic model was constructed based on the expression coefficients of each independent risk gene derived from the results of multivariate Cox analysis (Table 1. and Fig. 4a). The results showed the risk score of patients, in which below the median are low risk group (n = 172) while above the median are high risk group (n = 171). (Fig. 4b). The scatter plot represents alive in blue and dead in red, showing the mortality rate increased with the increase of patient risk (Fig. 4c). The K-M survival curve showed the survival status of low risk group in blue and high risk group in red, which indicated the predicted survival time of low-risk group was significantly longer than that of high-risk group (Fig. 4d, P < 0.001). We used the ROC curve for OS to assess the ability of the risk score to predict survival. The AUC value of the prognostic model was 0.807, which was significantly higher than that associated with age, gender, grade, stage, T stage, M stage, and N stage (Fig. 4e). These
results suggest that the risk signature has a better ability to predict the survival of HCC patients than these clinical factors.

### Table 1
Multivariate Cox regression analysis of necroptosis-related genes that can significantly affect OS.

<table>
<thead>
<tr>
<th>id</th>
<th>coef</th>
<th>HR</th>
<th>HR.95L</th>
<th>HR.95H</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP90AA1</td>
<td>0.0029</td>
<td>1.0029</td>
<td>1.0006</td>
<td>1.0053</td>
<td>0.0121</td>
</tr>
<tr>
<td>PPIA</td>
<td>0.0086</td>
<td>1.0087</td>
<td>1.0028</td>
<td>1.0146</td>
<td>0.0038</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>0.0025</td>
<td>1.0025</td>
<td>1.0013</td>
<td>1.0037</td>
<td>0.0001</td>
</tr>
<tr>
<td>USP21</td>
<td>0.0975</td>
<td>1.1024</td>
<td>1.0318</td>
<td>1.1779</td>
<td>0.00398</td>
</tr>
</tbody>
</table>

**Abbreviations:** HR, Hazardous Ratio.

The relationships between clinicopathological parameters and prognosis-related NRGs

Clinicopathological analysis to explore the association between clinical parameters and risk signature. The results showed that the signature was correlated with tumor stage (P = 0.002), T stage (P = 0.002), M stage (P = 0.014) and survival outcome (P = 0.007) (Fig. 5a-d). Furthermore, student's t-test analysis revealed that some signature related genes were similarly differentially expressed in different clinicopathological parameters (Supplementary Figure S2). To validate the independent predictive value of necroptosis-related features for OS, we performed univariate Cox regression analysis and multivariate Cox regression analysis. Univariate Cox analysis found that necroptosis-related features, tumor stage, T stages and M stages were all associated with survival of HCC patients (Fig. 5e). Further performed multivariate Cox analysis, the results demonstrated the predictive function of necroptosis-related features in prognosis (Fig. 5f). Therefore, our results confirm that necroptosis-related features can serve as independent prognostic factors in clinical practice.

Validation of the necroptosis-related features by an independent cohort

We calculated the risk score for each patient in the ICGA dataset using the same formula as an independent external validation cohort. The patients were divided into high-risk group and low-risk group according to the median risk score. OS in high-risk patients was shorter than that in low-risk patients as expected (Fig. 6a). The ROCs, with the AUC at 1 year value of 0.718; the AUC at 2 years value of 0.783; the AUC at 3 years value of 0.799, also proved the excellent survival prediction ability of the factor (Fig. 6b).

**Discussion**

Necroptosis plays a crucial role in the migration and invasion of a variety of cancers. Although the correlation between necroptosis and liver cancer has been reported, no systematic study has focused on necroptosis-related features as prognostic indicators for the prognosis prediction of patients with liver cancer including HCC.
To address the question, we reanalyzed the necroptosis-related genes of HCC patients derived from public databases via introducing an integrated bioinformatics strategy. At the discovery stage, a total of thirty-six differential NRGs were identified in the TCGA database, which were mainly involved in the signaling pathways of necroptosis and NF kappaB. Studies have shown that necroptosis may be associated with the inhibition of the NF-kB pathway (Zhu et al. 2018; Liu et al. 2016). Tumor necrosis factor (TNF) binds and activates TNF receptor 1, which in turn activates the NF-kB pathway triggering the expression of pro-survival genes to promote cell survival. However, when protein synthesis is inhibited, activation of TNFR1 turns into a death signal, inducing necroptosis of the cell. In parallel, studies found that NF-kB plays an important role in hepatocellular injury, liver fibrosis, and HCC development, and the effects of NF-kB on HCC largely depend on the degree of its activation or inhibition (Luedde and Schwabe 2011).

