Necroptosis-related lncRNAs and Hepatocellular Carcinoma Undoubtedly Secret

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Abstract

Background: The current study demonstrates that necroptosis is an important mechanism of carcinogenesis. However, the predictive value of necroptosis-associated long non-coding RNAs (lncRNAs) in hepatocellular carcinoma has not been demonstrated. The purpose of this study is to apply necroptosis-related lncRNAs to construct a predictive signature to predict the prognosis of hepatocellular carcinoma patients.

Methods: The clinical and RNA-seq data were downloaded using The Cancer Genome Atlas (TCGA) database. Univariate and multivariate Cox analysis was used to screen out suitable necroptosis-related lncRNAs, and then predictive signature was constructed. The TCGA data were randomly divided into high- and low-risk groups, and the Kaplan-Meier method was used to analyze the overall survival (OS) of the two groups to verify the predictive signature. Finally, a prognostic correlation model for predicting disease-free survival (DFS) in hepatocellular carcinoma (HCC) was constructed and validated.

Results: We had screened out 9 necroptosis-related lncRNAs (BACE1-AS, LINC01188, LUCAT1, PI3KCD-AS2, Z83851.1, AC009283.1, AC012360.2, AC015908.3, AC103760.1), which were used to construct a predictive signature, and draw the receiver operating characteristic (ROC) curve by the high- and low-risk group. It was found that the area under the curve (AUC) of risk score was 0.874, and the AUCs values of 1-, 3-, and 5-years were 0.8, 0.759, 0.787. The TCGA data were randomly divided into two cohorts. In two cohorts, The OS of the high-risk groups were significantly lower than that of the low-risk groups, and the AUC of the ROC curves of the two cohorts were 0.851, 0.804, 0.802 and 0.735, 0.716, 0.76 at 1-, 3-, and 5-years. After quantifying immune cell subsets and related functions, it was found that the infiltration of active dendritic cells (aDCs), macrophages, mast cells, natural killer cells (NK), T regulatory cells (Tregs) were significantly different, and the expressions of immune checkpoints CD86, LAIR1, CTLA4, VTCN1, TNFRSF18, CD80, CD276, HHLA2, TNFSF4, TNFRSF8, TNFRSF4, TNFRSF9, LGALS9, HAVCR2 and TNFSF15 were also significantly different.

Conclusion: This predictive signature can accurately predict the prognosis of hepatocellular carcinoma patients and provide guidance for the clinical treatment of hepatocellular carcinoma patients.

1. Background

Liver cancer is one of the most common malignant tumor in the world and the sixth most common cancer type that seriously threatens human health [1]. Traditional chemotherapy does not work well for patients with advanced liver cancer [2]. Although immunotherapy related studies CHECKMATE-040 [3] KEYNOTE-224 [4] have shown good efficacy, not all patients can benefit from immunotherapy. Therefore, better diagnostic and therapeutic methods for HCC need to be discovered. In 2014, Yuan et al. [5] found that the expression of long non-coding RNA (lncRNA) is related to the occurrence and development of hepatocellular carcinoma. Subsequently, more and more studies have found that lncRNAs are closely

related to tumor angiogenesis, tumor metastasis, and tumor treatment effects of hepatocellular carcinoma[6–10].

The main function of lncRNA is to regulate gene expression [11]. At present, people are mainly classified according to their positional relationship with adjacent coding genes [12]. According to whether lncRNAs overlap with the sequence of coding genes, they are divided into two categories: gene-related lncRNAs and intergenic lncRNAs. According to their transcription sites and molecular characteristics, they are divided into intergenic long non-coding RNAs, sense or reverse that overlap with protein-coding genes. Sense transcripts, enhancer RNAs, and promoter upstream transcripts [13]. These lncRNAs can bind to chromatin regulators [14]. It also can bind to DNA [15]. These functions are closely related to the occurrence and development of tumors.

Many lncRNAs have been reported to be involved in inducing necroptosis in HCC cells [16, 17]. At the same time, some studies have found that necroptosis is closely related to the development and treatment of liver cancer [18]. However, the predictive value of necroptosis-related lncRNAs in hepatocellular carcinoma has not been demonstrated. The purpose of this study was to apply necroptosis-related long noncoding lncRNAs to construct a predictive model to predict the prognosis of patients with hepatocellular carcinoma and validate it.

