ORIGINAL RESEARCH

Macrophage-Associated Genes for Predicting Prognosis and the Tumor Microenvironment in Patients with Hepatocellular Carcinoma

Zhiyuan Mao, PhD; Yinglin Li, MD; Li Bai, PhD

ABSTRACT

Background • Hepatocellular carcinoma (HCC) is a highly malignant tumor, which is difficult to treat and has a poor prognosis. Immunotherapy has been a hot topic in liver cancer treatment in recent years, and macrophages play an important role in liver cancer immunotherapy. In this paper, we will use bioinformatics to analyze the significance of macrophage-associated genes (Mags) in hepatocellular carcinoma. Our goal is to determine the impact of macrophage-related genes on the immunotherapy, prognosis, and tumor microenvironment of HCC patients.

Methods • 343 HCC patients with complete survival data were selected from RNA sequencing data from the Cancer Genome Atlas Hepatocellular carcinoma (TCGA-LIHC) database. Using univariate Cox regression analysis and Lasso regression analysis to identify macrophage-related genetic markers for prognostic HCC and constructed risk scores. Kaplan-Meier survival analysis helped to determine the relationship between genetic markers and overall survival (OS). Kaplan-Meier analysis was used to compare OS in stratified high-risk and low-risk groups. Risk scores and other clinical features were included to develop a prognostic profile of HCC. The accuracy of the model was evaluated by the receiver operating curve and calibration curve, respectively.

Results • A prognostic risk model consisting of 7 Mags was constructed to accurately predict OS in the TCGA cohort. In univariate and multivariate Cox regression analyses, risk scores were prognostic factors independent of other clinical factors. The prognostic histogram showed that risk score had a good prognostic effect on survival risk stratification. The expression of immunotherapy markers such as CTLA4 and TNFRSF9 was upregulated in high-risk patients, indicating an underlying immunotherapy response in these patients.

Conclusion • Our study constructs a macrophage-associated genetic marker for predicting OS in HCC patients, which may help guide clinical immunotherapy. (*Altern Ther Health Med.* 2023;29(8):337-341).

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly malignant liver tumor. it is the sixth most common cancer and the third leading cause of cancer-related death worldwide.[1] Traditional chemotherapy drugs have limited efficacy in treating HCC, but immunotherapy has become a hot topic. By activating the immune system, immunotherapy can achieve the purpose of treating liver cancer. This includes immune checkpoint inhibitors such as PD-1 and CTLA-4, cancer vaccines, oncolytic viruses, and adoptive immune cell transfusions.[2,3]

As one of the innate immune cells, macrophages can clear infectious pathogens, regulate inflammatory response and promote tissue repair[3]. In the tumor microenvironment, macrophages can differentiate into subtypes with pro-inflammatory (M1 type) or anti-inflammatory (M2 type) functions according to different signal stimuli.[4] M1-type macrophages have anti-tumor effects by releasing inflammatory factors, promoting the interaction between cytokines and receptors, and participating in leukocyte chemotaxis and other pathways.[3] In contrast, M2-type macrophages accelerate tumor progression by promoting tumor cell growth, metastasis, and immune escape,[5] and can

promote tumor development and metastasis.[6] This may be one of the mechanisms by which macrophage-related genes affect the prognosis of patients with hepatocellular carcinoma.

In addition, immune cells in the tumor microenvironment also play an important role in tumor development. Tumor-associated macrophages (TAMs) were negatively correlated with patient prognosis.[7] These findings suggest that macrophage-related genes may further influence the development of hepatocellular carcinoma by regulating the function of other immune cells in the tumor microenvironment. TAMs can provide a microenvironment of immune tolerance for the occurrence and development of liver cancer by directly secreting or recruiting immunosuppressive factors on the cell surface and immunosuppressive cells Tregs. TAMs regulate tumor blood vessels and secrete a large number of pro-angiogenic factors and enzymes that regulate angiogenesis to stimulate the angiogenesis of HCC blood vessels. Therefore, it is of great significance to study the role of macrophage-related genes in the prognosis and tumor microenvironment of hepatocellular carcinoma patients to discover new biomarkers and therapeutic targets.

In this study, we constructed a gene marker associated with HCC macrophages and demonstrated that it can predict the prognosis of HCC patients. In addition, we developed a graph that included risk scores and clinical factors, and further compared immune cell infiltration and potential response to immunotherapy in high-risk and low-risk groups with risk scores.

