Eight-Gene Metabolic Signature Related with Tumor-Associated Macrophages Predicting Overall Survival for Hepatocellular Carcinoma

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Abstract

**Background:** In recent years, the relationship between tumor associated macrophages (TAMs) and solid tumors has become a research hotspot. The study aims at exploring the close relationship of TAMs with metabolic reprogramming genes in hepatocellular carcinoma (HCC), in order to provide a new way of treatment for HCC.

**Materials and methods:** The study selected 343 HCC patients with complete survival information (survival time >= 1 month) in the Cancer Genome Atlas (TCGA) as the study objects. Kaplan-Meier survival analysis assisted in figuring out the relationship between macrophage infiltration level and overall survival (OS), and Pearson correlation test to identify metabolic reprogramming genes (MRGs) related to tumor macrophage abundance. Lasso regression algorithm were conducted on prognosis related MRGs screened by Univariate Cox regression analysis and Kaplan-Meier survival analysis to construct the riskscore, another independent cohort (including 228 HCC patients) from the International Cancer Genome Consortium (ICGC) were used for external validation regarding the prognostic signature.

**Results:** A risk score composed of 8 metabolic genes can accurately predict the OS of training cohort (TCGA) and testing cohort (ICGC). It is important that the risk score could widely used for people with different clinical characteristics, and is an independent predictor independent of other clinical factors affecting prognosis. As expected, high-risk group exhibited an obviously higher macrophage abundance relative to low-risk group, and the risk score presented a positive relation to the expression level of three commonly used immune checkpoints (PD1, PDL1, CTLA4).

**Conclusion:** Our study constructed and validated a novel eight-gene signature for predicting HCC patients’ OS, which possibly contributed to making clinical treatment decisions.

**Background**

Growing evidences showed that tumor progression and metastasis are closely related to tumor microenvironment[1, 2]. Once the tumor microenvironment is formed, many immune cells, such as T cells, myelogenic inhibitory cells, macrophages, etc, are chemotactic to form the tumor microenvironment[3]. Among them, Tumor associated macrophages (TAM) are macrophages derived from the infiltration of peripheral blood monocytes into solid tumor tissues[4], which is the most numerous inflammatory cell group in tumor stroma, accounting for about 30% - 50% of the total inflammatory cells[5, 6], and have attracted more and more attention.

In recent years, it has been confirmed that a large number of TAM in tumor microenvironment remarkably affect the tumor occurrence, growth, invasion and metastasis[7–9]. In order to evade the killing effect of macrophages, tumor forces its activation phenotype to change, so that it will evolve in the direction of tumor development. In this process, macrophages are no longer the effective cells of body defense. In turn, they express inflammatory factors, chemokines, angiogenic factors and lymphangiogenic factors[10–12]. However, how TAM can promote tumorigenesis, growth, invasion and metastasis, how
TAM can lead to immunosuppression, and how the biological behavior relationship between tumor cells and TAM remains unresolved.

In order to adapt to the decrease of nutrients and oxygen in tumor microenvironment (TME), and to maintain the high-speed proliferation and material synthesis of tumor cells, a series of changes have taken place in the metabolism mode of tumor cells, which lead to the increase of related metabolites in tumor microenvironment, such as lactate, nitrous oxide, active oxygen, prostaglandin and arachidonic acid, thus causing the inflammatory microenvironment[13, 14]. These changes affect the function of tumor associated macrophages (TAMs). Because of the change of TME composition, the metabolism pattern of TAMs will also change, mainly in glycolysis, fatty acid synthesis and nitrogen cycle metabolism[12, 15]. Changes in metabolism lead to changes in TAMs function, including changes in cytokines and angiogenic factors, thus promoting tumor progression and metastasis[15]. Therefore, it is necessary to understand the metabolic changes of tumor cells and TAMs and their intricate relationship.

