Developing And Verifying A Necroptic Gene-Based Prognostic Index In Patients With Hepatocellular Carcinoma

Yuhang Wang

Beijing University of Chinese Medicine

Shun Zhu

South-Central University for Nationalities

Xiaoxin Huang

Beijing University of Chinese Medicine

Chunguo Wang

Beijing University of Chinese Medicine

Tianhui Xuan

Beijing University of Chinese Medicine

Yong Liu (✉ yongliubucm@163.com)

Beijing University of Chinese Medicine <https://orcid.org/0000-0002-7486-3066>

Research Article

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Abstract

Hepatocellular carcinoma (HCC) is the sixth leading cancer throughout the world and ranks fourth in cancer mortality. Necroptosis modulates tumorigenesis, metastasis, and treatment resistance. However, gene sets related to necroptosis in HCC are rarely analyzed. Hence, it is vital to assess the clinicopathological influence of necroptosis in a large HCC patient population. Our goal in this research was to develop a novel HCC prognostic indicator based on necroptosis. To that end, we examined 132 necroptosis related genes (NRGs) and recognized 36 differentially expressed NRGs (DE-NRGs) in the Cancer Genome Atlas (TCGA) cohort. Using both uni- and multivariate Cox regression models, we selected 4 NRGs, which can be employed to categorize HCC cases into low- or high-risk groups. Based on our survival analysis, the overall survival (OS) duration of high-risk cases was markedly shorter, in comparison with low-risk cases. We also verified using COX regression analysis that NRGs can serve as stand-alone prognostic indicators of HCC prognosis. Moreover, the area under the curve (AUC) of the combined prediction model was 0.807. Lastly, we also verified the validity of our model using the Gene Expression Omnibus (GEO) dataset. In summary, a state-of-the-art NRG-based HCC prognostic indicator is established to accurately predict the outcome of HCC cases to aid clinicians in the efficient planning and designing of personalized anti-HCC therapy.

Introduction

In general, Liver cancer ranks sixth in the world and fourth among cancer-related mortality and the most prevalent sort of primary liver cancer is Hepatocellular carcinoma (HCC) [1]. According to the prediction of World Health Organization, by the year 2030 over a 1 million hepatocellular carcinoma patients will likely die [2]. Most HCC occur in patients with underlying liver diseases, and close to 80% are attributed to chronic hepatitis B and C. Any form of liver cirrhosis can enhance liver cancer risk, particularly those related to hepatitis B (HBV), hepatitis C (HCV), and non-alcoholic fat [3]. Among the other risk factors are aflatoxin exposure, alcoholism, diabetes, and obesity [4]. Currently, HCC treatments have been greatly enhanced in recent years. For example the enhanced efficacy of direct antiviral drugs that treat chronic hepatitis C viral infection is likely to positively impact HCC treatment and prevalence [4]. At present, HCC patients are encouraged to make choices that enhance their overall survival (OS), irregardless of the stage of disease.

On the contrary, the indicators for early monitoring and diagnosis of liver cancer have not massively improved over time. New treatment markers and targets are needed to achieve better HCC prognosis and it is possible that the novel treatment markers may play a role in clinical practice [5]. Identifying abnormal gene expression is gaining increasing attention in recent years, and the study of necroptosis is gaining momentum. Necroptosis is a kind of programmed necrotizing apoptosis, which acts as a gatekeeper to resist pathogenic invasion. It is mediated through receptor interacting protein 1 (RIPK1), RIPK3, and mixed pedigree kinase domain-like protein (MLKL) [6]. Multiple studies examined the significance of necroptosis in cancer. According to prior reports, cancer cells use necroptosis to facilitate metastasis. Therefore, suppressing necroptosis may be a means to preventing cancer metastasis. Alternately, some cancer cells

suppress mediators of necroptosis, indicating that, in specific cancer cells, necroptosis may exert an anti-tumorigenic role [7]. In fact, numerous cancers exhibit downregulation of multiple key necrotic pathway molecules, thus necrosis might be suppressed to proceed with tumorigenesis. RIPK3 levels, for instance, are illustrated to be scarcely expressed in multiple cancer cell lines [8, 9] and cancer tissues. Based on these evidences, RIPK3 may promote both anti-inflammatory and anti-tumor roles in cancer cells. Similarly, RIPK1 is reported to be down-regulated in head and neck squamous cell carcinoma [10]. It is worth noting that not all cancers show a down-regulation of necrotic factors. In fact, in some cancers, the expression of necrotic factors is up-regulated. The up-regulated RIPK1, RIPK3, FADD, and MLKL levels are associated with poor prognosis and accelerated tumorigenesis [11–13]. There is a very close relationship between necroptotic gene expression and cancer progression, but the relationship between necroptotic genes and HCC is not fully understood.

