Ferroptosis-related IncRNA NRAV affects the prognosis of hepatocellular carcinoma via miR-375-3P/SLC7A11 axis

Ke Zong (fcczongk@zzu.edu.cn)
Departments of Hepatobiliary Surgery, Zhengzhou, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China

Caifeng Lin
Shengli Clinical Medical College of Fujian Medical University, Fujian Medical University, Fuzhou, 350001, Fujian, China

Kai Luo
Departments of Hepatobiliary Surgery, Zhengzhou, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China

Yilei Deng
Departments of Hepatobiliary Surgery, Zhengzhou, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China

Hongfei Wang (zzuwang1989@126.com)
Departments of Hepatobiliary Surgery, Zhengzhou, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China

Jianfei Hu
Shengli Clinical Medical College of Fujian Medical University, Fujian Medical University, Fuzhou, 350001, Fujian, China

Shi Chen
Shengli Clinical Medical College of Fujian Medical University, Fujian Medical University, Fuzhou, 350001, Fujian, China

Renfeng Li (z17263883982@163.com)
Departments of Hepatobiliary Surgery, Zhengzhou, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China

---

Research Article

Keywords: IncRNA, ferroptosis, predictive models, NRAV, miR-375-3P/SLC7A11

Posted Date: March 1st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-1749498/v4
Abstract

Ferroptosis has essential value in cancer treatment. It is significant to explore the new ferroptosis-related IncRNAs (FRLs) prediction model in Hepatocellular carcinoma (HCC) and the potential molecular mechanism of ferroptosis-related IncRNAs. Using differentially expressed IncRNAs associated with ferroptosis, we developed a multi-IncRNA prognostic signature in HCC. We used qRT-PCR to detect IncRNA in HCC cells. We performed functional experiments in vitro and in vivo to determine the biological role of NRAV. Interaction between NRAV and miR-375-3P was confirmed by RNA immunoprecipitation (RIP) assay and dual luciferase reporter. We observe 6 differentially expressed IncRNAs related to the progression of HCC. According to Kaplan-Meier analyses, HCC with a high-risk IncRNAs signature has a poor prognosis. In addition, AUC values confirmed the utility of IncRNAs signature for predicting HCC prognosis. Further functional experiments show that the high expression of NRAV can strengthen the viciousness of HCC. Interestingly, we found that NRAV can enhance iron export and ferroptosis resistance. Further studies showed that NRAV attenuated the inhibition of SLC7A11 by miR-375-3P through competitive binding to miR-375-3P, influencing the prognosis of HCC patients. In conclusion, We developed a novel FRLs prognostic model with crucial predictive value for the prediction of hepatocellular carcinoma. NRAV is essential in ferroptosis induction through the miR-375-3P/SLC7A11 axis.

Introduction

The most common type of primary liver malignancy is hepatocellular carcinoma (HCC). The incidence of HCC is on the rise worldwide, causing about 690,000 deaths each year, ranking third among the causes of cancer death [1]. Treatments such as hepatectomy, liver transplantation, microwave ablation, and systemic therapy have progressed. However, due to the limited means of early detection, the diagnosis and curative effect of HCC are still not satisfactory, resulting in a 5-year survival rate of only 7% [2]. It is exciting that the success of immunotherapy in recent years has ushered in a new era of cancer treatment. Immunotherapy has also achieved a remarkable curative effect in hepatocellular carcinoma, including the application of PD-1 monoclonal antibody and CTLA-4 monoclonal antibody. Therefore, continuing research around immunity has positive implications for patients.

Regulatory cell death (RCD) is a mode of cell death controlled by specific signal transduction pathways. The most widely studied RCD types include apoptosis, pyroptosis, necrosis, autophagy, and ferroptosis, each of which has its unique molecular mechanism. Ferroptosis is a novel form of regulated cell death and is characterized by iron-dependent properties. The process of overexpression of iron-dependent unsaturated fatty acids on cell membranes, leading to lipid peroxidation and subsequent induction of cell death, is called ferroptosis. Exogenous or endogenous pathways can induce Ferroptosis. The exogenous pathway is initiated by inhibiting cell membrane transporters, and the endogenous pathway is initiated by blocking the activation of intracellular antioxidant enzymes [3]. The effect of ferroptosis on the efficacy of chemotherapy, radiotherapy, and immunotherapy has been determined [3, 4]. In the process of tumorigenesis, ferroptosis plays a dual role in promoting and inhibiting tumors, which depends on the release of damage-associated molecular patterns (DAMPs) can mediate immunogenic cell death that
could stimulate antitumor immunity, so the combined use of drugs for ferroptosis signals can improve the effectiveness of these therapies [5]. It is an indisputable fact that ferroptosis can enhance the therapeutic effect of immunotherapy [6]. However, many underlying molecular mechanisms remain unclear. Therefore, it is still necessary to strengthen the research on ferroptosis. Enhancing the study of ferroptosis is needed to improve clinical prognosis.