2. Material And Methods

2.1. Patients and datasets

Downloading the necroptosis-related pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and consulting the latest literature to obtain 123 necroptosis-related genes. Downloading the corresponding clinical and prognostic data for The Cancer Genome Atlas liver cancer (TCGA-LIHC) dataset and the fragments per kilobase of transcript per million mapped reads (FPKM)-standardized RNA-seq data from the TCGA website (<https://portal.gdc.cancer.gov/>); obtained the lncRNA expression of 374 hepatocellular carcinoma tissues and 50 normal liver tissues, and obtained the survival time and clinical data of hepatocellular carcinoma patients. We obtained disease-free survival (DFS) data for 310 hepatocellular carcinoma patients from the cBioPortal database (<https://www.cbioportal.org/>). Immune infiltration results from the TIMER database(<https://cistrome.shinyapps.io/timer/>). Immunohistochemical results from proteinatlas database(<https://www.proteinatlas.org/>). gene expression profile results from GEPIA database(<http://gepia.cancer-pku.cn/index.html>). All experimental procedures are shown in Fig. 1.

2.2. Functional enrichment analysis of differentially expressed necroptosis-related genes

Using $|\log_2 \text{fold change(FC)}| > 1$ and a false discovery rate (FDR) < 0.05 as screening criteria to obtain necroptosis-related differentially expressed genes (DEGs). We performed Gene Ontology (GO) and KEGG

analyses in the “ggplot2” package.

2.3. The necroptosis-related lncRNA predictive signature

Using the “limma” package to calculate the correlation between necroptosis-related genes. Using the correlation coefficient $p < 0.001$ and $|R^2| > 0.4$ as the screening criteria, a total of 765 necroptosis-related lncRNAs with expression values were obtained. Univariate Cox analysis was used to obtain necroptosis-related lncRNAs related to the prognosis of hepatocellular carcinoma patients, and then multivariate Cox analysis was used to obtain necroptosis-related lncRNAs, which laid the foundation for the construction of necroptosis-related lncRNAs Predictive Signature. The computational formula used for this analysis was as follows:

$$\text{Riskscore} = \sum_{i=1}^n (\text{Coef}_i \times x_i)$$

Coef represents the coefficient value, and x represents the selected necroptosis-related lncRNAs. This formula was used to calculate the risk score for each hepatocellular carcinoma patient.

2.4. Nomogram

A Nomogram that can predict 1, 3, and 5-year survival of hepatocellular carcinoma patients was constructed using risk scores and clinicopathological features such as age, gender, stage, and TNM stage. A calibration curve was used to test whether the predicted survival rate was consistent with the actual survival rate.

2.5. Functional enrichment analysis

Hepatocellular carcinoma patients were divided into high- and low-risk groups according to the median risk score. Which pathway genes were predominantly enriched were analyzed using Gene set enrichment analysis (GSEA). Nominal $p < 0.05$ and $\text{FDR} < 0.25$ were the thresholds for statistical significance.

2.6. Statistical analysis

All statistical analyses were performed with R software (version 4.1.2). The expression of necroptosis-related DEGs in cancer tissues and normal tissues were analyzed by Wilcoxon test. Univariate Cox regression was used to analyze the relationship between necroptosis-related lncRNAs and overall survival (OS), and multivariate Cox analysis was used to screen necroptosis-related lncRNAs to construct predictive signals. The Kaplan-Meier method and LOG-RANK test were used to analyze the OS of patients in high- and low-risk groups. The receiver operating characteristic (ROC) curve was drawn with the

"Survival ROC" software package, and the area under the curve (AUC) value was determined. SsGSEA uses the "GSVA" software package.