Herein, we extensively assessed the ability of necroptosis-related genes (NRGs) in predicting HCC patient prognosis. Based on our analysis, we recognized four NRGs that were associated with patient prognosis. Using these NRGs, we separated our HCC patient population into two groups that displayed markedly different molecular activities and disease prognosis, thereby indicating that the selected NRGs are strongly correlated with HCC developmental stage. Hence, we established a necroptosis-based prognostic index to assess HCC patient prognosis and identify high-risk patients. Using functional analysis, we revealed that high-risk patients have upregulation of necroptotic signaling, which corresponded with lymph node metastasis, degree of malignancy, and poor prognosis.

Materials And Methods

Human NRG sets

Our screening of the KEGG (KEGG, <https://www.kegg.jp/>) pathway aided in the identification of 132 NRGs, summarized in Table S1.

Sample and data collection

The HCC RNA-sequence (RNA-seq) information and corresponding clinical outcomes were acquired from the TCGA dataset. Overall, we collected 378 patient clinical data and 424 RNA-seq samples. Upon exclusion of healthy individuals and patients with less than 30 days of follow up, the combined data of 379 patients were extracted for analysis. In the meantime, a separate microarray HCC queue (login number: GSE54236) was retrieved from the GEO database (n=82). The expression data were analyzed using Log2 transform. Univariate analysis was then employed to identify NRGs associated with HCC patient OS for subsequent model generation.

Bioinformatics analysis

Consensus cluster assessment and principal component analysis were carried out with the R programming language (version 3.6.3) to validate the NRG-mediated modulation of HCC. The DE-NRGs were identified by R packet LIMMA (Table S2). Next, we performed GO, KEGG, and GSEA analyses to

establish primary biological functions of relevant genes. The GO tool package was employed for the visualization of enrichment items. Univariate assessment was used to evaluate the correlation between gene expression and OS. Subsequently, the significant genes determined through univariate assessment were entered into multivariate analysis, which, in turn, identified the stand-alone prognostic indicators. The hazard ratio (HR) and regression coefficient were computed through the Cox regression model.

Generation of the NRG-based prognostic model

Multivariate Cox regression analysis was employed for the identification of prognostic genes. Upon combination of specific gene expression values, a risk score was generated for individual patients, and weighted based on the predicted regression coefficient in multivariate analysis (Table S3). Next, using the median risk score as a cut-off point, each patient was assigned to either a high- or low-risk group. The Kaplan-Meier and log-rank methods were employed for comparing OS between low- and high-risk cases. Multivariate and stratified analyses were employed to verify the significance of risk scores in the accurate estimation of HCC patient prognosis. Receiver operating characteristic (ROC) curve was employed to examine model estimation accuracy.

Statistical analysis

To generate the survival curve, the method of Kaplan-Meier was employed, and the log-rank assessment was implemented for comparison between the two groups. The Cox proportional hazard model was employed for multi-factorial assessment. All data analyses were done in the R language. A two-tailed $P < 0.05$ was set as the significance threshold.

Results

Differentially expressed NRGs (DE-NRGs)

