Identification of Iron Metabolism-Related Genes Signature and Novel Role of FLVCR1 in Hepatocellular Carcinoma

Jianhui Chen  
Wenzhou Medical University First Affiliated Hospital

Chuan HU  
Qingdao University Medical College

Reguang Pan  
Wenzhou Medical University First Affiliated Hospital

Xuedan Du  
Wenzhou Medical University First Affiliated Hospital

Haotian Fu  
Wenzhou Medical University First Affiliated Hospital

Yunfeng Shan (✉ shanyunfeng@wmu.edu.cn)  
Wenzhou Medical University First Affiliated Hospital  https://orcid.org/0000-0003-4811-8932

Primary research

Keywords: HCC, signature, iron metabolism, FLVCR1

DOI: https://doi.org/10.21203/rs.3.rs-45663/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background: Hepatocellular carcinoma (HCC) is the main and highly malignant histological subtype of liver cancer. We tried to construct a novel signature with iron metabolism-related genes to provide new therapeutic targets and improve the prognosis for HCC patients.

Methods: The gene expression data of 70 iron metabolism-related genes and its relevant clinical information were obtained from The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) databases. Consensus clustering analysis was performed to determine clusters of HCC patients with different OS. Cox regression and LASSO regression analyses were used to establish a prognostic signature. Receiver operating characteristic (ROC) and Kaplan–Meier analyses were carried out to examine the predicted performance of the signature.

Results: Consensus clustering analysis determined two clusters of HCC patients with different OS (p<0.01), TNM stage (p<0.05) and pathological grade (p<0.05). A nine-gene prognostic signature established with iron metabolism-related genes can independently predicate the prognostic of HCC patients. The ROC curves showed a great performance of the signature. In addition, FLVCR1, a hub gene with the highest mutation frequency in our signature, showed the significantly prognostic value in HCC patients. High FLVCR1 expression was significantly associated with poor prognosis and aggressive progression in HCC patients. The promoter methylation level of FLVCR1 was lower in HCC samples with aggressive progression status. The FLVCR1 expression was positively correlated with the infiltration level of B cell, CD4+ T cell, macrophage, neutrophil and dendritic cell.

Conclusion: Our study first established a signature related to iron metabolism and identified FLVCR1 as a potential therapeutic target. These findings provided more treatment strategies for HCC patients.

Introduction

Hepatocellular carcinoma (HCC) is the most common histological subtype of liver cancer, accounting for >80% of primary liver cancer(1). It is estimated that HCC is the fourth most common cause of cancer-related deaths worldwide, and incidence and mortality continue to increase(2, 3). Approximately 85% of HCC cases are assessed to occur in developing countries or regions, especially in Eastern Asia and sub-Saharan Africa(4). Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are the main risk factors for HCC(5). Surgical resection, liver transplantation, tumor ablation, chemotherapy have been proven to be beneficial to the survival of HCC patients(6). However, most patients are diagnosed at an advanced stage, which leads to awfully poor prognosis for HCC patients.

Iron is an indispensable trace element for human health, and it plays a special role in the biological processes of cells, such as DNA synthesis, oxygen transport and cellular respiration (7). In general, iron homeostasis is maintained in healthy cells by balancing iron uptake, utilization, storage and export. Dysregulation of this balance is related to various diseases, including cancer(8). Numerous studies have indicated that iron has dual properties in the progression of cancer(9, 10). On the one hand, excess iron
provides fuel to meet the demands of the rapid growth of tumor cells(11). The aberrant expression of iron metabolism-related genes in cancer typically has the characteristics of upregulation of genes for iron uptake and downregulation of genes for iron outflow(12). Yet, excessive iron can produce reactive oxygen species (ROS) and destroy lipids in cell membranes, thereby triggering ferroptosis(13). Ferroptosis is a novel type of iron-dependent programmed cell death, which seems different from apoptosis, necrosis, and autophagy(14). In recent years, ferroptosis has been regarded as a new strategy of cancer treatment, and a variety of ferroptosis inducers have been applied(15, 16).