LncRNA is defined as a non-coding RNA of more than 200bp. Current research suggests that dysfunctional lncRNAs play an essential role in tumorigenesis through many biological processes associated with cancer, including apoptosis[7], cell cycle, metastasis, and DNA damage response[8, 9]. In recent years, there has been growing evidence that lncRNAs are strongly associated with ferroptosis. Wang et al. [10] found that LINC00336 could act as a ceRNA and thus inhibit ferroptosis in lung cancer. Overexpression of LINC00336 significantly reduced intracellular production of Fe2+, lipid ROS. Mao et al. found that P53RRA can interact with functional domains of signaling proteins in the cytoplasm by activating the p53 pathway, promoting ferroptosis, and playing a tumor suppressor role[11]. Therefore, studying lncRNA related to ferroptosis and HCC is very important for understanding the mechanism of tumor development.

Our study is based on two public databases, The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC), to construct a signature of FRLs that can be used as a stable marker to predict the prognosis of HCC patients, further verifying the model's potential role in immunotherapy to combine ferroptosis and immunotherapy and increasing the possibility of clinical transformation. The nomogram based on our gene signature and clinicopathological features improved HCC prediction and risk stratification. We then validated critical genes in the predictive model and experimentally verified the biological function of NRAV during ferroptosis in vitro and in vivo. Our findings establish a lncRNA prognostic model related to ferroptosis based on the expression of lncRNA, which complements the mechanism of lncRNA regulating ferroptosis in HCC.

**Methods**

**Data collection**

The RNA sequences of 422 patients (50 normal tissues and 374 tumor tissues) were extracted from the TCGA-HCC database, and the RNA sequences of 445 patients (202 normal tissues and 243 tumor tissues) were extracted from the ICGC-LIRI-JP database. Ferroptosis-related genes were acquired from FerrDb, an online source that supplies a comprehensive and updated checklist of ferroptosis markers, regulative molecules, and ferroptosis-disease associations[12]. In general, we identified 175 (Driver:76; suppressor:47; marker:75) ferroptosis-related genes (Table S1). Pearson correlation evaluation was used to identify the relationships between ferroptosis-related genes and lncRNAs. Connections with the value of correlation coefficients $|R| > 0.5$ as well as a $P$-value $< 0.001$ were taken into consideration as statistically substantial. The clinicopathological attributes of HCC people, consisting of age, gender, stage, TMN, survival condition, and survival time, were accumulated. In this study, we identified
significant differentially expressed lncRNAs (DElncRNAs) between HCC tumor and normal liver tissue (absolute log2FC >= 1 and FDR < 0.05). After screening differentially expressed genetics (DEGs), we performed functional enrichment evaluation and KEGG analysis using upregulated and downregulated ferroptosis-related differentially expressed genetics (DEGs). Enrichment analysis using metascape for EDGs data.

**Building of the ferroptosis-related lncRNAs prognostic signature**

Univariate Cox regression, LASSO, and multivariate Cox regression evaluations were used to establish the last variables for building the prognostic threat score model. stratified based on risk rating (Coefficient IncRNA1 × expression of IncRNA1) + (Coefficient IncRNA2 × expression of IncRNA2) + ⋯ + (Coefficient IncRNAn × expression IncRNAn). Based on the threat scores for OS, HCC clients with fibrosis were separated into high- or low-risk groups making use of the typical score as a cut-off, and also Kaplan-Meier contours were calculated for both teams.

**Predictive Nomogram building and validation**

To execute genetics established enrichment evaluations stabilized, all genetics matters were imported right into the GSEA software application as well as the Molecular Signature Database (MSigDB) Trademark, KEGG, and also Genetics Ontology gene libraries were utilized to identify enriched gene sets[13], which were then searched in the TCGA-HCC database. Enriched gene sets were selected on the basis of statistical significance (incorrect exploration rate FDR q value < 0.25, and normalized p-value < 0.05)[14]. We built a nomogram incorporating typical medical variables such as age, gender, grade, tumor stage, and the risk rating originated from the prognostic trademark to assess the possible 3- and 5-year OS of clients with HCC.

