A Prognostic Model for Hepatocellular Carcinoma Patients Based on Signature Ferroptosis-related Genes

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Abstract

Background

With the increasing number of HCC patients, it is necessary to accurately predicting the prognosis of these patients. Ferroptosis has been confirmed to be closely related to HCC progression. However, there is still a challenge in predicting the survival of HCC patients through ferroptosis-related genes.

Method

The RNA-seq data and corresponding clinical data of HCC from TCGA database were downloaded to establish a prognosis model, and data of ICGC and GSE14520 as the validation set. The risk score was constructed with 5 genes identified by univariate and LASSO Cox regression analysis. Then, risk score, TNM stage and cirrhosis were included to construct a nomogram, through univariate and multivariate Cox regression analysis.

Results

5 genes were identified from 70 ferroptosis-related DEGs to construct a gene signature to predict HCC patient survival from TCGA cohort. PCA and heatmap results show that there are obvious differences in patients with different score groups. Then, we included risk score, TNM stage and cirrhosis to construct a nomogram to further predict the overall survival of the patients. Survival analysis indicates that overall survival of the low- risk group is significantly higher than that of the high-risk group. Similarly, the data in the GSE14520 cohort also confirmed good performance for the nomogram. Furthermore, KEGG and GO functional enrichment analyses indicates the difference in overall survival between groups is closely related to immune-related pathways. Finally, through analyzing the immune status of all patients, we found that compared with patients in the low-risk group, “Macrophages M0”, “T cells CD8”, and “T cells regulatory” of the high-risk group were significantly higher.

Conclusion

The nomogram based on ferroptosis-related genes has a good performance for the prognosis of HCC patients. The model may provide a reference for evaluation of HCC patients by targeting ferroptosis.

Introduction

Liver cancer is one of the most common malignant tumours, which mainly develops from chronic hepatitis and cirrhosis¹. The latest report claims that liver cancer ranks 6th in global incidence and 3th among cancer-related deaths, causing more than 800,000 deaths each year². Among them, hepatocellular carcinoma (HCC) accounts for 75–85% of primary liver cancers. Although with the advancement of medical treatment, the condition of HCC patients has been improved, but their 5-year survival rate is still at a low level. The current treatment of liver cancer patients is mainly based on surgery, but it is greatly restricted by cost, donors, tumor metastasis, etc. Therefore, it is of great significance to explore the

potential mechanism of tumor occurrence and development, and to develop effective non-surgical treatments³.

Ferroptosis is an iron-dependent form of programmed cell death driven by accumulation of lipid reactive oxygen species, is characterized mainly by cell volume shrinkage and increased mitochondrial membrane density^{4, 5}. In the process of HCC, ferroptosis occurs in a variety of cells, including normal cells and tumor cells^{6–8}. Some targeted drugs are also trying to develop by regulating the iron death of tumor cells⁹.

In addition, many genes related to ferroptosis have also been identified, although their roles and functions are not consistent. CDGSH iron sulfur domain 1 (CISD1), nuclear factor erythroid 2-related factor 2 (NRF2), and metallothionein (MT)-1G has been proven to inhibited ferroptosis in HCC^{9–11}. In contrast, Glutathione S-transferase zeta 1 (GSTZ1), Quiescin sulfhydryl oxidase 1 (QSOX1), and retinoblastoma (Rb) positively regulate ferroptosis^{8, 12, 13}. However, the correlation between ferroptosis-related genes and the prognosis of HCC patients has not been revealed.

In this study, we analyzed the normal population and HCC patients in public databases based on machine learning. There identified differentially expressed ferroptosis-related genes were constructed into signature, followed used to construct a prognostic model for HCC patients. Finally, we explored the function enrichment and immunity related to the prediction model.

Materials And Methods

Data Collection

We downloaded the RNA sequencing (RNA-seq) data and corresponding clinical information of HCC patients including 347 tumor samples and 35 normal samples in The Cancer Genome Atlas (TCGA) database. (<https://portal.gdc.cancer.gov/repository/>). Expression profile data and clinical information of 232 tumor samples in Gene Expression Omnibus (GEO) database (GSE14520) (<https://www.genecards.org/>) are also downloaded and used. In additional, RNA-seq data of 265 tumor samples from the International Cancer Genome Consortium (ICGC) portal (<https://dcc.icgc.org/projects/LIRI-JP/>) was obtained. Finally, the 233 ferroptosis-related genes were collected, and are provided in Supplementary Table 1.