METHOD

Data Collection

The HCC RNA sequencing data are obtained (RNAseq) from the hepatocellular carcinoma cancer genome atlas (TCGA-LIHC) database (https://portal.gdc.cancer.gov/); The corresponding clinical data were obtained from the UCSC Xena website (https://xenabrowser.net/). Patients who lacked complete follow-up information survived for less than 30 days or lacked complete clinicopathological information were excluded. The molecular characteristics of the database search (MSigDB, http://www.gseamsigdb.org/gsea/msigdb/index.jsp), with "macrophages" as keywords, "C5 - gene ontology (GO)" as a condition of the filter, we got 35 macrophage related gene sets, which contained a total of 240 macrophage-related genes.

Differences in expression of macrophage-related genes between hepatocellular carcinoma and normal tissues

The macrophage-related differentially expressed gene (DEG) between tumor and normal tissue was identified. The absolute value of log2FC multiple changes was greater than 1, and the adjusted $P = .04$. The R package "limma" is used to visualize DEGs. DEG was then analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analysis. Then the intersection with the gene list of macrophage-related genes was obtained to obtain the differential macrophage-related genes.

Identify Key Features

Each patient's risk score was calculated by the following formula: Risk score = (0.01 × CDKN2A expression value) + (-0.003 ×IL1RL1 expression value) + (-0.039× IL33 expression value) + (0.215 × LPL expression value) + (0.128 ×MAPT expression value) + (0.067 × MDK expression) + (-0.255 × PDE2A expression). HCC patients were then divided into high-risk and low-risk groups based on a median risk score. We called this the macrophage predictive characteristic risk score model. To assess whether the risk score served as an independent prognostic indicator, clinical characteristics were evaluated using univariate and multivariate Cox regression analyses. Receiver operating characteristic curves (ROC) were used to assess the accuracy of the risk model. The distribution of patients with different risk scores was assessed and visualized using tSNE.

Construction of a line graph

By combining risk scores with clinical features, we used the R package "rms" to construct a column graph that predicted survival at 1, 3, and 5 years for HCC patients. Calibration curves are used to determine whether the predicted survival rate matches the actual survival rate.

Estimates of immune cell infiltration

Cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT) algorithm was used to estimate the infiltration proportion of 22 immune cell types in HCC samples. Samples with $P = .04$ were retained for further study. To determine if there was a significant difference in the proportion of immune cells between the low-risk and high-risk groups, the Wilcoxon rank sum test was used.

Immune checkpoint

The differential expression of 40 immune checkpoints between high-risk and low-risk groups was compared. The patients were grouped into four groups based on the risk score and immune checkpoint expression. Their relationships with overall survival were analyzed using Kaplan–Meier analysis. We considered a $P < .05$ as the threshold for significance.

Statistical Analysis

All statistical analyses were performed using R software (version 4.2.3, https: //www.rproject.org/). Wilcoxon test was used to compare the differentially expressed genes between tumor tissue and adjacent normal tissue. Univariate Cox and Lasso regression were used to identify macrophage differential genes associated with survival, and a polygenic prognostic risk model was constructed. Differences in OS between groups were compared using Kaplan-Meier survival analysis. The Spearman correlation coefficient was used to analyze correlation. The chi-square test was used to compare classified data. The macrophage-related differentially expressed gene (DEG) between tumor and normal tissue was identified. The absolute value of log2FC multiple changes was greater than 1, and the adjusted $P = .04$. Use R software (version 4.2.3, https: //www.r-project.org/) to draw. $P < 0.05$ is significant.

RESULTS

Macrophage-related DEG in HCC

Out of 424 HCC patients, 343 patients were eventually included in this analysis after excluding patients who lacked complete follow-up information, survived for less than 30 days, or lacked complete clinicopathological data. A total of 1702 DEGs were obtained, among which 922 were down-regulated and 780 up-regulated in HCC tumor tissues. GO analysis showed that the five most abundant categories were the negative regulation of inflammatory responses, the regulation of macrophage chemotaxis, the regulation of IL-6 production, and the positive regulation of IL-6 production and macrophage migration. These findings suggested that DEGs were mainly involved in regulating inflammatory responses, macrophage chemotaxis, and IL production. These DEGs were intersected with 240 Mags and 35 DeMaGs were detected in HCC.