**Gene expression and Immunity analysis**

At the same time, the CIBERSORT[15, 16], ESTIMATE[17], quanTIseq[18], xCell[19], MCP-counter (or mMCP-counter for mouse)[20], EPIC[21], and TIMER[22] formulas were analyzed the CC or cell sorts of immune reactions in heterogeneous examples among risky as well as low-risk teams based on FRLs trademark. The differences in immune feedback under various formulas were revealed using a Heatmap. In enhancement, ssGSEA was utilized to quantify the tumor-infiltrating immune cell subgroups between both groups and assess their immune function. Many possible immune check factor molecules were determined that could be targeted and incorporated with potential medication therapy[23].

**Tissue specimens**

86 matched hepatocellular carcinoma tissues and paracancerous tissues were obtained from patients with hepatocellular carcinoma diagnosed by Fujian Provincial Hospital and The First Affiliated Hospital of Zhengzhou University. All experiments involving human samples and clinical data were approved by the Accreditation Committee of Fujian Provincial Hospital and The First Affiliated Hospital of Zhengzhou University.
RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

According to the manufacturer's protocol, total RNA from HCC cells, tissues, and matched non-cancerous tissues was isolated using the TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using the PrimeScript RT Reagent Kit (Takara, Dalian, China). Bulge-loop™ miRNA RT-qPCR Primers were applied to determine the level of miRNAs. The real-time PCR reactions were performed using StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, MA, US). The program settings on temperature cycling were followed as instructed by the manufacturer. The sequences of primers are listed in Table S2.

Western blot analysis

In brief, proteins were isolated from HCC cells using RIPA buffer (Solarbio, Beijing, China) supplemented with proteinase inhibitors, and the protein concentration was determined with a BCA reagent (Beyotime, Beijing, China). Cell lysates were separated on SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). After the membranes were blocked in 5% skim powdered milk for 2 h, they were incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study included: ACSL4 (ET7111-43, HUABIO), GPX4 (ET1706-45, HUABIO), SLC7A11 (Abcam, ab175186), and GAPDH (Proteintech, 80570-1-RR). Next, the membranes were incubated with secondary antibodies (HUABIO, Hangzhou, China) at room temperature for 1h. After washing three times, the targeted proteins were visualized using enhanced chemiluminescence (ECL) reagent (Millipore, MA, USA). GAPDH was used as the loading control in this study.

RNA immunoprecipitation (RIP)

RNA immunoprecipitation (RIP) assay was performed using Magna RIP™ RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) following the manufacturer's protocol. The cell extract was incubated with magnetic beads conjugated with anti-Argonaute 2 (AGO2) or anti-IgG antibody (Millipore, Billerica, MA, USA) for 6h at 4°C. The beads were washed and incubated with Proteinase K to remove proteins. Finally, isolated RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), then the purified RNA was subjected to qRT-PCR analysis.

Dual-luciferase reporter assay

The full-length wild-type (WT) sequence of NRAV and the indicated mutant NRAV containing the predicted miR-375-3p binding sites were separately synthesized and cloned into the dual-luciferase reporter vector (Genechem, Shanghai, China). The resulting dual-luciferase reporter plasmids (WT or Mut) were co-transfected with the miR-375-3p mimic or inhibitor into hepg2 or HuH-7 cells, respectively, using Lipofectamine 3000. After 48h of incubation, the relative firefly luciferase activities concerning the corresponding Renilla luciferase activities were measured and analyzed using a Dual-Luciferase Assay System (Promega, Fitchburg, WI, USA) following the manufacturer's protocol.

Iron assay
Intracellular ferrous iron (Fe^{2+}) level was determined using the iron assay kit (ab83366, Abcam) according to the manufacturer's instructions. Cells were collected and washed in ice-cold PBS and homogenized in 5× volumes of iron assay buffer on ice, then centrifuged (13,000 × g, 10 min) at 4°C to remove insoluble material. The supernatant was collected, iron reducer was added to each sample before mixing, and incubated at room temperature for 30 min. Then, 100 μL of the iron probe was added to each sample, mixing and incubating the reaction for 1 h at room temperature in the dark. The absorbance at 593 nm was measured immediately using a colorimetric microplate reader.