Since the liver is the chief organ for storage of iron, excess iron is an important risk factor for HCC(17). Therefore, targeting iron metabolism is one of the promising therapeutic strategies in the treatment of HCC. In this study, we tried to first construct and validate a reliable prognostic model using iron metabolism-related genes for HCC patients. We suggested that our model can provide new targets and strategies for HCC treatment.

Method

1. Data Sources

Seventy iron metabolism-related genes were collected from the published literature(18). RNA-seq transcriptome and clinical data sets were downloaded from The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) databases. The expression data of FLVCR1 in Wurmbach and Guichard cohorts were obtained from the Oncomine database. Patients with unknown clinicopathological characteristics, survival time and status are excluded.

2. Consensus clustering analysis

Based on the expression pattern of iron metabolism-related genes, all tumor samples were clustered into several subgroups by using “ConsensusClusterPlus” package of R. The difference of overall survival (OS) of HCC patients in different subgroups were analyzed using the 'survival' package.

3. Functional analyses

GO (gene ontology) function and KEGG (kyoto encyclopedia of genes and genomes) pathway analyses were performed using clusterprofiler package, and P<0.05 was used as a threshold to screen the major enrichment functions and pathways. Gene set enrichment analyses (GSEA) were conducted to observe the top enriched functions and signal pathways in the Molecular Signatures Database with adj. p < 0.05.

4. Construction and validation of a prognostic signature

We first performed univariate analysis on the TCGA cohort to screen for genes associated with overall survival (OS) (p<0.05), and used LASSO regression analysis to further select them. Finally, the genes were identified, and their regression coefficient(β) were obtained using multivariate cox regression analysis. After calculating the risk score of each patient, all patients were divided into two risk groups according to
the median risk score. The predicated capability of the prognostic signature was evaluated using receiver operating characteristic (ROC) and Kaplan–Meier analysis in TCGA and ICGC cohorts.

5. Establishment of a nomogram

Cox regression analyses were conducted to estimate the prognostic value of multi-gene signature and clinicopathologic characteristics. Independent prognostic factors identified by multivariate Cox regression analysis were incorporated into the establishment of a prognostic nomogram. A calibration plot was conducted to evaluate predicated performance of the prognostic model.

6. Cell culture

The human liver cell line L-02 and HCC cell lines SMMC-7721, CSQT2, Huh7 and HepG2 were purchased from the Shanghai Institute of Biosciences Cell Resource Center, Chinese Academy of Sciences. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum, and placed at 37 °C in a cell culture incubator with 5% CO2.

7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Collected cells were thoroughly ground by adding an appropriate amount of TRIzol (Invitrogen Corporation), and RNA was extracted based on the kit directions. cDNA was finally obtained by a reverse transcription kit (PrimeScript RT reagent kit, TaKaRa Corporation). The specific primers used were as follows: FLVCR1 (F:5'-GAGCAGAGATGTCACATGGCGAAG-3', R:5'-CTCATGGCTTGGTGCTGTCCTTG-3').

8. Immune infiltration

TIMER (https://cistrome.shinyapps.io/timer/) is a website for systematically analyzing immune infiltration across diverse cancer types. In this research, relationships between the infiltration level of tumor immune infiltration cells (TIICs) and FLVCR1 expression or copy number variation were conducted.

9. Statistical analyses

SPSS 21.0, Graph Prism7.0 and R software 3.6.1 were used for all statistical analyses. In general, p<0.05 is considered a statistically significant difference.

Results

1. The molecular mechanism of iron metabolism-related genes

To explore the molecular mechanism of 70 iron metabolism-related genes, the gene enrichment analyses were performed. GO analysis demonstrated that iron metabolism related mechanisms such as iron ion homeostasis, transition metal ion homeostasis, cellular iron ion homeostasis, cellular transition metal ion homeostasis, iron ion transport and iron ion transmembrane transport etc. were enriched (Figure1a).
Ferroptosis, mineral absorption, porphyrin and chlorophyll metabolism, ABC transporters and vitamin digestion and absorption were associated with these genes in KEGG analysis (Figure 1b).