Differentially Expressed Gene Analysis

“limma” package from Bioconductor in R software (version 4.1.0) used to compared tumor tissues to normal tissues. Differentially expressed genes (DEGs) were identified with the criteria: false discovery rate (FDR) < 0.05 and log2FoldChange > 1.

Construction and Validation of the Prognostic Ferroptosis-related gene Signature

The prognostic value of ferroptosis-related genes was determined by univariate Cox regression analysis where $P < 0.05$ was considered statistically significant. Then least absolute shrinkage and selection operator (LASSO)-penalized Cox regression analysis with “glmnet” R package, which is a widely used machine learning algorithm that deals with multicollinearity¹⁴, was used to further identify ferroptosis-related genes related to the prognosis of HCC. Penalty parameter (λ) for the model was determined by the lowest partial likelihood deviance. The prognostic risk score was determined using a

linear combination of the regression coefficient (β) in the LASSO model and the expression levels of the genes. Patients were divided into low-score and high-score groups according to the cutoff values. Based on the risk score of identified genes, principal component analysis (PCA) by the “prcomp” function of the “stats” R package was performed. Kaplan-Meier survival curves were performed to evaluate the predictive performance of the prognostic ferroptosis-related gene signature.

Establishment of Nomogram Prognosis Prediction Model

Nomogram is widely used to predict the prognosis of cancer, which is an intuitive visualization of the model. Based on the variables identified by the univariate and multivariate Cox regression analysis, we established a nomogram using “rms” R package to predict the survival of patients. According to the median of the nomogram score, we divide HCC patients into high-risk groups and low-risk groups. The concordance index (C-index) and receiver operating characteristic (ROC) curve were used to evaluate the discrimination ability of the nomogram.

Functional Enrichment Analysis

Based on DEGs between the high-risk and low-risk groups, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses were performed using “clusterProfiler” R package¹⁵. The pathways were regarded to be significantly enriched with $P < 0.05$.

Immune Profile Analysis

To analyze the immune status of each sample, Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) used to calculate the relative proportions of 22 immune cells in HCC patients, including seven T cell types, naïve and memory B cells, plasma cells, NK cells, and myeloid subsets^{16, 17}. The CIBERSORT was applied to convert mRNA data into the infiltration fractions of non-tumor cells in the tumor microenvironment, through using standard annotation files to organize gene expression characteristics.

Quantitative Real-time RT-PCR (qRT-PCR)

For real-time PCR analysis, PCR was performed with a reaction mixture containing cDNA template, primers, and TB Green™ Fast qPCR Mix (TaKaRa) in a Step One Plus Real-Time PCR System (Thermo Fisher Scientific). The relative abundances of the target genes were obtained by comparison against a standard curve.

Statistical Analysis

Continuous variables were tested for normality, and if they follow a normal distribution,

the mean $\pm$ standard deviation standard is provided, and Student's *t* test was performed for comparisons between groups. Kaplan-Meier method was used to estimate the overall survival of different groups, and log-rank was used to test the significance of the difference between different groups. Survival analysis was completed by the survival and "survminer" R package. Statistical analyses were performed using SPSS (Version 25.0) and R software (Version 4.1.0). A two-side *P* value < 0.05 was considered statistically significant.

Results

Identification of Ferroptosis-Related DEGs

5266 DEGs obtained through comparing tumor and normal tissues in TCGA database (Fig. 1A). Correspondingly, genes expression in normal tissues and tumor tissues are also significantly different (Fig. 1B). Then, combining the list of ferroptosis-related genes, we obtained 70 ferroptosis-related DEGs by taking the intersection (Fig. 1C). Further, Protein-Protein Interaction (PPI) networks was established through the STRING database for analyzing and predicting protein functional connectivity and protein interactions. Figure 1D indicated that HMOX1, G6PD, LOX, JUN, SRC, etc. were the hub genes.