We downloaded the RNA-seq of 374 tumor and 50 non-tumor tissue specimens from the TCGA website. Overall, 377 primary HCC patients were eligible for this research and were monitored for over one month. We examined the expression profiles of 132 NRGs, and identified 36 DE-NRGs, namely IL1B and CAMK2B, which were down-regulated, and FTL, TYK2, CHMP3, CASP8, FTH1, HSP90AA1, RNF31, SLC25A6, DNM1L, IL1A, CAMK2G, CHMP4C, PPIA, PARP1, CAPN2, BIRC3, PLA2G4B, RBCK1, JMJD7-PLA2G4B, BAX, SHARPIN, PYCARD, HSP90AB1, SQSTM1, PLA2G4C, TRAF2, USP21, PLA2G4E, PYGB, ALOX15, TRAF5, RNF103-CHMP3, GLUL, and PLA2G4F, which were up-regulated. Our DE-NRG identification criteria was $P < 0.05$ and $[\log_2(\text{fold change})] > 1$ (Fig. 1a,d). Fig. 1b presents a scatter plot that illustrates the DE-NRGs in the tumor and non-tumor samples. Owing to their importance in tumorigenesis, we next examined their genetic variations and identified truncated and missense mutations as the most prevalent mutations (Fig. 1c). Overall, 10 genes exhibited mutation rates $\geq 3\%$, among which, USP21 and SHARPIN were most commonly mutated (10%).

Functional enrichment of DE-NRGs

We next employed functional enrichment analysis to determine the physiological function of these genes. Data from the GO functional enrichment and KEGG pathway enrichment analyses are presented in Fig. 2. The GO-rich biological processes included I-kappaB kinase or NF-kappaB cell signal transduction pathways and their regulation, and cytokine-mediated signal transduction pathways; the cellular components included secretory granule cavity, cytoplasmic vesicle cavity, and vesicle cavity; in terms of molecular functions, the genes were mainly enriched in ubiquitin-like protein ligase binding, ubiquitin protein ligase binding, and cytokine receptor binding. In terms of biological process, GO was mainly enriched in the NF-kappaB cell signal transduction pathway, which regulates expression of the adhesion molecule ICAM-1. Aberrant expression of these adhesion molecules can lead to cancer. The cellular components included secretory granule cavity, cytoplasmic vesicle cavity, and vesicle cavity. In respect of molecular functions, the genes were enriched in the binding of ubiquitin protein ligase. The KEGG pathway enrichment assessment divulged that these genes were strongly correlated with necroptosis, nod-like receptor signaling pathway, and shigellosis-related pathways. The scores of Z for most enrichment pathways were greater than zero, suggesting that a majority of the networks were enhanced.

Generation of a HCC prognostic model using the four NRGs

The NRGs that demonstrated statistical significance in univariate assessments were subsequently entered into multivariate test. Multivariate analysis revealed four genes that were significantly related to HCC patient prognosis. Next, on the basis of the multi-factorial Cox proportional hazard regression model, the independent risk gene expression coefficients were obtained. The prognostic prediction model based on these four genes was as follows: prognostic index (PI) = $(0.0029 \times \text{HSP90AA1 levels}) + (0.0087 \times \text{PPIA levels}) + (0.0025 \times \text{SQSTM1 levels}) + (0.0975 \times \text{USP21 levels})$.

Next, we computed the individualized risk score of each case and separated the cases into either a high-risk (nude 171) or low-risk group (nude 172), with the median risk score as the cut-off point. The heat maps of the four NRGs and the curve of Kaplan-Meier for each risk score are presented in Fig. 3a-d. A marked difference was observed in OS rates between the low- and high-risk patients. High-risk cases demonstrated remarkably shorter OS, compared to low-risk cases (the 5-year rates of survival were 57.30% and 73.26%, accordingly, $p < 0.001$). The OS ROC curves were employed to determine the predictability of the risk scores of the four NRGs (Fig. 4d). The AUC value was 0.807, which was markedly elevated, compared to those related to sex, age, grade, tumor stage, tumor N stage, tumor T stage, and tumor M stage. These results suggest that risk characteristics are more likely to accurately predict OS of HCC patients than clinical factors.

NRGs can act as stand-alone prognostic indicators for HCC patients.

Clinicopathological analysis was employed to examine the correlation between clinical features and risk factors (Fig. 4a-c). Based on our results, NRGs are strongly associated with OS (p-value 0.007), T stage (p-value 0.008), and tumor stage ($p < 0.001$). Furthermore, we conducted both uni- and multivariate analyses to validate the independent nature of NRG-mediated HCC prognosis prediction. Using univariate

analysis we revealed that necrosis-related indexes, tumor stage, M stage, and T stage were all associated with OS in HCC patients (Fig. 3e). Hence, we entered these factors into multivariate analysis and revealed that these can be used as stand-alone prognostic indicators for HCC prognosis (Fig. 3f). Given these evidences, we validated that there is a strong correlation between necroptosis and HCC stage. As such, it can be used to predict prognosis in HCC patients. Therefore, the achieved outcomes confirm that the signal related to NRGs can be employed as an independent prognostic factor in clinical practice.