2. Association between iron metabolism-related genes and HCC prognosis

Based on the expression pattern of 70 iron metabolism-related genes, we determined the optimal specimen clustering number as two via consensus clustering analysis (Figure 2a). The Kaplan–Meier curve revealed that the HCC patients in cluster 2 had a distinctly worse OS than those in cluster 1 (p = 0.006) (Figure 2b). Then, the relationship analyses of clinicopathological characteristics between the two clusters showed significant differences for the stage and grade (p < 0.05) (Figure 2c). These findings indicated that the clustering results were associated with the prognosis and malignancy of HCC.

Then, we further explored the difference between the two clusters. Setting adjust p = 0.05 and |log2FC| = 0.5, we screened the differentially expressed genes in cluster 2 compared to cluster 1 (Figure 3a). Subsequently, we annotated the biological functions of these genes using functional enrichment analyses. GO enrichment showed that differential genes were significantly enriched in terms associated with cell junction, such as cell-cell junction, collagen-containing extracellular matrix and bicellular tight junction etc. (Figure 3b) In addition, multiple cancer-related pathways were found in KEGG pathway analysis, such as PPAR, TGF-beta and AMPK signaling pathways (Figure 3c). Circle plots visualized the Top significantly altered GO functions and KEGG pathways (Figure 3d, e).

3. Construction and validation of an iron metabolism-related prognostic signature

To explore the prognostic role of 70 iron metabolism related genes for patients with HCC, Cox regression analyses and LASSO regression analysis were conducted in TCGA cohort. First, we used univariate Cox analysis to identify 22 genes closely related to the prognosis of HCC patients (Figure 4a). Then, after LASSO regression analysis and stepwise multivariate Cox analysis, an optimal nine-gene prognostic signature (FBXL5, SLC48A1, BMP6, HAVCR1, ALAS1, CD163, PCBP2, FLVCR1, ABCB6) was established (Figure 4b, c). Functional enrichment analysis was performed again, and the results showed that the nine-gene prognostic signature was closely related to the biological processes and signaling pathways of iron metabolism (Figure 4d). The risk coefficients obtained by multivariate Cox regression analysis were used to calculated risk score for each patient in TCGA cohort and ICGC cohort, The calculation formula of the risk score was as follows: risk score = (-0.035734629) * FBXL5 + (0.056492195) * SLC48A1+(0.078465689) * BMP6+ (0.035704907) * HAVCR1+(-0.002130707)* ALAS1+(0.03201534) * CD163+(0.01923491) * PCBP2+(0.126735075) * FLVCR1+ (0.171470322) * ABCB6.

All patients in TCGA and ICGC cohorts were divided into two risk groups based on the risk score. The survival curves displayed that patients with high risk had shorter OS time than those with low risk (p<0.001). Consistent with the result was found in the ICGC cohort(p<0.001) (Figure 5a). The distribution and survival status of patients for the nine-gene prognostic signature were shown in the TCGA and ICGC cohorts (Figure 5b). The number of deaths increased as the risk score increased. ROC analysis was performed to assess the predictive capacity of the prognostic signature at 1, 2, and 3 years. The results
showed that AUCs for 1-, 2- and 3-year OS reached 0.806, 0.781 and 0.750 in the TCGA cohort and 0.747, 0.772 and 0.813 in the ICGC cohort, respectively (Figure 5c).