Hepatocellular carcinoma (HCC) contributes to more than 90% of all liver cancer cases. Liver cancer ranks 2nd in leading to death resulted from cancer worldwide[16]. It has been reported that metabolic reprogramming has a significant effect on the prognosis of HCC[17, 18]. However, there is no definite answer to the relationship between TAMs and metabolic reprogramming in HCC.

In this study, we used the data of tumor immune cell infiltration in Tumor Immune Estimation Resource (TIMER) database for exploring the effect of the infiltration level of six kinds of immune cells in HCC on prognosis. The results showed that the patients with higher macrophage infiltration level had poor overall survival rate. Then, we further identified differential expressed metabolic related genes (DEMRGs) between high and low infiltration level of macrophage in HCC patients and made use of them building a prognostic signature.

**Materials And Methods**

2.1 Data collection

The data of immune infiltration of the Cancer Genome Atlas (TCGA) tumors were obtained from the TIMER(Tumor Immune Estimation Resource) website(http://timer.cistrome.org/)[19]. The RNA-sequencing of TCGA dataset came from the Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/) (including 343 HCC cases) and corresponding clinical data were acquired from UCSC Xena website(https://xenabrowser.net/). Samely, The RNA-sequencing data together with corresponding clinical information were obtained from the International Cancer Genome Consortium (ICGC) (https://icgc.org/) (including 228 HCC cases). With the purpose of eliminating the impact exerted by perioperative factors on patients’ survival, our study did not include patients with a survival time of less than one month. Sequence data of TCGA and ICGC database utilized in this research were based on the same Illumina HiSeq_RNA-Seq platform and we also used R-package “combat” to remove batch effect. The above data are publicly available, the approval of the local ethics committee is not required.
During the process of our research, we strictly abide by the regulations for the use of the above database. Figure 1 displays the workflow.

2.2 Identification of TAM related metabolic genes (TRMGs)

According to the median value of immune cell infiltration in 343 patients with HCC in TCGA data set, we divided them into high infiltration level group and low infiltration level group. We extracted previously published 2752 genes related to metabolism that encode all the known metabolic enzymes as well as transporters of human being to perform following analysis[20]. Then, we used wilcoxon test in R package “limma” to identified differential expressed metabolic related genes (DEMRGs) between two groups. Pearson correlation test was used for this study for further investigating the association of estimated immune infiltrates with gene expression. The genes with pearson correlation coefficient over 0.4 and p less than 0.001 were defined to have a significant correlation with the TAM. The R package “clusterprofiler” applied in performing the Kyoto Encyclopedia of Genes as well as Genomes (KEGG) pathway enrichment analysis regarding the TRMGs, with the items recognized based on the threshold of P < 0.05.

2.3 Identification of the prognostic TRMGs

Univariate Cox regression analysis together with Kaplan-Meier survival analysis were applied for screening TRMGs associated with prognosis in the TCGA database. P-value < 0.05 in both methods was recognized as the selection criteria.

2.4 Construction of riskscore related to overall survival in TCGA cohort

Lasso regression algorithm were conducted on prognosis related TRMGs screened by Univariate Cox regression analysis and Kaplan-Meier survival analysis to construct the riskscore, which involved confirming the optimal penalty parameter $\lambda$ related to the smallest 10-fold cross-validation in TCGA dataset. Calculation of the risk score specific to the signature was conducted following the formula: Risk score = the sum of each mRNA coefficient X each mRNA expression. The formula assisted in computing the risk score specific to each patient in TCGA and ICGC datasets. The median risk score of TCGA cohort was confirmed as the unified cutoff for splitting patients into high-risk and low-risk groups. The prediction efficiency of our risk signature for 0.5/1/3/5-year survival was assessed by receiver operating characteristic (ROC) curves generated via the R package “survival ROC”. Kaplan–Meier curves from the R package “suvminer” to compare patients’ OS in different groups were tested by the log-rank test. The R package “glmnet” utilized in this research to conduct lasso regression analysis.