**Lipid ROS measurement**

Lipid ROS level was analyzed by flow cytometry using BODIPY-C11 (GLP BIO, GC40165) dye. Cells were seeded at 2.5 × 10^5 per well in a six-well dish and grown overnight for 12 h. Cells were washed once with PBS. Cells were then stained with 2 mL medium containing 5 μM of BODIPY-C11 and incubated at 37°C for 20 min in the dark. Cells were washed twice with PBS to remove excess labeling mixture, followed by resuspending in 500 μL fresh PBS (DPBS, Gibco). The cell suspension was filtered through a 0.4 μM cell filter and subjected to flow cytometric analysis to detect the amount of intracellular lipid ROS. The fluorescence intensities of cells per sample were determined by flow cytometry using the BD FACS Aria cytometer (BD Biosciences). A minimum of 10,000 cells was analyzed for each sample. Data analysis was evaluated using the FlowJo Software.

**Subcutaneous xenograft model**

The male nude mice (BALB/c, aged 4-6 weeks, 18-20 g) were randomly divided into two groups and inoculated with cells as follows: sh-NC stable transfected hepG2 Cell (1 × 10^7 cells); sh-NRAV stable transfected hepG2 Cell (1 × 10^7 cells); Cells were mixed with matrigel (1:2) and inoculated subcutaneously at the right rear back region. Tumors were measured weekly with calipers, and tumor volume was calculated as \( V = \frac{L \times W^2}{2} \).

**Statistical analysis**

The experimental data were analyzed using statistical analysis software, including GraphPad Prism 8.0 software (GraphPad) and the R package (V.3.3.4). Data are reported, including estimation of variation within each group. Unpaired t-test or one-way ANOVA was used to measure differences between groups. Chi-square \( (\chi^2) \) tests compared categorial variables. Statistical significance was determined at \( P < 0.05 \).

**Results**

**Identification of robust DEGs**

The FRLs signature was constructed following the flowchart shown in **Figure 1A**. We identified 174 DEGs associated with ferroptosis, including 38 down-regulated and 136 upregulated genes (Fig 1B-D, Table S1).
Enrichment analysis of overexpressed genes showed that they are mainly related to ferroptosis, cellular response to chemicals, oxidative and oxidative stress. (Fig. 1E)

**Construction and validation of FRLs**

Then a predictive risk assessment model (FRLs) based on 6 ferroptosis-related genes was constructed using the LASSO model with an optimal penalty parameter ($\lambda = -1.8$). Figures 2A, B. show the cvfit and lambda curves of the model. Kaplan-Meier curve analysis showed statistically significant differences in patient survival between high and low-risk groups ($P < 0.001$, Fig. 2C). Figure 2D demonstrated the variation in risk scores between high and low groups was confirmed, as well as more deaths and fewer years of survival in the high-risk group. As shown in Figure 2E, the expression of 6 RNAs associated with ferroptosis was higher in the high-risk group than in the low-risk group. Meanwhile, the AUC of the signature IncRNAs was 0.795. Then, Our data suggest that FRLs are superior to traditional clinicopathological factors in predicting the prognosis of HCC patients and are an independent prognostic factor for HCC patients (Fig. 2F, G). The AUC predictive values of the FRLs feature for predicting patient survival at 1, 3, and 5 years were 0.795, 0.749, and 0.748, respectively (Fig. 2H).

Subsequently, we used univariate Cox analysis, a Robust likelihood-based survival model, and multivariate Cox analysis to construct a signature composed of IncRNAs and tumor stage, independent prognostic factors of OS in HCC patients ($P < 0.001$; Fig. 3A, B). Figure 3C shows the relationship between FRLs and Ferroptosis-related genes. In addition, the relationship between FRLs and clinicopathological factors was shown by heat map (Fig. 3D). And a hybrid nomogram including clinicopathological features and FRLs features was constructed (Fig. 3E). The results are stable and accurate and, therefore can be applied to the clinical management of HCC patients. Gene set enrichment analysis (GSEA) showed that high-risk groups based on FRLs typing were mainly associated with cell cycle and tumor-related pathways (Fig. 3F). On the other hand, the low-risk groups based on FRLs typing are mainly associated with multiple metabolic processes in cells. (Fig. 3G). The immune heat map algorithm of Figure S3A is based on CIBERSORT, ESTIMATE, quanTIseq, xCell, MCP-counter (or mMCP-counter for mouse), EPIC, and TIMER. To further investigate the association between risk scores and immune cells as well as function, we used the ssGSEA R package to quantify the enrichment scores of 16 immune cell types and their associated biological functions, which revealed that APC co-stimulation, cytolytic activity, MHC class I, type I INF response and statistically significant difference in type II INF response between high and low-risk groups (Fig. S3B). Knowing that checkpoint inhibitors are essential for immunotherapy, we further explored the differences in immune checkpoint expression between high and low-risk groups. We found that the two groups' presentations of HHLA2, TNFRSF14, and CTLA4 significantly differed. (Fig. S3C).