3. Results

3.1. Functional enrichment analysis of differentially expressed necroptosis-related genes

67 necroptosis-related DEGs were obtained by differential analysis of the data screened from the TCGA database, including 63 up-regulated genes and 4 down-regulated genes (Fig. 2A, S1, S2). We further analyzed the regulatory relationship between these differentially expressed genes (Fig. 2B). These genes were then subjected to KEGG pathway and GO enrichment analysis. The KEGG enrichment analysis showed that the DEGs were mainly enriched in Necroptosis, NOD-like receptor signaling pathway, Linoleic acid metabolism, Inflammatory mediator regulation of TRP channels, alpha-Linolenic acid metabolism, Lipid and atherosclerosis, GnRH signaling pathway, Shigellosis, Arachidonic acid metabolism (Fig. 2C, S3, S4). GO enrichment analysis showed that in the biological process category, the DEGs were mainly enriched in regulation of I-kappaB kinase/NF-kappaB signaling, I-kappaB kinase/NF-kappaB signaling, programmed necrotic cell death, in the cellular components category, the DEGs are mainly enriched in CD40 receptor complex, autolysosome, secondary lysosome, in the molecular function category, the DEGs are mainly enriched in phospholipase A1 activity, calcium-dependent phospholipase A2 activity, ubiquitin protein ligase binding (figure 2D, S5, S6).

3.2. The necroptosis-related lncRNA predictive signature

The lncRNAs were isolated from the DEGs, and finally 765 necroptosis-related lncRNAs (Table S1) were obtained, and then univariate Cox regression analysis was used to obtain 94 lncRNAs related to prognosis, and multivariate Cox analysis was used to obtain the final 9 lncRNAs. The expression of these 9 necroptosis-related lncRNAs were visualized (figure 2E), Then using Cytoscape and the ggalluvial R software package to find co-expression mRNAs for further visualization (figure 2F, $|R2| > 0.4$ and $p < 0.001$). 31 genes co-expressed with these 9 lncRNAs. Among them, BACE1-AS, LINC01188, LUCAT1, PI3KCD-AS2 and Z83851.1 were risk factors, AC009283.1, AC012360.2, AC015908.3 and AC103760.1 were protective factors (Fig. 2G). Then we analyzed the expression of these co-expressed genes in cancer tissues and adjacent tissues. The results indicated that BAX, CDKN2A, FTH1, H2AF, FTL, HSP90AB1, HSPA4, PLK1 and TRAF2 were highly expressed in tumor tissues, 6 of these genes were significantly associated with the prognosis of hepatocellular carcinoma (Fig. 2H-J, Immunohistochemical results from protein atlas database (<https://www.proteinatlas.org/>)). Gene expression profile from GEPIA database (<http://gepia.cancer-pku.cn/index.html>)). The formula for calculating the risk score is: Risk score = $(0.597 \times \text{BACE1-AS expression}) + (-0.537 \times \text{AC015908.3 expression}) + (0.342 \times \text{PIK2HD-AS2 expression}) + (-0.491 \times \text{AC012360.2 expression}) + (0.520 \times \text{Z83851.1 expression}) + (-0.664 \times \text{LINC01138 expression}) + (-0.457 \times \text{AC103760.1 expression}) + (-1.112 \times \text{AC009283.1 expression}) + (0.340 \times \text{LUCAT1 expression})$.

3.3. Validation of the necroptosis-related lncRNA predictive signature

Calculating the risk score of all patients, Then we divided patients into two groups by the median risk score. As risk scores increased, so did the number of patient deaths (Fig. 3A). We then performed PCA principal component analysis, which showed that patients grouped according to risk lncRNA was more specific than all gene, all lncRNA, and necroptosis-gene (Fig. 3B). Kaplan-Meier analysis method was used to analyze the OS time of high-risk group and low-risk group to determine the value of risk score in predicting the prognosis of patients with hepatocellular carcinoma. The result showed that the OS of the high-risk group was significantly shortened (Fig. 3C, $p < 0.001$). Subsequently, Univariate Cox regression analysis showed that tumor stage, T grade, N grade and risk score were the factors affecting the prognosis of patients (Fig. 3D, $p < 0.05$). Multivariate Cox regression analysis showed that only risk score was an independent risk factor affecting the prognosis of patients (Fig. 3E, $p < 0.05$). According to the drawn ROC curve, it was found that the AUC of the risk score was 0.874, indicating that the risk score is better than other clinical features for the prognosis of hepatocellular carcinoma patients (Fig. 3F). And the AUCs of 1-year, 3-year and 5-year were 0.8, 0.759, 0.787, indicating that the risk score has good accuracy in predicting survival time (Fig. 3G). Differences in lncRNA expression between high- and low-risk groups were analyzed according to clinical characteristics (figure S7). A nomogram was constructed by clinical features and risk score, which predicts 1-, 3-, and 5-year prognosis in patients with hepatocellular carcinoma (Fig. 3H). Calibration curves show good agreement between predicted 1-, 3-, and 5-year survival rates and patients' actual OS (Fig. 3I).