By integrating univariate and multivariate Cox regression analysis, four NRGs (HSP90AA1, PPIA, SQSTM1 and USP21) were found to have the strong association with the prognosis parameters of HCC patients. Furthermore, we observed that the subjects with HCC could be clearly divided into the high-risk and low-risk groups according to the median risk score using the four NRGs. Of note, the patients in the high-risk group had a shorter OS time compared with that of the low-risk group, which indicated that the expression levels of HSP90AA1, PPIA, SQSTM1 and USP21 were strongly correlated with the survival outcomes and tumor stage of HCC patients.

In previous studies, the genes (HSP90AA1, PPIA, SQSTM1 and USP21) have been reported to be strongly related to cancer. HSP90AA1 belongs to the category of heat shock proteins and is a member of the HSP90 family, which involved in maintaining proper folding of client proteins, playing an important role in regulating protein synthesis / degradation as well as localization balance (Xiao et al. 2021; Jego et al. 2013). Many client proteins regulated by HSP90AA1 are proto oncogene products or important signal transducers during tumor pathogenesis, which are closely related to tumor development and progression (Xiang et al. 2018; Shi et al. 2020). Besides, PPIA was found to be significantly elevated in HCC, which was in accordance with previous reports (Lee and Kim 2010; Ye et al. 2013). A study conducted by Wang et al. (2019) demonstrated that PPIA was highly correlated with survival in patients with liver cancer. According to previous studies, overexpression of PPIA played a central role in a wide of pathological processes, such as aging, cancer metastasis, and the progression of inflammatory diseases (Nigro et al. 2013). SQSTM1 is a macroautophagy/autophagy receptor protein that can be degraded by selective autophagy. Studies have found that increased accumulation of SQSTM1 activation is frequently observed in various cancers including HCC. This may be related to the involvement of SQSTM1 in biological processes such as autophagy, defense against oxidative stress, and protein aggregation as well as apoptosis (Chao et al. 2022). USP21 belongs to the ubiquitin specific peptidases (USPS) subfamily and plays an important role in tumorigenesis (Chen et al. 2017). Yang et al. (2020) found that USP21 was elevated in HCC and promoted the disease progression by targeting mir-637.

Based on ROC analysis, the panel comprising HSP90AA1, PPIA, SQSTM1 and USP21 showed a high AUC of 0.807, suggesting that it had the capacity of distinguishing the prognostic progression of HCC.
Furthermore, the diagnostic value of this panel was verified using another cohort of patients, in which its AUCs were over 0.7.

In conclusion, we depicted a necroptosis-related gene signature that had a high predictive value for the prognosis of HCC patients which could be further developed as a novel potential diagnostic biomarker for assessing the clinical outcomes and precise progression of this disease in future.

**Declarations**

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**Author Contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Junyan Lin and Zhiwei Wang. Data validation and visualization were undertaken by Yonghong Jiang and Huihui Su. The first draft of the manuscript was written by Zhandong Yang. All authors carefully revised the manuscript and approved the final version.

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**Competing Interests** The authors have no relevant financial or non-financial interests to disclose.


**References**


Figures
Figure 1

Study Design Workflow
Figure 2

**Identification of differential NRGs between HCC and normal liver tissue.** (a) Identification of differential NRGs in HCC by the volcano plot with FDR<0.05 and log2 FC>1. (b) The heatmaps of Thirty-six differential NRGs. (c) Expression levels of differential NRGs. N indicates non-tumor tissues; T indicates tumor tissues; FC is fold change.
Figure 3

**Functional analysis of differential NRGs.** (a) The top 30 significant terms of GO function enrichment. BP biological process, CC cellular component, MF molecular function. (b) The top 30 significant terms of KEGG analysis.
Figure 4

Construction of a NRGs-related prognostic signature. (a) Heatmap of the expression profile of the four NRGs. (b) The number of patients in different risk groups. (c) Survival status of patients in different groups. (d) Kaplan-Meier curves of OS in the high-risk and the low-risk groups stratified by the necroptosis-related signature in the cohorts. (e) The Survival-dependent receiver operating characteristic (ROC) analysis of OS for the signature and the clinicopathologic parameters.
Figure 5

Clinicopathological significance of the NRG signature in the cohorts. The necroptosis-related signature in the cohorts stratified by survival outcome (a), tumor stages (b), T tumor (c) and M tumor stages (d). (e) A forest plot of univariate Cox regression analysis in the cohorts. (f) A forest plot of multivariate Cox regression analysis in the cohorts.
Figure 6

Validation of the necroptosis-related signature in the ICGC database. a. ROC analysis in ICGA. b. Kaplan Meier curve of OS in high-risk and low-risk groups stratified by necroptosis-related features in ICGA.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure.docx
- SupplementaryTable.docx