Correlation between key gene characteristics and prognosis of HCC patients

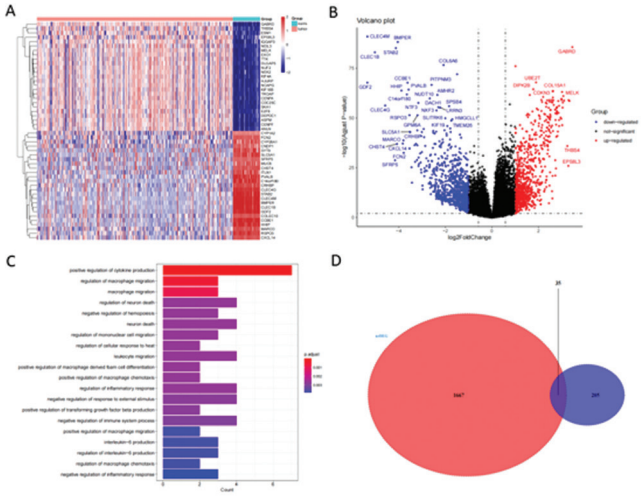
To identify the differentially expressed macrophage-related genes associated with prognosis in HCC, eight genes were obtained by univariate Cox regression analysis, and then a seven-gene prognostic risk model was constructed by LASSO regression analysis. Each patient's risk score was calculated by the following formula: Risk score = (0.01 × CDKN2A expression value) + (-0.003 × IL1RL1 expression value) + (-0.039 × IL33 expression value) + (0.215 × LPL expression value) + (0.128 × MAPT expression value) + (0.067 × MDK expression) + (-0.255 × PDE2A expression). The formula was used to calculate the risk score of each patient, and the patients were divided into two groups according to the median risk score, including 172 patients in the high-risk group and 171 patients in the low-risk group. Kaplan-Meier analysis showed that the OS time in the high-risk group was significantly shorter than that in the low-risk group. We then evaluated the relationship between risk score and different clinicopathological parameters, and univariate and multivariate Cox regression analysis showed that risk score was an independent prognostic risk factor for HCC patients. The AUCs of 1-year, 3-year, and 5-year ROC curves were 0.750, 0.692, and 0.658, respectively, indicating that the risk score had a relatively good prognostic performance.

Construction of a line graph

Combining the risk scores and clinicopathologic stage, a histogram was established to guide clinical prognosis assessment to estimate the prognosis of HCC patients.

To further investigate the relationship between risk scores and immune cell infiltration, we used the CIBERSORT algorithm to estimate the proportions of 22 immune cell types in the HCC tumor immune microenvironment. Here, memory CD4 T cells ($P < .01$), T cell follicular helper cells ($P = .04$), M0 macrophages ($P < .001$), and neutrophils ($P = .04$) were activated. There were significant differences between patients with high-risk and low-risk scores, and

Figure 1. Identification of macrophage-associated DeGs. **A**. Heat maps of 1702 DeGs in normal and tumor HCC tissues. **B.** Volcanic maps of 1702 differential genes in normal and HCC tumor tissues. **C.** GO analysis of DEG. **D.** Venn diagram of differential macrophage-related genes.



Abbreviations: N, normal tissue; T, tumor tissue; DEG, differentially expressed gene.

Figure 2. Prognostic value of risk score as determined by risk score prediction model. **A.** Kaplan-Meier curves for OS in high-risk and low-risk groups were stratified by median risk scores determined by seven gene signatures. **B.** A risk curve based on the risk score for each sample, where red dots represented high risk and blue dots represented low risk. **C.** Forest maps of univariate Cox regression analysis and multivariate Cox regression analysis. **D.** Time-dependent ROC curves for 1-year, 3-year, and 5-year survival of predicted characteristics.

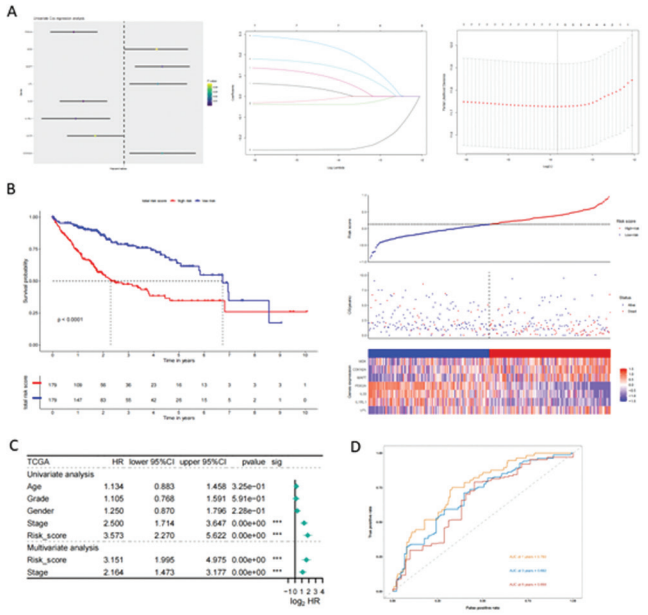


Figure 3. Construction and validation of a column diagram. **A.** The 1-year, 3-year, and 5-year survival probabilities of HCC patients were predicted by a graph combining clinicopathological variables and risk scores. **B.** Calibration curves to evaluate the predictive performance of line charts.3.4 Immune microenvironment and HCC immunotherapy in different risk populations.