3.4. The accuracy of the predictive signature in predicting the prognosis of hepatocellular carcinoma patients with different clinical characteristics

In order to verify the accuracy of the Predictive Signature in predicting the prognosis of hepatocellular carcinoma patients with different clinical characteristics, hepatocellular carcinoma patients were grouped according to age, gender, stage, T stage and grade. The OS of low-risk group were significantly higher than the high-risk group (Fig. 4A). These results suggest that predictive features can predict the prognosis of patients with hepatocellular carcinoma regardless of clinical features.

3.5. Validation of predictive signature

To verify the validity of the predictive signature, the TCGA data were randomly divided into two cohorts, and the clinical characteristics of two cohorts were shown in Table 1. The results were the same as expected, and the OS of patients in the high-risk group in the first cohort was significantly lower than that in the low-risk group (Fig. 4B, $p < 0.01$). Another cohort also showed the same result (Fig. 4C, $p < 0.01$). And the AUCs of the ROC curve for the first cohort at 1-, 3-, 5- year were 0.851, 0.804, 0.802 (Fig. 4D). The AUCs of the ROC curve for another cohort at 1-, 3-, 5- year were 0.735, 0.716, 0.765 (Fig. 4E). Box plots were then

drawn according to different clinical characteristics and risk scores(Fig. 5A), This suggests that there are differences in risk scores for different clinical features.

Table 1

Variables	Entire	Validation cohort	
	TCGA dataset(n = 342)	First cohort(n = 172)	second cohort(n = 170)
Age(%)			
<=65	216(63.16)	109(63.37)	107(62.94)
> 65	126(36.84)	63(36.63)	63(37.06)
Gender(%)			
Female	109(31.87)	55(31.98)	54(31.76)
male	233(68.13)	117(68.02)	116(68.24)
Grade			
G1	53(15.5)	25(14.53)	28(16.47)
G2	161(47.08)	87(50.58)	74(43.53)
G3 + 4	123(35.96)	58(33.72)	65(38.24)
Unknow	5(1.46)	2(1.16)	3(1.76)
Stage(%)			
I + II	238(69.59)	115(66.86)	123(72.35)
III + IV	83(24.27)	45(26.16)	38(22.35)
Unknow	21(6.14)	12(6.98)	9(5.29)
T(%)			
T1 + 2	252(73.68)	121(70.35)	131(77.06)
T3 + 4	87(25.44)	50(29.65)	37(21.76)
TX + Unknow	3(0.88)	1(0.58)	2(1.18)
M(%)			
M0 + 1	247(72.22)	121(70.35)	126(74.12)
MX + Unknow	95(27.78)	51(26.95)	44(25.88)
N(%)			
N0	242(70.76)	120(69.77)	122(71.76)
NX + Unknow	100(29.24)	52(30.23)	48(28.24)

3.6. Gene enrichment analysis

Differences in prognosis of patients in high- and low-risk groups were investigated using GSEA. We found that the high-risk groups had cell cycle pathway, complement and coagulation cascades pathway, drug metabolism cytochrome p450 pathway, metabolism of xenobiotics by cytochrome p450 pathway, oocyte meiosis pathway, peroxisome pathway, fatty acid beta oxidation, azurophil granule, aromatase activity and purine metabolism pathway significant enrichment (Fig. 5B, 5C), It shows that high-risk patients are closely related to tumors and a variety of substances metabolism-related pathways.