4. Independent prognostic and predictive value of the multi-gene prognostic signature

To examine whether the prognostic signature can independently predict the prognosis of HCC patients, the Cox regression analyses were carried out again. After univariate Cox regression analysis, stage and risk score were found to be closely related to OS in HCC patients (Figure 6a). After multivariate Cox regression analysis, stage and risk score were identified as independent prognostic factors (Figure 6c). Consistent results were obtained in the ICGC cohort (Figure 6b, d). Then, all independent prognostic factors determined by Cox regression analyses were used to construct a nomogram for predicting the 1-, 2- and 3-year OS (Figure 6e). The result showed, as the risk score increased, the 1-, 2- and 3- years OS time of patients decreased. The calibration plots approached 45 degrees, which indicated that the nomogram model had a great performance (Figure 6f). The relationship analysis of clinicopathological characteristics between the two risk groups demonstrated significant differences for the stage, grade and gender (p < 0.05) (Figure 6g). The subgroup analysis indicated that patients with high risk in all subgroups had dramatically shorter OS time than those with low risk (p < 0.05) (Figure 7a). Top 5 significantly altered GO function and KEGG pathways in two risk groups were performed using GSEA in TCGA cohort (Figure 7b). In addition, we analyzed whether our iron metabolism-related signature was associated with immune infiltration of TILCs in HCC. The results showed that our prognostic signature was positively correlated with CD8+ T cells (r = 0.119, P = 0.022), dendritic cells (r = 0.168, P = 0.001), neutrophils (r = 0.244, P < 0.001), and macrophages (r = 0.265, P < 0.001) (Figure 7c).

5. FLVCR1 gene expression in HCC and its impact on prognosis

PPI network showed that FLVCR1, ABCB6 and ALAS1 was hub genes of the prognostic signature (Figure 8a). In addition, we examined the changes in genetic alterations of the nine genes and eventually found that amplification was the most common type of mutation (Figure 8b). Among them, the FLVCR1 gene had the highest mutation frequency. These results implied that FLVCR1 had a special role in the progression of HCC.

As the results showed, FLVCR1 expression was abnormally upregulated in several cancers, such as BLCA, HCC and STAD (Figure 8c). The upregulated expression of FLVCR1 in HCC was verified again based on the ICGC, Wurmbach and Guichard cohorts (Figure 8d). The RT-qPCR analysis revealed that mRNA expression levels of FLVCR1 were higher in HCC cells than LO2 cells (Figure 8e). The relationship analysis between clinicopathological characteristics and FLVCR1 showed significant difference for the age (p = 0.0015), T (p = 0.0058), stage (p < 0.001) and grade (p < 0.001) (Figure 9a). Promoter methylation levels of FLVCR1 were significantly lower in primary HCC samples than normal samples (p < 0.001). Patients with TP53 mutation, higher tumor grade and stage had higher promoter methylation levels of FLVCR1 (Figure 9b). In addition, survival curves demonstrated that high FLVCR1 expression was related to poor OS, DFI, DSS and PFI in TCGA cohort (Figure 9c). High FLVCR1 expression was closely related to poor OS in ICGC cohort.
6. Potential role of FLVCR1 in HCC

Many studies reported that TIICs are closely related to prognosis of cancer patients. Therefore, we explored the relationship between FLVCR1 expression and immune infiltration using TIMER. The results indicated that FLVCR1 expression was positively associated with tumor purity and the infiltration level of B cell, CD4+ T cell, macrophage, neutrophil and dendritic cell (Figure 10a). In addition, copy number alteration of FLVCR1 significantly correlated with infiltration level of TIICs. Elevated arm-level gain of FLVCR1 results in lower infiltration levels of CD8+ cells, macrophage, neutrophil and dendritic cell compared with normal samples (Figure 10b). Furthermore, GSEA revealed that FLVCR1 was significantly involved in some cancer-related pathways, including cell cycle, fatty acid metabolism, glucose metabolism, MYC targets, E2F targets, WNT signaling pathway and PI3K signaling pathway (Figure 10c).

Discussion

Disordered iron metabolism is one of the hallmarks of malignancies, in which iron is required for the growth, metastasis and survival of tumor cells (19, 20). Previous studies have shown that iron plays a significant role in the development of HCC (21). HCC requires excess iron to meet the demands of rapid proliferation and DNA duplication (22). However, excessive iron can lead to ferroptosis, which has great potential in the treatment of HCC. As a commonly used chemotherapy drug for the treatment of advanced HCC, sorafenib has been found to kill HCC cells by inducing ferroptosis (23). Given the dual role of iron metabolism in HCC, it is valuable to find new targets in iron metabolism for predicting the prognosis of HCC patients more accurately and providing new treatment strategies.