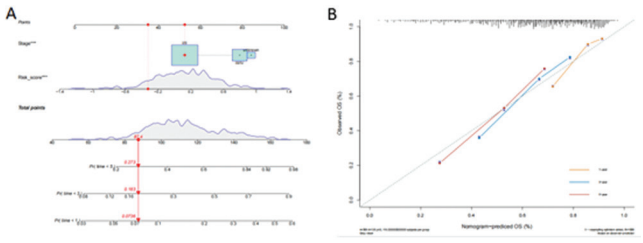
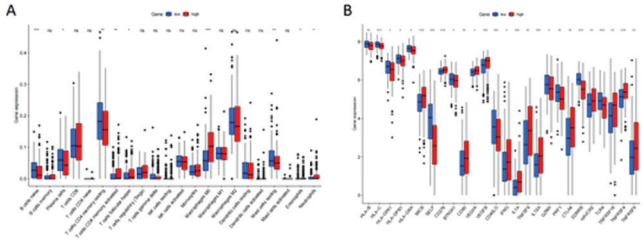


Figure 4. Immune microenvironment and HCC immunotherapy in different risk populations. **A.** The proportion of 22 immune cell types in the HCC tumor immune microenvironment. **B.** Biomarkers associated with immune checkpoints in genes differentially expressed in high-low-risk groups



these differences provided potential treatment guidelines for immunotherapy.

Studying whether risk model-related biomarkers related to immune checkpoints, high-risk and low-risk groups of differentially expressed genes associated with immune checkpoints have 26 biomarkers, including MICB, SELP, previously undescribed genes, CD80, VEGFA, VEGFB, IFNG, IL1A, TNFSF9, IL12A, CTLA4, HAVCR2, TNFRSF18, TNFRSF4, and TNFRSF9 were expressed at higher levels in the high-risk group, suggesting a potential immunotherapy response in high-risk patients.

DISCUSSION

Macrophage chemotaxis refers to the migration of macrophages to the site of inflammation or tumor under the action of chemokines CCL2, CCL5, and CXCL10.[10] In HCC, chemotaxis enables macrophages to rapidly gather into the tumor microenvironment and play a role in phagocytosis and regulation of tumor cells. However, chemotaxis may also lead to aggregation of M2-type macrophages, thus promoting tumor growth and metastasis. In HCC, IL produced by macrophages plays a key role in regulating tumor development and inflammatory responses. In addition, IL-6 produced by macrophages can promote the growth, invasion, and metastasis of liver cancer cells.[11]

In HCC treatment, targeting the macrophage DEG enrichment pathway can regulate the polarization of macrophages: by promoting the transformation of macrophages from M2 type to M1 type, it helps to inhibit tumor growth and metastasis and enhance the attack ability of immune cells on tumors. Inhibition of macrophage chemotaxis reduces the aggregation of M2-type macrophages in the tumor microenvironment and reduces the risk of tumor growth and metastasis by intervening in specific chemotaxis signaling pathways, such as the CCL2/CCR2 pathway.[12] It can also regulate the production of IL, and regulate the growth, invasion, and immune escape of hepatocellular carcinoma cells by inhibiting the expression of pro-inflammatory cytokine IL-6 or enhancing the expression of anti-inflammatory cytokine IL-10, thus providing a new strategy for HCC treatment. The treatment and prognosis of hepatocellular carcinoma depend on many factors, including the patient's underlying disease, tumor characteristics, and treatment methods.[13] Risk score is one of the independent prognostic risk factors for HCC patients, which is used to evaluate HCC patients' basic conditions, tumor characteristics, treatment methods, and other factors to determine the prognosis of patients, and guide the selection of treatment strategies for patients. In the diagnosis and treatment of HCC, the rational use of the risk scoring system to predict the survival and progression-free survival of patients has important clinical significance for the development of personalized treatment plans.