Construction and Validation of Prognostic Gene Signature

In order to clarify the association between these ferroptosis-related DEGs and patient prognosis, the 70 genes that have been identified are analyzed by univariate Cox regression analysis, followed 12 genes are selected with $P < 0.05$ and entered into LASSO Cox regression (Fig. 2A). Then, a 5-gene signature was established based on the optimal λ value (Fig. 2B). The five genes included G6PD, HMOX1, LOX, SLC7A11, and STMN1. Based on the β value of each gene that derived from the LASSO Cox regression, the prognostic risk score was calculated as follows: $(0.235 * \text{expression level of G6PD}) + (0.039 * \text{expression level of HMOX1}) + (0.009 * \text{expression level of LOX}) + (0.029 * \text{expression level of SLC7A11}) + (0.042 * \text{expression level of STMN1})$. According to the median of risk score, patients were stratified into high-score group ($n = 174$) and low-score group ($n = 173$). PCA revealed that the patients in these two subgroups were distributed in discrete directions (Fig. 2C). Similarly, these selected genes are also significantly different between the high-score group and low-score group (Fig. 2D). Furthermore, Kaplan-Meier analysis showed that the overall survival of HCC patients in the low-score group was significantly higher than that of the high-risk group ($P < 0.001$) (Fig. 2E)

Next, we introduced ICGC database to further validation this model. We also divided HCC patients into high-score groups and low-score groups in ICGC database according to risk score. The results show that the two subgroups were significantly different, and the survival rate of the low-score group is significantly higher than that of the high-score group ($P < 0.001$) (Supplementary Fig. 1A-C).

Risk Score Is an Independent Prognostic factor for HCC

In order to better predict the overall survival of HCC patients, risk score and clinical characteristics such as age, gender, TNM stage, etc., are included univariate and multivariate Cox regression analyses. As we expected, risk score was identified as an independent prognostic factor for survival of patients. In addition, TNM stage and cirrhosis have also been identified as independent prognostic factors with $P < 0.05$ (Fig. 3A-B). Then, patients were classified according to TNM stage and cirrhosis, and survival analysis indicate that the survival of patients in low-score group was still higher than that of the high-score group (Fig. 3C-D).

Construction and Validation of the Prognostic Prediction Nomogram

As visualization of the prognostic model, nomogram was established to show the survival of all patients more intuitively, including TNM stage, cirrhosis, and risk score (Fig. 4A). Nomogram shows that the risk score has the greatest influence on 1-year, 3-year, and 5-year survival of HCC patients, followed by TNM stage. Then, discrimination power of the nomogram was evaluated by the C-index values and ROC curves. The C-index for the prediction of 1-year, 3-year, and 5-year overall survival in patients were 0.732, 0.705, and 0.774, respectively. Correspondingly, the areas under ROC curve (AUC) of the 1-year, 3-year, and 5-year were 0.745, 0.740, and 0.756, and was better than the traditional HCC marker alpha-fetoprotein (AFP) (Fig. 4B). The calibration ability of the model was also indicated by the calibration curve, which has good consistencies between the prediction and observation (Fig. 4C). Furthermore, patients were divided into high-risk and low-risk group according to the median of the total points. Compared with the HCC patients in high-risk group, the low-risk group has a significantly different direction of dispersion, and the overall survival is also higher ($P < 0.001$) (Fig. 4D-E).

GSE14520 dataset was introduced to validation this nomogram. Supplementary Fig. 2A-D show that this model had good discrimination and calibration ability for HCC patients in GEO. Interesting, this nomogram has the best predict for the 3-year survival rate of this cohort, which is different from the TCGA cohort.

Functional Enrichment Analysis

To elucidate the underlying pathways that were associated with the risk score, KEGG functional enrichment analyses was performed using DEGs between the high-risk and low-risk groups. The DEGs mainly enriched in “cell cycle”, “IL-17 signaling pathway”, and “HIF-1 signaling pathway” (Fig. 5A). GO functional enrichment analyses also was performed. Results show that the DEGs almost mapped to the immune-related GO terms, such as “humoral immune response”, “B cell mediated immunity”, and “immunoglobulin complex”, “antigen binding”, and “immunoglobulin receptor binding” (Figs. 5B-D), indicating that survival differences between subgroups may be related to immune status of patients.

Immune Status of HCC Patients with Different Groups

Based on the immune-related terms displayed by the enrichment results, we explored the association between nomograms established by gene features and immune. CIBERSORT algorithm used to estimate

the immune infiltration differences between

22 immune cells in HCC patients in high and low-risk groups, including LM22 feature

matrix. Figure 6A-B summarized the results of immune cell infiltration in HCC patients, show similarities and differences between subgroups. Immune cells that differ between the high-risk group and the low-risk group were mainly “Macrophages M0”, “Macrophages M2”, “T cells CD8”, and “T cells regulatory” (Fig. 6C), may representing the intrinsic characteristics of individual differences. The correlation between the infiltration of type of immune cells is different (Fig. 6D).