3.7. The correlation between risk scores and immune cells and functions

To further explore the correlation between risk scores and immune cells and function, we quantified immune cell subsets and associated functions. The results showed that active dendritic cells (aDCs), macrophages, mast cells, natural killer cells (NK), T regulatory cells (Tregs) were different between high-risk and low-risk groups. salience (Fig. 6A). Likewise, there were significant differences in immune function between the two groups (Fig. 6B). Then we analyzed the differences of different immune checkpoints between the two groups, and found that the expressions of CD86, LAIR1, CTLA4, VTCN1, TNFRSF18, CD80, CD276, HHLA2, TNFSF4, TNFRSF8, TNFRSF4, TNFRSF9, LGALS9, HAVCR2 and TNFSF15 were significantly different (Fig. 6C). The immune cell infiltration between co-expressed genes was analyzed online using the TIMER database, and the results showed that they were closely related to a variety of immune cell infiltration (figure S8, immune infiltration level from the TIMER database(<https://cistrome.shinyapps.io/timer/>)).

3.8. Analysis of Gene Mutation in Patients with Hepatocellular Carcinoma

In order to further explore the reasons for the occurrence and development of hepatocellular carcinoma, we conducted gene mutation analysis (Fig. 6D). First, we analyzed the genes with the highest mutation rates. The results show that these mutated genes were significantly associated with necroptosis-related genes (Fig. 6E). We also analyzed the bases of gene mutation and found that the most common T base changed to A base (Fig. 6F). These mutated genes are enriched in RTK-RAS, WNT, NOTCH, PI3K and other pathways (Fig. 6G). Subsequent drug-mutated gene correlation analysis showed that the therapeutic effect of DRUGGABLE GENOME and CLINICALLY ACTIONABLE may be better (Fig. 6H). Finally, we found that the heterogeneity of hepatocellular carcinoma is more obvious, which suggests that the drug resistance of liver cancer will be very strong (Fig. 6I).

3.9. the necroptosis-related lncRNA predictive signature for DFS

Then we constructed the necroptosis-related lncRNA predictive signature for DFS. We collected DFS data on hepatocellular carcinoma patients from the cBioPortal database, including 310 patients. After univariate Cox regression analysis, 33 necroptosis-related lncRNAs were found to be significantly associated with DFS in patients with hepatocellular carcinoma. After multivariate Cox regression analysis, 7 necroptosis-related lncRNAs were obtained, which were used to construct predictive signatures. Risk score = $(0.357 \times \text{ZFPM2-AS1 expression}) + (-0.332 \times \text{AC026401.3 expression}) + (0.596 \times \text{SREBF2-AS1 expression}) + (0.293 \times \text{AL391427.1 expression}) + (0.405 \times \text{MIR4458HG expression}) + (0.523 \times \text{AC005229.4 expression}) + (0.512 \times \text{LYRM4-AS1 expression})$. The risk score for each patient was calculated according to the formula, and the patients in the entire dataset were divided into high-risk and low-risk groups based on the median. Kaplan-Meier survival curve analysis showed that the DFS in the high-risk group was significantly shorter than that in the low-risk group (figure S9, $p < 0.001$). The AUCs for 1-, 3-, and 5-year survival were 0.677, 0.728, and 0.726 (figure S10). To validate the predictive signature, 310 patients were randomized into two cohorts. The validation results were the same as expected, and the DFS of patients in the high-risk group in the first cohort was significantly lower than that in the low-risk group (figure S11, $p < 0.01$). Another cohort also showed the same result (figure S12, $p < 0.01$). And the AUCs of the ROC curve of the first cohort at 1-, 3-, 5-year were 0.726, 0.749, 0.734 (figure S13). The AUCs of the ROC curve of another cohort could reach 0.602, 0.719, 0.766 at 1-, 3-, 5-year (figure S14).

4. Discussion

Hepatocellular carcinoma is the main pathological type of primary liver cancer [19]. The occurrence of hepatocellular carcinoma is related to factors such as chronic hepatitis virus infection, long-term exposure to carcinogens (such as aflatoxin, etc.), excessive drinking, non-alcoholic fatty liver disease, hemochromatosis, and α -1 antitrypsin deficiency [20]. In addition, studies have shown that necroptosis plays a key role in the development of hepatocellular carcinoma. Our study also demonstrated that necroptosis-related lncRNAs can accurately predict the prognosis of HCC patients.