2.5 Internal validation of the prognostic signature in TCGA cohort

We performed Kaplan-Meier survival analysis on people with different clinical features (race, gender, age, body mass index (BMI), vascular invasion, histological grade, ajcc-tnm stage, previous malignancy history, presence of new tumors after initial treatment, and individual tumor status) to verify whether the prognostic model could be capable of used for predict different populations’ prognosis.
2.6 External validation of prognostic signature in ICGC cohort

We calculated the risk score of each patient in the ICGC cohort using the same formula obtained from TCGA cohort, and assigned patients into high-risk and low-risk groups with the uniform cutoff. We also performed Kaplan-Meier survival analysis on people with different clinical features (including gender, age, stage, previous malignancy).

2.7 Independence validation of the prognostic signature

Risk score together with clinicopathological features were evaluated by univariate and multivariate Cox regression analyses for verifying if risk score can be regarded as an independent predictor for prognosis of HCC.

2.8 Correlation analysis between risk score and clinicopathology

We conducted chi-square tests on different risk groups for correlation analysis regarding clinical features. P less than 0.05 represented statistical significance.

2.9 Estimation of immune infiltration

Two algorithm were carried out for estimation of immune infiltration in different risk groups: TIMER, which is a calculating method for estimating the abundance exhibited by 6 types of tumor-infiltrating immune cell (B cell, CD4 T and CD8 T cells, neutrophil, macrophage, as well as dendritic cell)[21]; CIBERSORT-ABS, which is a methodology on the basis of the gene expression profile for evaluating the absolute abundance exhibited by 22 immune cell populations[19].

2.10 Statistical analysis

R v.3.6.1 (https://www.r-project.org/) and SPSS Statistics 25 (https://www.ibm.com/products/software) were employed for all the statistical analyses. Unpaired Student's t-test and Mann–Whitney U-test assisted in comparing two groups containing variables with normal distribution and two groups containing variables with non-normal distribution, respectively. To compare the three groups, one-way analysis served as the parametric method and Kruskal–Wallis tests of variance served as the nonparametric method. Fisher's exact tests or chi-square test assisted in analyzing the contingency table variables. Kaplan–Meier method was applied to the survival analysis, with results compared through the log-rank test. A univariate Cox proportional hazards regression model assisted in estimating the hazard ratios exhibited by univariate analyses. A two-tailed P value less than 0.05 exhibited statistical significance [22].

Results

3.1 Identification and annotation of TRMGs
We observed that patients with high abundance of macrophage infiltration had a poor prognosis (Fig. 2a), which provided a clue for us to find prognostic biomarkers of HCC according to the degree of macrophage infiltration. We identified 1190 metabolic genes with different expression between high macrophage infiltration and low macrophage infiltration patients (Fig. 2b), among which 192 genes were significantly related to macrophage infiltration (cor > 0.4, p < 0.001) (Fig. 2c). GO and KEGG enrichment analysis showed that above genes involved many aspects of metabolism, involving glycoprotein, sulfur compound, coenzyme, carbon, purine, glycolysis, glycogenesis, etc (Fig. 3).

### 3.2 Construction of prognostic signature

A total of 87 TRMGs screened out by Univariate Cox regression analysis and Kaplan-Meier survival analysis and they were all risk factors for the overall survival of HCC (Fig. 4a). The lasso regression model included 8 metabolic genes associated with prognosis (Fig. 4b-c), namely G6PD, GNPDA1, LDHA, ELOVL1, SLC25A24, CAD, GTDC1, and AMD1. The risk score = 0.0045*expression of G6PD + 0.0010*expression of GNPDA1 + 0.0018*expression of LDHA + 0.0042*expression of ELOVL1 + 0.0025*expression of SLC25A24 + 0.0519*expression of CAD + 0.0847*expression of GTDC1 + 0.0030*expression of AMD1.

The median value of risk score 0.731 defined as the critical value of dividing high-risk group and low-risk group. The relationship of 8 genes’ expression to macrophage cell infiltration is shown in Fig. 5.