**Identification of NRAV in HCC**

Next, to further confirm the role of IncRNAs in the HCC prognostic model. By analyzing the data of 424 cases of liver cancer in TCGA, the expression levels of 6 FRLs are shown in Figure 4A. Through the prognostic analysis of three molecules in the Gepia database, we found that NRAV was significant for OS (Fig. 4B), while DANCER, MKLN1-AS1, and ZFPM2-AS1 showed little difference (Fig. S4A-C), and no
difference between AL137186.2 and LNCSRLR. So we selected NRAVs with the most significant difference in HCC prognosis for further validation. NRAV is located on chromosome 12. In different liver cancer cell lines, the expression of NRAV is shown in Figure 4C. Compared with Paracancer, NRAV is generally upregulated in cancer and can be stably expressed in various commercial liver cancer cells (Fig. 4D). Nuclear and cytoplasmic separation experiments detected the subcellular localization of NRAV in hepatoma cells, and it was found that NRAV was located not only in the cytoplasm but also in the nucleus (Fig. 4E). With the deepening of the research, researchers have found that many non-coding RNAs can also work by translating into proteins. To study the action mode of NRAV, we use software to predict and analyze the protein-coding ability of NRAV. ORF reading frame prediction shows a reading frame, and ORF12 is greater than 300bp (Fig. S4D), arousing our intense curiosity. However, it is a pity that coding-potential computer (CPC) analysis shows that the coding possibility of NRAV is very little (Fig. S4E), and we can't detect any notable protein bands through in vitro translation experiments. In contrast, the control gene can catch the band (Fig. 4F), which also supports that NRAV is a non-coding RNA.

**NRAV promotes HCC tumorigenesis in vitro and in vivo**

Next, we studied the carcinogenic function of NRAV in HCC. To evaluate the biological function of NRAV in HCC cells, we designed and constructed three siRNA targeting NRAV to change their expression specifically. QRT-PCR results showed that all three siRNAs effectively silenced NRAV (Fig. 5A). In addition, the introduction of the recombinant human NRAV gene successfully realized the overexpression of the gene (Fig 5B). The results of CCK-8 and clone formation experiments showed that knockout of the NRAV gene effectively inhibited the proliferation of hepG2, while up-regulation of NRAV significantly enhanced the proliferation of HuH-7 (Fig. 5C-F). Transwell's experiment showed that the down-regulation of NRAV significantly decreased the migration ability of hepG2, while the up-regulation of NRAV enhanced the migration ability of HuH-7 cells (Fig. 5G, H). Next, we constructed a CDX model to examine the carcinogenic function of NRAV in vivo. Compared with the control group, the tumor volume and weight in the knockout group decreased significantly, and the tumor growth rate slowed down (Fig. 5I-K). To sum up, these experimental results show that NRAV has a carcinogenic function in HCC.

**NRAV promotes iron export and ferroptosis resistance as a sponge of miR-375-3P**

Iron accumulation was increased in hepG2 cells infected with sh-NRAV lentivirus compared with control and decreased in HuH-7 cells infected with oe-NRAV compared with Vector (Fig. 6A). Analysis of ROS by flow cytometry showed a similar trend as the iron accumulation results (Fig. 6B, C). These results suggested that NRAV plays a negative regulatory role in ferroptosis in HCC. Furthermore, western blot analysis in Figure 6D revealed that SLC7A11 and GPX4 expressions were decreased in hepG2 cells infected with sh-NRAV lentivirus compared with control and increased in HuH-7 cells infected with lv-NRAV compared with Vector. The opposite trends were observed in ACSL4 expressions, and the most obvious change is SLC7A11. By Exploring downstream of NRAV through annolnc2 database and upstream of SLC7A11 through starbase, we found that miR-375-3P played a key role in this process. miR-375-3P was also reported to play a role in regulating the key molecule SLC7A11 of ferroptosis in other cell
lines[24]. However, the specific mechanism by which NRAV regulates the miR-375-3P/SLC7A11 axis has not been explored. Through bioinformatics methods, we discovered the binding site of NRAV and miR-375-3P (Fig. 6E). We conducted immunoprecipitation assays using the AGO2 antibody and found that the AGO2 antibody was able to enrich both endogenous miR-375-3p and NRAV (Fig. 6F). To further verify the binding of NRAV and miR-375-3p, we constructed two NRAV luciferase reporter plasmids: wild type and miR-375-3p binding site mutant (Fig. 6G). The luciferase activity of the wild type can be significantly inhibited by miR-375-3p mimics, but the luciferase activity of the mutant has no significant change (Fig. 6H).