Verification of necroptosis related signatures using independent database

We used the same formula to calculate the risk score of individual cases in the GEO database GSE54236, in order to verify our prior findings. We first separated patients into a high- and low-risk group, according to the median risk score. Next, using GSEA assessment, we revealed cellular networks associated with necroptosis in the GSE54236 dataset. The significant necroptosis-based pathways were cell adhesion molecule (CAM), calcium signaling pathway, autoimmune thyroid disease, and VEGF signaling pathway (Fig. 5a-d). Using Kaplan-Meier assessment, we next demonstrated the predictive performance of our prognostic signature (Fig. 5e). Similar to our prior findings, high-risk cases represented remarkably shorter OS duration, compared to low-risk cases (2-year rate of survival = 57.50% vs 80.62% $P < 0.001$). Our ROC curve also validated the superior predictability of our signature, with AUC values of 0.827, 0.734, and 0.713 at 1-year, 2-year, and 3-year, accordingly (Fig. 5F). Unfortunately, owing to the absence of clinical information, we were not able to analyze other clinical factors via ROC. Overall, our data suggests the outstanding capacity of our newly developed risk signature in predicting HCC patient prognosis.

Discussion

Necroptosis involves cell death via lysis. This separates it from programmed cell death and general necrosis, as it does not depend on caspase activity. Upon the drug- or viral inhibitor-induced blockage of caspase-8, TNFR1, TLR and other receptors become stimulated, and this initiates necroptosis, which involves the phosphorylation of serine / threonine protein kinase 1 (RIPK1). This, in turn, activates RIPK3, and stimulates two distinct signal transduction pathways: phosphorylation and activation of MLKL in necroptosis executors and pore formation in the cell membrane, as well as the stimulation of transcription factors like NF- kappa B and IRF1, which induce inflammatory gene expression [14]. Hence, necroptotic dysregulation is a main contributor to inflammatory diseases. Emerging evidences suggest that necroptosis strongly modulates tumorigenesis and metastasis. Therefore, targeting necroptosis has great potential as a novel anti-tumor therapy [15].

Chronically injured livers almost always turn cancerous, due to the varying types of cell death that occur like necrosis, apoptosis or necroptosis. We demonstrated that oncogene-activated hepatocytes eventually develop cholangiocarcinoma, in the presence of excess necroptosis. However, hepatocytes with the same carcinogenic drivers can lead to HCC if they are not adjacent to dying hepatocytes [16]. In Sod1KO mice [17], enhanced oxidative stress induces necroptosis and related inflammation, which accelerates the

formation of chronic liver disease (CLD), and eventually liver cancer. Similarly, in human CLD, inflammation and necroptosis are markedly elevated, suggesting a role of necroptosis in CLD formation. Hence, necroptosis is a possible candidate for future anti-CLD therapy [18].

Thus far, very limited information is available regarding necroptosis and HCC pathogenesis. Massive databases like TCGA and GEO facilitate an efficient exploration of genetic characteristics. Herein, we employed the TCGA database to identify 36 DE-NRGs between HCC patients and healthy volunteers. Using GO and KEGG analyses, we revealed that most DE-NRGs were enriched in the modulation of NF- κ B signaling, necrotic pathway, necroptosis, and shigellosis. To recognize DE-NRGs that accurately predict HCC patient prognosis, we employed both uni- and multivariate analyses to identify four prognosis-related DE-NRGs, which were then used to generate a prognostic index that allowed the separation of HCC cases into high- and low-risk groups. According to our data, the high-risk patients demonstrated considerably shorter OS duration, compared to the low-risk cases. HCC patients often experience recurrence and metastasis. In fact, the liver to lymph node metastasis occurs in about 25-30% patients [19]. Moreover, lymph node metastasis is strongly correlated with unfavorable patient outcome. Furthermore, the employed TCGA cohort was missing half of the HNSCC patient M-phase data. Hence, they were excluded from our analysis. Using Cox regression analysis, we verified the four NRGs as stand-alone indicators of HCC prognosis. The four NRG-associated risk score corresponded to OS, tumor size, and lymph node metastasis. Given these evidences, our newly developed prognostic index is an accurate reflection of HCC diagnosis and patient prognosis.