There are various ways of cell death, including apoptosis, autophagy, ferroptosis and necroptosis. Different studies have proved that these cell death ways are related to the occurrence of tumors [21, 22]. However, unlike apoptosis, necroptosis is a necroptosis complex formed by the combination of RIPK1 and RIPK3 after apoptosis is inhibited, which induces high expression of MLKL and forces cells to lose cell membrane integrity [23]. Necroptosis can promote tumor cell extravasation and migration. For example, tumor cells use the expressed amyloid precursor as a mediator and induce necroptosis of endothelial cells through DR6 on endothelial cells, leading to tumor cell extravasation and metastasis [24]. When RIPK1/RIPK3 mediates tumor necroptosis, the released soluble factor CXCL1 promotes tumor growth and invasion through an immunosuppressive tumor microenvironment induced by suppressive macrophages [25]. Genetic inactivation of RIPK3 or TNFR1 alleviates clinical symptoms of colitis and cancer, suggesting that RIPK3-mediated necroptosis promotes chronic inflammation and colorectal cancer development [26, 27]. In addition, sorafenib is a multi-targeted kinase inhibitor, which is mainly used to treat kidney cancer, liver cancer, thyroid cancer and some leukemia patients. Sorafenib targets the necroptotic pathway in addition to the Braf/Mek/Erk and VEGFR pathways and alleviates RIPK1/3-

mediated pathology in acute inflammation [28]. Our experiments also demonstrated a close relationship between necroptosis and hepatocellular carcinoma.

Many studies have demonstrated that lncRNAs can act as key regulators to affect tumor invasion and metastasis[29–31], tumor cell proliferation and apoptosis[32–35], tumor angiogenesis[34, 36–38] and regulate tumor drug resistance[39, 40]. More and more studies have shown that abnormally expressed lncRNAs are key factors in cancer detection and can be used as non-invasive tumor markers to play an irreplaceable role in the early diagnosis of hepatocellular carcinoma[7, 41–43]. Further research has found that lncRNA can be used as a key breakthrough point in molecular targeted therapy. Silencing, blocking, destroying oncogenic lncRNA or restoring the function of tumor suppressor lncRNA can effectively inhibit the malignant biological behavior of hepatocellular carcinoma cells and regulate tumor resistance, which are all important for the treatment of hepatocellular carcinoma[10]. A large number of studies have confirmed that abnormally expressed lncRNA is closely related to the prognosis of liver cancer. It can be used as a potential molecular marker to predict the degree of tumor tissue differentiation, lymph node metastasis, TNM stage, and tumor size, which is of great significance for the clinical diagnosis and treatment of hepatocellular carcinoma[10]. Recent studies have reported that necroptosis-related lncRNAs play a key role in hepatocellular carcinoma[18]. Our study also demonstrated that necroptosis-related lncRNAs are closely related to the prognosis of patients with hepatocellular carcinoma, and can even construct a model that effectively predicts the prognosis of patients. This proves that there is enough space for research in this direction, and the author's team will continue to pay attention and report on this direction.

The results of immune cell and immune function analysis showed that the high-risk group had higher infiltration levels of Treg cells, higher infiltration levels of NK cells, and higher infiltration levels of macrophages. This is consistent with the results of previous studies. Previous studies have found that hepatocellular carcinoma tissue forms a specific immune microenvironment at an early stage, and Treg cells can promote the proliferation and metastasis of tumor cells. Patients with higher levels of Treg cell infiltration have poorer prognosis [44–46]. Studies have shown that TAMs isolated from the liver cancer microenvironment are mainly M2 type, and more evidence supports that TAMs with M2 phenotype promote tumor progression through complex autocrine and paracrine pathways closely related to tumor malignant proliferation, invasion and metastasis[34, 47]. There is also a clear relationship between immune cells and immune function and patient prognosis, and some studies have even established an immune prognosis model to identify high-risk patients with low survival rates[48–50]. Our study also found that there were significant differences in the expression of immune checkpoints CD86, LAIR1, CTLA4, VTCN1, TNFRSF18, CD80, CD276, HHLA2, TNFSF4, TNFRSF8, TNFRSF4, TNFRSF9, LGALS9, HAVCR2 and TNFSF15 between the high-risk group and the low-risk group. Related studies can also be conducted to explore whether these immune checkpoints can be used as diagnosis and treatment's target.