In our work, based on the expression pattern of iron metabolism-related genes, we identified two clusters of HCC subgroups using consensus clustering analysis. The results indicated that there is a significant difference in OS, stage and grade between the two clusters, which suggested that the expression pattern of iron metabolism-related genes was closely related to the development of HCC. Previous research found that iron metabolism-related genes could influence numerous biological processes and signaling pathways, such as cell cycle (24), epithelial to mesenchymal transition (25), JAK/STAT (26) and ERK signaling pathways (27). Hence, we performed functional analyses of the differentially regulated genes between the two clusters of HCC. Similarly, the results showed that these genes are significantly related to cancer-related biological processes and signaling pathways. Combined with these results, it could be determined that iron metabolism-related genes play a critical effect in regulating the malignant process of HCC.

Iron metabolism is associated with the prognosis of cancer patients, including HCC patients (21). Therefore, we performed Cox regression analysis and LASSO regression analysis to construct a nine-gene prognostic signature with iron metabolism-related genes, which consists of FBXL5, SLC48A1, BMP6, HAVCR1, ALAS1, CD163, PCBP2, FLVCR1 and ABCB6. The high- and low-risk groups divided based on the median risk score showed significant difference in OS, and the signature had a great performance for predicating the prognosis of HCC patients. Then, the results of multivariate Cox regression analysis
further indicated that this prognostic signature could independently predict the prognosis of HCC patients. In addition, a distinct difference of OS between low-and high-risk groups was observed in all subgroups stratified by age, gender, stage and grade. Patients with high risk in all subgroups had shorter OS time than those with low risk. These results indicated that our nine-gene prognostic signature has reliable predictive performance and great clinical value.

In this nine-gene prognostic signature, FLVCR1 was identified as a hub gene with the highest mutation frequency, which implied FLVCR1 plays a vital role in the progression of HCC. Feline Leukemia virus subgroup C receptor (FLVCR1) functions as a mammalian cell heme exporter. FLVCR1a and FLVCR1b are two different isoforms of FLVCR1, which are expressed on the plasma membrane and mitochondria, respectively(28). FLVCR1 is involved in a variety of biological processes, including cell proliferation, cell apoptosis, oxidative stress response, cellular differentiation and metabolism(29–31). In addition, the carcinogenesis of FLVCR1 in several malignancies has gradually been uncovered. For example, Changliang Peng found FLVCR1 can promote the proliferation of synovial sarcoma through regulating autophagy(32). Although, Xianli Wei, reported the expression of FLVCR1 in HCC tumor tissues was upregulated compared with the adjacent non-tumor tissues(33), the role of FLVCR1 in progression of HCC is still unclear. In our study, GSEA showed that FLVCR1 might affect the malignant progression of HCC by regulating some biological processes such as cell cycle, metabolic pathways and cancer-related pathways. This result is consistent with the previous reports. Furthermore, we found that FLVCR1 expression was positively associated with the infiltration level of some TIICs. In the tumor microenvironment, TIICs were educated by tumor cells to adopt an iron-donor phenotype, which promotes tumor proliferation and metastasis. The mechanism by which FLVCR1 affects the progress of HCC may also regulate the iron outflow of TIICs. This requires further to explore in vitro and vivo experiments, but the interaction between iron metabolism genes and the tumor microenvironment is a potential strategy for HCC treatment.

Our study also has several potential limitations. First, this is a retrospective study, and multi-center prospective trials are still required to validate our conclusion. In addition, in vivo and in vitro experiments are needed to further verify the function and mechanism of FLVCR1 in HCC.

Conclusion

In summary, this study is the first to use large-scale data to construct an iron metabolism-related prognostic signature that predicts the prognosis of HCC patients and reveal the potential role of FLVCR1 in the progression of HCC. These findings could improve risk management and provide new therapeutic targets for HCC patients.