Our data also revealed that all indicator genes are closely associated with cancer. In fact, they are all HCC driver genes: HSP90AA1, PPIA, SQSTM1 and USP21. Among them, USP21 has the strongest driving effect, and its HR is 1.1. The gene encodes a family of ubiquitin-specific proteases. In prior studies [20, 21], USP21 was shown to be a pathogenic gene for human pancreatic adenocarcinoma, which accelerates non-small cell lung cancer formation. Moreover, using GSEA analysis, we further analyzed the differences between the low- and the high-risk cases, based on necroptosis index. We identified cellular networks that were particularly enriched in high-risk patients, namely, cell adhesion molecule (CAM), calcium signaling pathway, autoimmune thyroid disease, and VEGF signaling pathway. It was suggested that necroptic gene-based signatures may be related to biological pathways associated with HCC, and their dysregulation may accelerate HCC occurrence. In recent years, the relationship between cell adhesion molecules and tumor occurrence, invasion and metastasis has garnered increasing attention. Prior studies revealed that cell adhesion hinders development of endometrial carcinoma[22][23], particularly, the differential expression of cell adhesion molecules may be intricately related to the tissue differentiation of endometrial carcinoma. Being a unique second messenger, calcium (Ca^{2+}) serves an essential function in modulating biological processes like cell proliferation, survival, apoptosis, and immune response. Moreover, it mediates the functioning of various types of ion channels, Ca^{2+} transport in and out of cells, and Ca^{2+} binding to a variety of intracellular proteins. Furthermore, Ca^{2+} signaling is closely related to multiple tumors, autoimmune diseases, and viral infections [24]. VEGF interacts with its receptors, namely, VEGFR1, VEGFR2, and VEGFR3 to stimulate intracellular signaling pathways that

modulate tumor angiogenesis. In addition, VEGF also modulates numerous immunity-related cells, including dendritic cells, T cells, regulatory T cells, and myeloid derived suppressor cells [25]. The Kaplan-Meier curve, analyzed by OS and ROC of the GSE54236 cohort further confirmed that our newly developed necroptotic signature can indeed serve as a stand-alone predictor of HCC patient prognosis.

To sum up, we developed an NRG index model that can accurately predict HCC patient prognosis. Moreover, we validated our results using an independent patient population using the GEO database. Our conclusion paves way for the enhanced comprehension of the necroptotic state of HCC cells, and further the application of this marker in clinical settings. We recommend additional investigations to confirm the efficacy of our NRG index.

Statements And Declarations

Compliance with Ethical Standards:

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Not applicable.

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Supplementary Tables

Supplementary Tables S1-S3 are not available with this version

Figures

Figure 1

Differential expression of 36 necrosis-related genes (DE-NRGs). a: Heat maps; b: Scatter plot; c: Mutations; d: Volcanic map; N: signifies non-tumor tissue; T: signifies tumor tissue.

Figure 2

GO and KEGG analyses of differentially expressed NRGs. a, c: The first 30 important pathways in GO and KEGG analysis: BP: biological process, CC: cellular component, and MF: molecular function; b, d: The GO and KEGG circles represent the scatter plot of the log FC of a specific gene, and the larger the Z value, the more the expression of the enrichment pathway.

Figure 3

The advancement of a prognostic index on the basis of NRGs. a: Expression profiles of the four differentially expressed NRGs; b: Overall survival curve between the four differentially expressed genes; B: Overall survival curve between low- and high-risk cases; c: Quantity of cases in each risk group; d: Living conditions of cases in each risk group; E: Forest map of univariate assessment; F: Forest map of multivariate assessment.

Figure 4

Validating the HCC prognostic signature in the cohorts. a: Necroptosis related signals in the survival finding group b: necroptosis related signals in the lymph node metastasis group; c: necroptosis related signals in tumor stage group; d: ROC analysis of the clinical parameters and risk characteristics throughout duration of study or until patient expiration.

Figure 5

Validation of the prognostic signature in the GEO dataset. a-d: GSEA analysis of pathways related to necroptosis; e: Overall survival curve of high- and low-risk cohorts; f: Receiver Operating Characteristic analysis.