However, our study also has certain limitations. We only use data from the TCGA database for validation, so we also need real-world clinical data for validation to test the accuracy of the predictive model.

Second, the mechanism of action of necroptosis-related lncRNAs in hepatocellular carcinoma needs further experimental verification. In conclusion, the necroptosis-related lncRNA model can independently predict the prognosis of hepatocellular carcinoma patients, and provide a basis for exploring the possible mechanism and clinical efficacy of necroptosis-related lncRNA in hepatocellular carcinoma.

5. Conclusions

Our study has identified the prognostic value of necroptosis-related lncRNAs in hepatocellular carcinoma and established a prognostic prediction signature. The verification results are the same as expected. Our study has also found that there are significant differences in the expression of immune checkpoints IDO2, TNFRSF14, BTNL2, TNFRSF25, and CD276 between the high-risk group and the low-risk group. Related studies can also be conducted to explore whether these immune checkpoints can be used as diagnosis and treatment's target.

Abbreviations

lncRNAs

Long non-coding RNAs

TCGA

The Cancer Genome Atlas

OS

Overall Survival

GSEA

Gene set enrichment analysis

DFS

Disease-free survival

HCC

Hepatocellular carcinoma

ROC

Receiver operating characteristic

AUC

Area under the curve

aDCs

active Dendritic cells

NK

Natural killer cells

Tregs

T regulatory cells

KEGG

Kyoto Encyclopedia of Genes and Genomes

TCGA-LIHC

The Cancer Genome Atlas liver cancer

FPKM

Fragments per kilobase of transcript per million mapped reads

FC

Fold change

FDR

False discovery rate

DEGs

Differentially Expressed Genes

GO

Gene Ontology

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have agreed to publish this manuscript.

Availability of data and materials

All data are available in the main text or the supplementary materials.

Competing interests

The Authors declare no conflicts of interest regarding this study.

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Authors' contributions

Jingyuan Ning and Keran Sun conceived and wrote the paper. Keran Sun, Jingyuan Ning, Keqi Jia, Xiaofeng Wang, Xiuli Wang, Xiaoqing Fan analyzed the materials, and drafted the manuscript. Cuiqing Ma and Lin Wei revised the whole paper. All authors have reviewed the final version of the manuscript and approved to submit to your journal.

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Figures

Figure 1

Figure 1

Experimental flow chart

Figure 2

Figure 2

67 necroptosis-related DEGs were obtained by differential analysis of the data screened from the TCGA database, including 63 up-regulated genes and 4 down-regulated genes (figure 2A). We further analyzed the regulatory relationship between these differentially expressed genes (figure 2B). The KEGG enrichment analysis showed that the DEGs were mainly enriched in Necroptosis, NOD-like receptor signaling pathway, Linoleic acid metabolism, Inflammatory mediator regulation of TRP channels, alpha-Linolenic acid

metabolism, Lipid and atherosclerosis, GnRH signaling pathway, Shigellosis, Arachidonic acid metabolism (figure 2C). GO enrichment analysis showed that In the biological process category, the DEGs were mainly enriched in regulation of I-kappaB kinase/NF-kappaB signaling, I-kappaB kinase/NF-kappaB signaling, programmed necrotic cell death, In the cellular components category, the DEGs are mainly enriched in CD40 receptor complex, autolysosome, secondary lysosome, In the molecular function category, the DEGs are mainly enriched in phospholipase A1 activity, calcium-dependent phospholipase A2 activity, ubiquitin protein ligase binding—figure 2D—. The expression of these 9 necroptosis-related lncRNAs were visualized—figure 2E—. Using Cytoscape and the ggalluvial R software package to find co-expression mRNAs for further visualization—figure 2F—|R2|>0.4 and $p<0.001$ —. Among them, BACE1-AS, LINC01188, LUCAT1, PI3KCD-AS2 and Z83851.1 were risk factors, AC009283.1, AC012360.2, AC015908.3 and AC103760.1 were protective factors

(figure 2G). The results indicated that BAX, CDKN2A, FTH1, H2AF, FTL, HSP90AB1, HSPA4, PLK1 and TRAF2 were highly expressed in tumor tissues, 6 of these genes were significantly associated with the prognosis of hepatocellular carcinoma (figure 2H-J), Immunohistochemical results from proteinatlas database (<https://www.proteinatlas.org/>). gene expression profile from GEPIA database (<http://gepia.cancer-pku.cn/index.html>).