### 3.3 Prognostic assessment of the signature in TCGA cohort

Kaplan-Meier survival showed that the patients of high-risk group with obviously worsen overall survival (OS) outcome compared to low-risk group (log-rank test p < 0.001) (Fig. 6a). The AUC for 1, 3, and 5 OS was 0.786, 0.727, and 0.693, respectively (Fig. 6b). The survival status distribution map also showed that the number of dead patients increased as the growing of risk score (Fig. 6c-e). Only risk score can be regarded as an independent prognosis predictor for OS as demonstrated by univariate and multivariate Cox regression analysis (Fig. 7a-b).

### 3.5 Internal validation of the prognostic signature in TCGA cohort

Patients were assigned into 20 groups according to different clinical characteristics. Compared with low-risk group, high-risk group exhibited poorly prognostic outcome in each subgroup based on the Kaplan-Meier analysis. The p values obtained from log-rank test in each subgroup were all < 0.05 (Fig. 7c).

### 3.6 External validation of the prognostic signature in ICGC cohort

228 patients with complete survival information served as external validation cohort. Each patient acquired their own risk score computed with the formula obtained from TCGA, we separated them into high-risk and low-risk groups following the same cutoff (0.731). High-risk group with a bad survival outcome demonstrated by Kaplan-Meier survival analysis (p < 0.01), which consistent with the result of TCGA cohort (Fig. 8a). The AUC for OS at 1, 3, and 5 years OS was 0.775, 0.713, and 0.761, respectively (Fig. 8b). Univariate and multivariate Cox regression analysis indicated that risk score with independent ability to predict overall survival (Fig. 9a-b). We also carried out Kaplan-Meier survival
analysis on patients assigned by clinical features (8 subgroup), patients with high-risk group exhibited a worse OS in each subgroup (Fig. 9c). These results further confirmed the robustness of the signature in prediction for overall survival of HCC.

3.7 Relationship between riskscore and clinical features

According to the results of Chi square test, the high-risk group has higher histopathological grade, later clinical stage, more prone to vascular invasion, higher probability of new tumor after initial treatment, and is usually in the state of carrying tumor, which may helped to explain the poorly prognosis of high-risk patients (Table 1–2).
Table 1
The chi-square test of the relation between risk score and clinical features in TCGA cohort

| Clinical feature           | Risk Score | c2    | P    |
|----------------------------|------------|-------|------|
|                            | High risk n(%) | Low risk n(%) |
| Age                        | 0.931      | 0.335 |
| ≥ 65                       | 59(46.46%) | 68(53.54%)   |
| ≤ 65                       | 112(51.85%) | 104(48.15%)  |
| BMI                        | 1.275      | 0.259 |
| ≥ 25                       | 71(46.41%) | 82(53.59%)   |
| ≤ 25                       | 86(52.76%) | 77(47.24%)   |
| Family cancer history      | 0.149      | 0.7   |
| NO                         | 29(23.58%) | 94(76.42%)   |
| YES                        | 19(26.03%) | 54(73.97%)   |
| Gender                     | 2.032      | 0.154 |
| female                     | 61(55.45%) | 49(44.55%)   |
| male                       | 110(47.21%)| 123(52.79%)  |
| Tumor status               | 10.08931   | 0.001 |
| tumor free                 | 73(40.78%) | 106(59.22%)  |
| with tumor                 | 87(58.39%) | 62(41.61%)   |
| New tumor event            | 4.85       | 0.028 |
| no                         | 71(43.83%) | 91(56.17%)   |
| yes                        | 94(55.95%) | 74(44.05%)   |
| Prior Malignancy           | 2.815      | 0.093 |
| no                         | 160(51.28%)| 152(48.72%)  |
| yes                        | 11(35.48%) | 20(64.52%)   |
| Histologic Grade           | 14.724     | 0.001 |
| G1-2                       | 90(42.06%) | 124(57.94%)  |
| G3-4                       | 79(63.71%) | 45(36.29%)   |
| Stage                      | 13.911     | 0.001 |
### Table 2
The chi-square test of the relation between risk score and clinical features in ICGC cohort