The expression of immune checkpoints has become indicator of the timing of immunotherapy for HCC patients. We found that the expression levels of CTLA4, LAG3, TIGIT and IDO1 in the high-risk group were significantly higher than

those in the low-risk group ($P < 0.001$) (Supplementary Fig. 3), indicating that the poor prognosis of high-risk patients was partly due to the immunosuppressive microenvironment.

Discussion

In this study, to better assess the survival of HCC patients, we established a

prognostic model based on panel of 5 ferroptosis-related genes and clinical characteristic using machine learning methods to analyze the data of a large number of HCC patients. The model was established and validated through multiple databases, showing good discrimination and calibration in predicting survival. Finally, the connection between the model and immunity has been partially confirmed.

As a major leading cause of cancer-related mortality worldwide, HCC causes a major health burden for society¹⁸. Considering the increasing number of HCC patients, it is very important to predict the survival of patients. The ferroptosis proposed by Dixon et al.⁴, as a new type of programmed cell death, has been considered to be closely related to HCC^{19,20}. Although some studies have shown that several genes might regulate drug-induced ferroptosis in HCC, but their relationship with the prognosis of HCC patients is still unknown. Therefore, it has great potential to construct a models based on ferroptosis-related genes to predict the overall survival of HCC.

Through the differential gene analysis of HCC patients, we identified 70 ferroptosis-related DEGs from 5266 DEGs between normal and tumor tissues. Then, 5 genes were obtained, after univariate and LASSO Cox regression analysis. These genes have been confirmed to be closely related to cancer. G6PD, which is involved in the pentose phosphate pathway, has been reported to involved in erastin-induced ferroptosis in non-small cell lung cancer cells⁴. Similarly, inhibition of SLC7A11, a subunit of system Xc to import cystine in the cell, sensitized fibrosarcoma cells to erastin-induced death⁴. Furthermore, we constructed these 5 genes into a signature to better predict the overall survival, and the superiority of the model was confirmed by the HCC patients in TCGA and ICGC.

The clinical characteristics of HCC patients are closely related to the prognosis. In order to better optimize the model and strengthen the survival prediction of patients, TNM stage and cirrhosis were selected, through univariate and multivariate Cox analysis. TNM stage is an important criterion for the current staging of cancer patients and is widely considered to be the standard approach for predicting prognosis in most solid tumour systems^{21, 22}. In addition, cirrhosis, as a major risk factor for HCC, has been confirmed to be closely related to the occurrence and development of HCC²³. Every year, a large number of cirrhosis patients infected with viral hepatitis progress to HCC, which greatly shortens the life span of patients²⁴.

Based on these meaningful variables closely related to the prognosis of HCC, we have incorporated clinical information combined with gene signature to construct a nomogram. In order to understand the performance of the model, we evaluated its discrimination and calibration ability. C-index and ROC curve are used to evaluate the discrimination ability of nomogram. In predicting 1-year, 3-year, and 5-year survival of HCC patients in TCGA, the C index is 0.732, 0.705, and 0.774 respectively. At the same time, AUC is 0.745, 0.740, and 0.756. Similarly, in the HCC patients in the GEO database, these indexes also perform relatively well, indicating that the model has a good discrimination ability for predicting survival. The calibration curve for evaluating calibration also shows that the nomogram has good calibration, whether in TCGA or GEO database.

Although the mechanism of ferroptosis in HCC has been extensively studied, the relationship between ferroptosis and tumor immunity is not yet clear. We performed functional enrichment analysis on the DEGs between the high-risk group and the low-risk group. Interestingly, most of the enriched items are immune-related pathways. This may be closely related to the difference in survival rates between subgroups. With this purpose, we compared the composition of different types of immune cells between the groups. The results show that tumor-associated macrophages and Treg cells, clued to poor prognosis in HCC patients due to their role in immune invasion²⁵⁻²⁷, are higher in the high-risk group. This may be that the ferroptosis-related genes affect tumor cells by affecting immune cells. Moreover, the expression of immune checkpoint, including CTLA4, LAG3, TIGIT, and IDO1, of the HCC patients in high-risk group was significantly higher than that in the low-risk group, indicating that lower overall survival of patients may be due to immunosuppressive microenvironment.