Moreover, the knockdown of NRAV significantly increased miR-375-3P expression, while the overexpression of NRAV significantly reduced the expression of miR-375-3p (Fig. 6I, J). Western blot analysis confirmed that the miR-375-3P inhibitor and mimic reversed the alterations in SLC7A11 caused by alterations in NRAV. In summary, these results prove that NRAV promotes iron export and ferroptosis resistance through the regulation of the miR-375-3p/SLC7A11 axis.

**High expression of NRAV is associated with poor prognosis in HCC patients**

Through the analysis of q-PCR results of clinical samples, the results showed that the expression of NRAV was negatively correlated with miR-375-3P and positively correlated with SLC7A11 (Fig. 7A, B). To further study the clinical significance of NRAV in HCC, the clinical characteristics of patients with high NRAV expression in our hospital showed that the clinical outcome of HCC patients with high NRAV expression was worse than that of patients with low NRAV expression (Fig. 7C, D). The pattern of ferroptosis regulated by NRAV is shown in figure 7E. These results suggest that the high expression of NRAV may predict a poor prognosis in patients with HCC.

**Discussion**

In recent years, significant progress has been made in the clinical treatment of HCC. It is still one of the leading causes of cancer-related deaths worldwide, suggesting an urgent need to develop new therapies for HCC [4]. Ferroptosis are thought to play an essential role in immunotherapy. Many kinds of IncRNA are thought to have abnormal expression and participate in cancer progression by binding to genetic material and encoding proteins or other small molecular peptides[25, 26]. Therefore, we developed a IncRNA prognostic risk model combined with ferroptosis. In this study, we first identified 84 genes and then identified 52 significant IncRNA associated with ferroptosis. Finally, 6 IncRNA characteristic models related to ferroptosis were established, most of which have been reported to affect the progression of liver cancer and are associated with poor prognosis, including NRAV[27]. He et al. found that ZFPM2-AS1 acts as a miRNA sponge and promotes cell invasion through regulation of miR-139/GDF10 in hepatocellular carcinoma [28], Ma et al. Found that DANCR was upregulated in tumor tissues and plasma of patients with HCC, and its expression was highly correlated with microvascular, and liver capsule invasion of HCC [29], MKLN1-AS[30], LNCSRLR[31] and AL137186.2[32] also be associated with the prognosis of patients with HCC, which are consistent with our findings. Moreover, after correcting for
traditional clinical risk indicators, the six-FRLs signature model was shown to be an independent predictive factor for HCC. This result suggested that the six-FRLs signature could reliably predict the prognosis of HCC patients.

Although adjuvant chemotherapy, immunotherapy, and other anticancer therapies have shown encouraging results in early cancer treatment, the middle and later stages of treatment are challenging in most cases. Therefore, it is imperative to find a more effective cancer treatment strategy. Ferroptosis has long been proven to be closely related to immunotherapy[33, 34], so we further explored the connection between the model and immunity. We studied the difference in immune checkpoint expression between the two groups and found that there were significant differences not only in T cell function between the two groups but also in other genes expressed, including checkpoint (suppression), cytolysis, HLA, inflammatory regulation, co-stimulation, co-inhibition, and type II INF response. We further discussed the differences in the expression of immune checkpoint genes between the two high and low-risk groups. The results showed significantly different expressions of CD44, PDCD-1 (PD-1), HHLA2, CTLA4, etc., between the two groups. And most of them play a key role in immunotherapy.