| Clinical feature        | Risk Score | $c^2$ | P    |
|-------------------------|------------|------|------|
|                         | High risk n(%) | Low risk n(%) |      |
| Age                     | 0.899      | 0.343|
| ≤65                     | 61(43.57%) | 79(56.43%) |      |
| ≤65                     | 44(50%)    | 44(50%)  |      |
| Gender                  | 0.001      | 0.978|
| female                  | 28(45.91%) | 33(54.10%) |      |
| male                    | 77(46.11%) | 90(53.89%) |      |
| Prior Malignancy        | 0.292      | 0.589|
| no                      | 93(46.73%) | 106(53.27%) |      |
| yes                     | 12(41.38%) | 17(58.62%) |      |
| Stage                   | 9.806      | 0.002|
| I-II                    | 53(37.32%) | 87(62.68%) |      |
| III-IV                  | 52(59.09%) | 36(40.91%) |      |

#### 3.8 Relationship between risk score and immune infiltration
The outcome of TIMER algorithm showed that high-risk group presented a higher infiltration abundance regarding 6 types of immune cells, namely, B cell, CD4 T and CD8 T cells, macrophage, neutrophil as well as dendritic cell, relative to low-risk group (Fig. 10a). Among them, the risk score has the strongest correlation with macrophages and neutrophils (Fig. 10b). The above two immune cells were also estimated to be with higher infiltration level in high-risk group used by CIBERSORT-ABS algorithm (Fig. 10c-d). At the same time, we found that there was co expression relationship between 8 genes and a variety of immune checkpoints and risk score was positively related to expression of CD274, PDCD1 and CTLA4 (Fig. 10e-f).

Discussion

Hepatocellular carcinoma (HCC) acts as a representative primary liver cancer. Despite the improved treatment recently, its still holds a low five-year survival [23]. The mechanism of occurrence and development of HCC remains to be further clarified. In recent years, it has been found that the occurrence and development of HCC is not only related to the characteristics of tumor itself, but also closely related to its microenvironment [3]. Tumor and stromal cell, various immune inflammatory cells, chemokines and cytokines together constitute the tumor microenvironment [24], where tumor associated macrophages (TAMs) play the role of significant inflammatory cells. As shown by more and more studies, TAMs promote the generation, metastasis and immunosuppression of HCC [25, 26]. With the development of genomics and proteomics, we can better study the molecular mechanism of TAMs in the process of tumorigenesis and development, clarify the relationship between TAMs and tumor, and provide new clues for tumor biotherapy. It has been reported that abnormal metabolism of malignant tumor cells can not only induce changes in the phenotype and function of TAMs, but also change the metabolic mode of TAMs, causing it to exert immunosuppressive function, and finally promote the development and metastasis of tumor [15]. On that account, it is of huge interest to identify new metabolic genes in cancers for predicting its death risk.

The study found that patients with high level of macrophage infiltration had poor prognosis. We further screened out metabolic-related genes whose expression level with closely correlated with infiltration of macrophage following this clue. A prognostic signature contained 8 genes built eventually in TCGA dataset used by lasso regression analysis. A prognostic signature contained 8 genes built used by lasso regression analysis could accurately assess the overall survival of HCC patients in both TCGA and ICGC cohort. It is worth noted that the signature could applicable for people with different clinical features demonstrated this signature with strongly robustness. Meanwhile, the signature could be regarded as an independent predictor for overall survival of HCC confirmed by cox analysis. The immune microenvironment of different risk group were also evaluated, immune infiltration has been reported to be correlated with clinical outcome of many kinds of cancer previously [27]. In this study we found that the high-risk people who has unfavorable prognosis with high level of Tumor-infiltrating macrophages, which consistent of the results of Li’s report [28]. In addition, high-risk group also exhibited a higher infiltration level of Tregs and Neutrophils relative to low-risk group, the two kinds of immune cells’s infiltration were also have been reported correlated with prognosis of HCC previous [29, 30]. PDL1(CD274), PDCD1, CTLA4
were three kinds of immunosuppressive agents commonly used in clinic[31], which may be used by tumors to form immune escape. They control the progress of cell cycle by controlling extracellular and intracellular signals. which expression level were positively related to riskscore, implicated that metabolic reprogramming gene may have a significant effect on tumor immune microenvironment.