The important finding in this paper is that the nomogram constructed based on the risk score related to ferroptosis genes can well predict the prognosis of HCC patients. Although some prognostic models have been used to predict the overall survival of HCC patients²⁸⁻³⁰, there are few nomograms based on ferroptosis-related genes. This model incorporates a large number of HCC patients to construct, and has been validated by multiple databases, showing a good ability to predict survival. At the same time, the 5 ferroptosis-related genes identified can also be used as biomarkers for predicting the survival of HCC patients.

However, there are still some limitations to our study. Due to the lack of information in the database, we did not include the cause of HCC. The number of HCC patients caused by non-alcoholic fatty liver disease

and alcoholic liver is gradually increasing, though the number of HCC patients induced by viral hepatitis is gradually decreasing. For HCC caused by different causes, the prognosis and treatment of patients are different^{31–34}. The lack of relevant information led us to not include HCC-related imaging data and serum indicators into the nomogram. The inclusion of more indicators helps increase the specificity and sensitivity of the prognostic model. In addition, the RNA-seq data included in this model are all derived from liver specimens, not blood and other easier-to-obtain specimens, which increases the difficulty of sampling.

In conclusion, our study defined a novel prognostic model of 5 ferroptosis-related genes. This model proved to have good discrimination and calibration ability, by analyzing a large number of HCC patients from multiple databases, providing a new insight into the evaluation the prognosis of HCC patients.

Declarations

Author Contributions

SW and YL contributed equally to this study. SW designed and wrote the manuscript. YL screened the literature and collected data. ML analysed the data. BW critically revised the manuscript.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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Figures

Figure 2

Identification of the candidate ferroptosis-related genes to establish a prognostic gene signature for HCC patients in The Cancer Genome Atlas (TCGA) cohort. (A) Univariate Cox regression confirmed that ferroptosis-related genes associated with HCC prognosis. (B) LASSO Cox regression was performed to identify the ferroptosis-related genes significantly related to the prognosis of HCC. (C) Principal component analysis (PCA) plot of HCC patients based on the expression profiles of the signature genes

in different score groups. (D) Heatmap of mRNA expression of five selected ferroptosis-related genes in high and low-score samples. (E) Kaplan-Meier survival curves indicated that the overall survival in the low-score group was markedly higher than that in the high-score group.

Figure 3

Independent prognostic factor analysis of risk scores and clinical parameters. (A-B) Univariate and multivariate Cox regression confirmed that the risk score, TNM stage, and cirrhosis were independent

prognostic factors. (C) Kaplan-Meier survival curves indicated that the overall survival in high and low-score samples with different TNM stage. (D) Kaplan-Meier survival curves indicated that the overall survival in high and low-score samples with cirrhosis.

Figure 4

Construction a nomogram to predicts the survival of HCC patients in The Cancer Genome Atlas (TCGA) cohort. (A) The nomogram for predicting the overall survival of HCC patients at 1-, 3-, and 5-year. (B) The

receiver operating characteristic (ROC) curves of the nomogram and AFP for the survival prediction of HCC patients at 1-, 3-, and 5-year. (C) The calibration curves of the nomogram for 1-, 3-, and 5-year survival probabilities. (D) Principal component analysis (PCA) plot of HCC patients based on the expression profiles of the signature genes in different risk groups. (E) Kaplan-Meier survival curves indicated that the overall survival in the low-risk group was markedly higher than that in the high-risk group. ROC, receiver operating characteristic; AUC, areas under receiver operating characteristic curve; AFP, alpha-fetoprotein; OS, overall survival.

Enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) for differentially expressed genes between high-risk group and low-risk group. (A) KEGG enrichment analysis of differentially expressed genes between high-risk group and low-risk group. (B-D) (A) GO enrichment analysis of differentially expressed genes between high-risk group and low-risk group.

Immune profiles between different risk groups. (A) Relative proportion of immune cells of HCC patients in different groups. (B) Heatmap of immune cells expression profiles in high and low-risk samples. (C) Comparison between the fractions of immune cells in the high and low-risk group. (D) Correlation network between the immune cells. * $P < 0.05$; *** $P < 0.001$.

Supplementary Files

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