To further study the regulation of IncRNA on ferroptosis, we selected the IncRNA NRAV with the most pronounced effect on prognosis for validation. It has been proved that NRAV has a clear relationship with the occurrence and development of hepatocellular carcinoma. Wang et al. have confirmed that NRAV can promote the event and development of HCC through Wnt/ β-catenin signal pathway[27]. In addition, several HCC-related prognostic models have shown that NRAV is related to immunity and cell death[35-37]. Our results show that the change of NRAV expression can significantly affect the ferroptosis-related proteins SLC7A11, GPX4, and ACSL4, especially SLC7A11, and can change the Fe²⁺ content and ROS level in HCC cells. Through a series of tests, we found that NRAV can change the expression of SLC7A11 by binding to mir-375-3P. Our results show that NRAV promotes the occurrence and development of hepatocellular carcinoma and affects ferroptosis in hepatocellular carcinoma through the mir-375-3P/SLC7A11 axis.

Compared with other IncRNA prognostic models related to ferroptosis in HCC [38-40], this study has more in-depth basic research, larger sample size, and more vital prediction accuracy. Through lasso regression analysis and the performance of IncRNA in liver cancer, we selected the most representative FRLs-NRAV and verified it, and first found the regulation of NRAV via miR-375-3P/SLC7A11 axis on ferroptosis. Still, this study also had some defects, including the lack of research depth, and the mechanism research is relatively simple. At the same time, due to the difficulty of clinical data collection and verification, the number of related clinical samples is relatively small. In the future, we will continue to study the role of NRAV in immunity to improve the mechanism of ferroptosis and immunotherapy.

**Conclusion**

In conclusion, we developed a new model that can be used for clinical prognosis and demonstrated that NRAV could affect ferroptosis in HCC cells through the mir-375-3P/SLC7A11 axis and then influence the
onset and progression of HCC. Our findings expand the mechanism of ferroptosis regulation in HCC and provide new markers for HCC prognosis.

**Abbreviations**

HCC: Hepatocellular carcinoma;

GEO: Gene Expression Omnibus;

ICGC: International Cancer Genome Consortium

RIP: RNA immunoprecipitation;

RCD: Regulatory cell death;

TCGA: The Cancer Genome Atlas;

DEGs: Differentially expressed genes;

KEGG: Kyoto Encyclopedia of Genetics and Genomes;

GO: Genetics Ontology;

MSigDB: Molecular Signature Database;

ECL: Enhanced chemiluminescence;

CDX: Cell line-derived xenograft;

OS: Overall survival;

DFS: Disease-free survival;

CCK-8: Cell Counting Kit-8;

FRLs: Ferroptosis-related IncRNAs;

GSEA: Gene set enrichment analyses;

CPC: Coding-potential computer;

**Declarations**

**Ethical Approval and Consent to participate**
All participants provided informed written consent. This study was approved by the Human Research Ethics Committees of the Fujian Provincial Hospital and was carried out following the principles embodied in the Declaration of Helsinki. The patients provided their written informed consent to participate in this study. All in vivo animal experiments were approved by the Committee on the Ethics of Animal Experiments at the Shanghai University of Traditional Chinese Medicine.

Consent for publication

All authors have given their consent for publication.

Availability of supporting data

All data in our study are available upon request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by The Key Scientic Research Project Plan of Henan University (Grant No.20A320037)

Authors' contributions

RF. L. and S. C. designed the study. K.Z. performed the study and wrote the paper. CFL. and JF. H. conducted experiments and collected the samples and clinical data. K. L. participated in data analysis. YL. D. assisted with experiments. HF. W. collected the samples and clinical data. All authors read and approved the final manuscript. No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Yi Huang for permitting us to conduct our experiments at the Center for Experimental Research in Clinical Medicine for this study. This manuscript has been preprinted [Ke Zong, Caifeng Lin, Kai Luo et al. Ferroptosis-related IncRNA NRAV affects the prognosis of hepatocellular carcinoma via miR-375-3P/SLC7A11 axis, 12 August 2022, PREPRINT (Version 3) available at Research Square [https://doi.org/10.21203/rs.3.rs-1749498/v3]. And as shown in the Supplemental Files [41].

Authors' information

Ke Zong, Cai-Feng Lin, Shi Chen, and Ren-Feng Li contributed equally to this work.