Lactate dehydrogenase A (LDHA) is a metabolic enzyme that can produce lactate in human body, and has become an important indicator of clinical tumor diagnosis. It has been reported that it can mediate tumor immune escape by inhibiting the activity of T cells and NK cells[32]. It was found for the first time that LDHA expression level was positively related to the macrophage abundance in HCC, which also provided a new clue for the further study of the tumor immune mechanism regulated by LDHA. Human solute carrier protein 25 family is a superfamily of human solute carrier protein family, which plays a role in the molecular transport, oxidative phosphorylation and iron metabolism related to urea and citric acid cycle. Recently, more and more researches have found that SLC25 family members can affect tumor occurrence and development [33]. The study for the first time demonstrates the correlation between SLC25A24 and prognosis of HCC and macrophage infiltration. Glycosyltransferase played an critical role in glycosylation, which can catalyze the transfer of active glycosyl from glycosyl donor to glycosyl receptor and form glycosidic bond[34]. David Kessel et al found that levels exhibited by three plasma glycosyltransferases could affect cancer patients' neoplasia especially for patients with tumor metastasizing to liver [35]. However, the mechanism of glycosyltransferase like domain containing 1 (GTDC1) in HCC remains to be elucidated. After HCC leads to unlimited proliferation of hepatocytes, it begins to slow down the development speed. After that, the most common metabolic phenomenon observed in HCC cells is the increase of glycolysis rate, which is called Warburg effect[36]. glucose effect 6-phosphotetahydrodredogenase (G6PD) is an important metabolic enzyme in glycolysis pathway, which has been proved to be related to the proliferation and apoptosis of HCC[37]. This study first found the correlation between 6-phosphotetahydrodredogenase and the abundance of tumor macrophages, which provides a new direction for further exploring the development mechanism of G6PD regulating HCC. The rapid growth of tumor also depends on the content of polyamine in cells. The abnormal metabolism of polyamine can also cause malignant transformation of cells[38]. The metabolism of polyamine has draw the special attention by tumor research field. S-adenosylmethionine decarboxylase 1 (AMD1), also known as SAMDC, is the rate limiting enzyme regulating polyamine metabolism[39, 40]. In lymphoma, AMD1 plays the role of tumor suppressor gene by regulating the post-translational modification regarding eukaryotic translation initiation factor 5A (eIF5A)[39]; in prostate cancer, AMD1 affects tumor cell proliferation by regulating the mTOR pathway, thus promoting tumor development[41]; AMD1 also remarkably affects breast cancer occurrence and development[42]. However, the expression and role of AMD1 in HCC are rarely reported. The role of the remaining three genes: CAD, GNPDA1 and ELOVL1 in tumor remains unclear.

Our study is the first attempt to identify the metabolic genes with prognostic significance for HCC from the perspective of immune infiltration. Our results further showed that there is a significant relationship between metabolic reprogramming and tumor immune microenvironment, and metabolic disorders may affect tumor development by mediating tumor immune regulation. which provided theoretical support for
exploring the non-metabolic mechanism of metabolic genes in the future. However, there are still some limitations in our research, because we have not carried out further experiments to explore the immune mechanism of the above metabolic genes, which we need to add in our future work. Our research preliminarily demonstrated that it is feasible to explore the immune activity of tumor from the perspective of metabolizing reprogramming. Therefore, it is necessary to continue to carry out multi-center prospective research on this subject.