Figure 3

Figure 3

Divided patients into two groups by risk score. As the risk score increases, the number of patient deaths also increases (figure 3A). The OS time of the high-risk group was significantly shortened (figure 3C, $p < 0.001$). PCA principal component analysis showed that patients grouped according to risk lncRNA was more specific than all gene, all lncRNA, and necroptosis-gene (figure 3B). Univariate Cox regression analysis showed that tumor stage, T grade, N grade and risk score were the factors affecting the

prognosis of patients (figure 3D, $p \leq 0.05$). Multivariate Cox regression analysis showed that only risk score was an independent risk factor affecting the prognosis of patients (figure 3E, $p \leq 0.05$). According to the drawn ROC curve, it was found that the AUC value of the risk score was 0.748, indicating that the risk score is better than other clinical factors for the prognosis of hepatocellular carcinoma patients (figure 3F). And the AUCs of 1 year, 3 years and 5 years were 0.8, 0.759, 0.787 respectively, indicating that the risk score has good accuracy in predicting survival time.(figure 3G). A nomogram was constructed using clinical features and risk score, which predicts 1, 3, and 5-year prognosis in patients with hepatocellular carcinoma (figure 3H). Calibration curves show good agreement between predicted 1, 3, 5-year survival rates and patients' actual OS (figure 3I).

A

B

C

D

E

Figure 4

Figure 4

The OS of low-risk groups were significantly higher than the high-risk groups (figure 4A). The OS of patients in the high-risk group in the first cohort was significantly lower than that in the low-risk group (figure 4B, $p < 0.01$). Another cohort also showed the same result (figure 4C, $p < 0.01$). And the AUCs of the ROC curve for the first cohort at 1, 3, 5- years was 0.851, 0.804, 0.802 (figure 4D). The AUCs of the ROC curve for another cohort at 1, 3, 5- years was 0.735, 0.716, 0.765 (figure 4E).

Figure 5

Figure 5

Box plots were then drawn according to different clinical characteristics and risk scores (figure 5A). This suggests that there are differences in risk scores for different clinical features. GSEA analysis found that the high-risk groups had cell cycle pathway, complement and coagulation cascades pathway, drug metabolism cytochrome p450 pathway, metabolism of xenobiotics by cytochrome p450 pathway, oocyte

meiosis passway, peroxisome passway, fatty acid beta oxidation, azurophil granule, aromatase activity and purine metabolism passway significant enrichment (figure 5B, 5C).

Figure 6

Figure 6

active dendritic cells (aDCs), macrophages, mast cells, natural killer cells (NK), T regulatory cells (Tregs) were different between high-risk and low-risk groups. salience (figure 6A). Likewise, there were significant

differences in immune function between the two groups (figure 6B). The expressions of CD86, LAIR1, CTLA4, VTCN1, TNFRSF18, CD80, CD276, HHLA2, TNFSF4, TNFRSF8, TNFRSF4, TNFRSF9, LGALS9, HAVCR2 and TNFSF15 were significantly different (figure 6C). We conducted gene mutation analysis (figure 6D). First, we analyzed the genes with the highest mutation rates. The results show that these mutated genes were significantly associated with necroptosis-related genes (figure 6E). We also analyzed the bases of gene mutation and found that the most common T base changed to A base (figure 6F). These mutated genes are enriched in RTK-RAS, WNT, NOTCH, PI3K and other pathways (figure 6G). Subsequent drug-mutated gene correlation analysis showed that the therapeutic effect of DRUGGABLE GENOME and CLINICALLY ACTIONABLE may be better (figure 6H). Finally, we found that the heterogeneity of hepatocellular carcinoma is more obvious, which suggests that the drug resistance of liver cancer will be very strong (figure 6I).

Supplementary Files

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