Currently, the commonly used HCC risk scoring system includes Barcelona Clinic Liver Cancer, BCLC), the American Association for the Study of Liver Diseases (AASLD), and the Japanese Society of Hepatology (Japan Society of Hepatology, JSH). These scoring systems took into account factors such as liver function, tumor size and number, serum tumor marker levels, and pathology.[14] 35 DeMaGs were detected in HCC in this study. To identify the differentially expressed macrophage-related genes associated with prognosis in HCC, 8 genes were obtained by univariate Cox regression analysis, and then 7-gene prognostic risk models were constructed by LASSO regression analysis. Several studies have shown that high-risk scores are associated with poor outcomes in HCC patients. For example, one study of BCLC staging found that both overall survival and progression-free survival were significantly shorter with increasing BCLC staging.[15] At the same time, other studies have also found that a high-risk score is associated with an increased risk of complications such as liver cancer metastasis and liver insufficiency.[16,17] In addition, risk scores have been used to guide treatment strategy selection for HCC patients. For example, in BCLC staging, treatment strategies include surgical excision, liver transplantation, trans-arterial interventional therapy, targeted therapy, and optimal supportive therapy. Treatment strategies for patients with different stages of BCLC are also different. Therefore, the accuracy of risk scores is very important to guide patients' treatment choices.

Immune cell infiltration refers to the presence of various immune cells, such as lymphocytes, macrophages, and dendritic cells, around or inside the tumor. They can control

the growth and spread of liver cancer by directly killing or inhibiting the growth of cancer cells and promoting the immune response of T cells. Therefore, immune cell infiltration is considered to be one of the important predictors of liver cancer immunotherapy.[16] Studies have shown that the degree of immune cell infiltration is closely related to the prognosis of HCC patients.[17,18] For example, large numbers of tumor-infiltrating lymphocytes (TILs) can predict a better prognosis, while low levels of TILs are associated with a poorer prognosis. In addition, the degree of infiltration of immune cells such as macrophages and dendritic cells is also related to the prognosis of HCC patients.[17]

There was also a strong relationship between risk score and immune cell infiltration. In terms of the relationship between risk scores and immune cell infiltration, we evaluated the proportions of 22 immune cell types in the HCC tumor immune microenvironment. Activated memory CD4 T cells, T cell follicular helper cells, M0 macrophages, and neutrophils differed significantly between patients with high and low-risk scores, and these immune cells provide potential therapeutic guidelines for immunotherapy. The study found that patients with abundant immune cell infiltration in liver cancer tissue had a relatively low liver cancer risk score, suggesting that immune system activity could inhibit the development of liver cancer.[18] On the contrary, liver cancer patients with a lack of immune cell infiltration had a relatively high liver cancer risk score, indicating that insufficient immune system activity could not effectively inhibit the development of liver cancer. Therefore, comprehensive consideration of liver cancer risk score and immune cell infiltration can more comprehensively evaluate the prognosis of liver cancer.

Immunotherapy is a new technique for the treatment of liver cancer that has attracted much attention in recent years, which aims to destroy tumor cells by activating or enhancing the patient's immune response. The main therapies include immune checkpoint inhibitors, tumor-associated antigen vaccines, and CAR T cell therapy.[19] Immune checkpoints are a balance between T cell activation and suppression. Liver cancer cells can evade the attack of the immune system by regulating the balance of T-cell activation and inhibition. The antitumor effects of the natural immune system in patients with liver cancer can be restored through the use of immune checkpoint inhibitors. Studying whether risk-model-related biomarkers related to immune checkpoints, high and low-risk groups of differentially expressed genes in MICB, SELP, previously undescribed genes, CD80, VEGFA, VEGFB, IFNG, IL1A, TNFSF9, IL12A, CTLA4, HAVCR2, TNFRSF18, TNFRSF4, TNFRSF9. These genes have higher expression levels in the high-risk group, suggesting the potential efficacy of immunotherapy in high-risk patients.[18] The single use of an anti-PD-1/PD-L1 antibody in the treatment of advanced liver cancer patients can achieve certain therapeutic effects and prolong survival. In addition, the combination of multiple immunotherapy drugs may benefit high-risk patients more.

CONCLUSION

In this study, we identified macrophage-related gene markers for prognostic HCC through bioinformatics and constructed risk scores. We studied the correlation between risk models and biomarkers associated with immune checkpoints. It can be further verified in basic and clinical experiments to provide more accurate and individualized solutions for the diagnosis and treatment of liver cancer.

DATA AVAILABILITY

The data could be obtained by contacting the corresponding author.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Li Bai designed the study. Zhiyuan Mao wrote the original draft. Yinglin Li collected raw data. Zhiyuan Mao performed statistical and bioinformatics analyses. Li Bai supervised the study.

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