Supplementary Materials

Figure S1. A. Heatmap for immune responses based on CIBERSORT, ESTIMATE, quanTLseq, xCell, MCP-counter, EPIC, and TIMER algorithms among the high and low-risk groups. B. The correlation between immune cell subpopulations and related functions was evaluated using ssGSEA. C. Expression of
common immune checkpoints between the high and low-risk HCC patients. Figure S2. A-C. Overall survival of HCC patients (N=362) from the Gepia project with high or low expression levels. D. The protein-coding potential analysis of NRAV was performed using the ORF finder from NCBI. E. Using CPC 2.0 to predict the coding capability of NRAV. Table S1. The list of 174 ferroptosis-related DEGs. Table S2. The list of primer sequences. (Supplementary Materials)

References


**Figures**
Figure 1

A. The study flowchart. (B-E) A screen of the ferroptosis-related differentially expressed genes in hepatocellular carcinoma.
Figure 2

FRLs signature for prognostic prediction. (A, B) LASSO regression model of the prognostic FRLs. C. Kaplan-Meier curves resulted from the log-rank test for survival analyses. D, E. Risk survival status plot. F. The AUC values of the risk models. G. The DCA of the risk models. H. The AUC is for predicting the 1, 3, and 5-year survival rates of HCC patients.
Figure 3

Figure 4

Identifying NRAV. A. Expression levels of 6 selected genes between tumors and peritumor tissues in TCGA database. B. Overall survival of HCC patients from geopia with high or low NRAV expression levels. C. The expression levels of NRAV in HCC cells (HuH-7, SKhep1, hep3B, hepG2) D. Quantitative real-time PCR was performed to examine the expression of NRAV in tumors and matched non-tumor tissues from HCC patients. E. RT-PCR was performed to examine cytoplasmic or nuclear NRAV RNA levels in hepG2 cells. F. The constructs were transfected into HEK293T cells for 48h with an N-terminal Flag tag. Cell lysates were harvested and subjected to Western blotting with Flag antibody. Flag-SRSF5 served as a positive control. Results are presented as mean ±SD. *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 5

NRAV functions as an oncogene in HCC. A. The expression of NRAV in hepG2 cells was analyzed by qRT-PCR after transfection with three siRNAs or the control siRNA (si-NC). B. The levels of NRAV in HuH-7 cells were analyzed by qRT-PCR after stable transfection with the NRAV overexpression or the control vector. C-F. Cell proliferation was assessed by CCK-8 (C, D) and colony formation (E, F). G-H. Transwell assays confirmed that the migration ability of the HCC cell line was suppressed with NRAV knockdown (G) and enhanced with NRAV overexpression (H). I-K. Representative images of tumors from nude mice inoculated with HCC cells and NRAV knockdown decreased tumor volume and tumor weight of nude mice. Scale bars, 50 μm. Results are presented as mean ± SD. *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 6

NRAV induces iron export and ferroptosis resistance via sponging miR-375-3P in HCC. A. Iron content increased after NRAV knockdown and decreased after NRAV overexpression. B-C. Flow cytometry of ROS levels in HCC cells after NRAV knockdown or overexpression. D. Western blot showed expressions of the ferroptosis-related proteins (ACSL4, SCL7A11, and GPX4) in hepG2 Cell with NRAV knockdown and HuH-7 Cell with NRAV overexpression as well as control. E. NRAV and MIR-375-3P binding to the secondary structural diagram from AnnoLnc. F. RIP assay confirmed the interaction of miR-375-3P with NRAV in HCC cells. G. Schematic illustration of the NRAV-WT and NRAV-Mut luciferase vectors. H. Relative luciferase activities in hepG2 and HuH-7 cells co-transfected with NRAV-WT or NRAV-Mut and the miR-
375-3P mimic, inhibitor, or corresponding negative control. I-J. The expression level of miR-375-3P in each group transfected was verified through RT-qPCR. K. The SLC7A11 protein levels in HepG2 and HuH-7 cells from different groups were determined by western blot analysis. GAPDH was used as a control. Results are presented as mean ± SD. *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 7
Overexpression of NRAV is associated with poor prognosis in HCC patients. A-B. The correlation between NRAV and miR-375-3P or SLC7A11 in HCC was analyzed by Pearson correlation analysis. C-D. Overall, disease-free survival analyses were performed to assess the impact of NRAV expression high and low in HCC patients. E. Schematic illustration of the NRAV/miR-375-3P/SLC7A11 axis in HCC cells. Results ar

**Supplementary Files**

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

- SupplementFigure.docx
- FigS3.tif
- FigS4.tif
- tableS1.xls
- tableS2.docx