Declarations

Acknowledgements
Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Funding
No funding.

Availability of data and materials
All data are available from the sources listed in the manuscript—the TCGA, ICGC and Oncomine data portal.

Competing Interests
The authors have declared that no competing interest exists.

Authors’ contributions
JC, CH, YS conceived and designed the present study. XD, RP, HF analyzed the data. JC interpreted the data and wrote the manuscript. YS revised the manuscript. All authors read and approved the final manuscript.

References


**Figures**
Figure 1

Functional enrichment analyses of iron metabolism-related genes in HCC. a. Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment study; b. Results of Gene ontology (GO) enrichment study.
Figure 2

Clinical outcome of HCC patients in the two clusters. a. Unsupervised cluster analysis of the iron metabolism-related genes expression profile in TCGA cohort; b. The survival curve for the patients in two clusters; c. Heatmap and clinicopathologic characteristics of the two clusters. *p<0.05, **p<0.01, ***p<0.001
Figure 3

Functional enrichment analyses of the differentially expressed genes in two clusters. a. Volcano map of differentially expressed genes for the two clusters; b. GO enrichment results of differentially expressed genes; c. KEGG pathway enrichment results of differentially expressed genes; d. Top 5 terms of GO enrichment and its related genes; e. Top 3 terms of KEGG pathway enrichment and its related genes.
Figure 4

Construction of a prognostic signature with iron metabolism-related genes. a. Forest plot of univariate Cox regression analysis results in TCGA cohort; b. Results of LASSO Cox regression analysis; c. Forest plot of multivariate Cox regression analysis results in TCGA cohort; d. GO and KEGG pathway enrichment results of nine-gene prognostic signature.
Figure 5

Survival risk assessment of prognostic signature in TCGA and ICGC cohort. a. Kaplan–Meier survival curves between the high- and low-risk groups. b. The distribution of survival status, risk score and corresponding expression profiles. c. ROC curves at 1, 2, and 3 years.
Figure 6

Identification of the independent prognostic factors and construction of a nomogram. a, b. Univariate Cox analysis of the risk score and clinicopathological characteristics in TCGA and ICGC cohorts; c, d. multivariate Cox analysis of the risk score and clinicopathological characteristics in TCGA and ICGC cohorts; e. A nomogram constructed with independent prognostic factors; f. Calibration curves for nomogram model at 1, 2, and 3 years; g. Heatmap and clinicopathologic characteristics of the two risk groups. *p<0.05, **p<0.01, ***p<0.001
Figure 7

Functional enrichment analyses, immune microenvironment and subgroup analysis for two risk groups in TCGA cohort. a. The survival curves for the low- and high-risk subgroups stratified by age, gender, grade and stage; b. Top 5 significantly altered GO and KEGG pathways in high- or low-risk patients; c. Correlation between our signature and TIICs.
Figure 8

Expression of FLVCR1 in HCC. a. PPI network of nine-gene prognostic signature; b. Genetic alteration of nine gene in signature; c. Expression pattern of FLVCR1 in 33 types of tumors based on TCGA cohorts; d. FLVCR1 expression in HCC and adjacent normal tissues based on ICGC, Wurmbach and Guichard cohorts; e. FLVCR1 expression in LO2 cells and HCC cell lines.
Prognostic value of FLVCR1 in HCC. a. Correlation between FLVCR1 expression and clinicopathological characteristics in TCGA cohort; b. Correlation between promoter methylation levels of FLVCR1 and clinicopathological characteristics in TCGA cohort; c. FLVCR1 expression significantly correlated with overall survival (OS), disease-specific survival (DSS), disease-free interval (DFI), progression-free interval (PFI).
Figure 10

Potential role of FLVCR1 in HCC microenvironment. a. Correlation between FLVCR1 expression and infiltration level of TIICs in TCGA cohort; b. Correlation between copy number alteration of FLVCR1 and infiltration level of TIICs; c. Several cancer-related pathways associated with FLVCR1 expression displayed by GSEA.