**Conclusion**

Our study established a novel riskscore to evaluate the prognosis of HCC by identifying the metabolic genes related to tumor macrophages. Considering the heterogeneity of HCC, the prognostic model may further improve the prognosis evaluation system of HCC, At the same time, our study also provides theoretical support for further elucidating the complex relationship between metabolic reprogramming and tumor immune mechanism.

**Abbreviations**

HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; ICGC, International Cancer Genome Consortium; TAMs, tumor associated macrophages; TRMGs, TAM related metabolic genes; DEGs: differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic; OS, overall survival.

**Declarations**

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**Data availability statement**

The datasets analysed for this study were obtained from The Cancer Genome Atlas (TCGA) ([https://portal.gdc.cancer.gov/](https://portal.gdc.cancer.gov/)) and International Cancer Genome Consortium (ICGC) ([https://icgc.org/](https://icgc.org/)) and TIMER ([https://cistrome.shinyapps.io/timer/](https://cistrome.shinyapps.io/timer/)) and UCSC Xena website ([https://xenabrowser.net/](https://xenabrowser.net/)).

**Author contributions**
Junyu Huo and Liqun Wu designed this study, Junyu Huo, Hongjing Dong, Xiaoqiang Liu, Fu He, and Xiao Zhang analyzed the data in this study and interpreted the findings and drafted the manuscript. Liqun Wu, Yunjin Zang carried out, data management and revised the manuscript. All authors reviewed the final version of the manuscript.

**Declaration of Competing Interest**

The authors have no conflicts of interest to declare.

**Ethics approval and consent to participate**

Not applicable

**Declarations**

Not applicable

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Figures

The work flow chart of this study
Figure 2

Identification of Tumor associated macrophage-related metabolic genes (a) Kaplan–Meier survival curve regarding macrophage abundance and overall survival (b) Heat map of differentially expressed metabolic-related genes between high and low macrophage abundance hcc patients (c) Venny plot of Tumor associated macrophage-related metabolic genes screened by pearson correlation analysis
Figure 3

Functional enrichment analysis of Tumor associated macrophage-related metabolic genes (TRMGs) (a-b) GO enrichment analysis of TRMGs (c-d) KEGG enrichment analysis of TRMGs
Figure 4

The building process of the prognostic signature (a) Forrest plot of prognostic metabolic genes identified by Univariate cox and Kaplan–Meier survival analysis (b) Lasso regress analysis performed on prognostic metabolic genes
Figure 5

Eight genes that make up the prognostic model (a) Correlation analysis between expression level of 8 genes and Infiltration level of macrophage (The image downloaded from https://cistrome.shinyapps.io/timer/) (b) Boxplot of expression level of 8 genes between tumor and normal tissues (The image downloaded from http://gepia.cancer-pku.cn/) (c) Kaplan–Meier survival curve regarding expression level of 8 genes and overall survival (The image downloaded from http://gepia.cancer-pku.cn/)
Figure 6

Construction of the prognostic model in TCGA cohort (a-b) Kaplan–Meier survival analysis and time dependent ROC analysis of predicting overall survival for patients in TCGA cohort used by riskscore (c-e) The heatmap of the eight genes and the distribution of risk score and the survival status of patients
Figure 7

Internal validation of the prognostic model in TCGA cohort (a-b) Univariate and multivariate regression analysis of the relation between the RS and clinicopathological characteristics regarding overall survival in the TCGA cohort (green represents univariate analysis, and red represents multivariate analysis) (c) Subgroup survival analysis depending on different clinical features
Figure 8

External validation of the prognostic model in ICGC cohort (a-b) Kaplan–Meier survival analysis and time dependent ROC analysis of predicting overall survival for patients validated in ICGC cohort used by riskscore (c-e) The heatmap of the eight genes and the distribution of risk score and the survival status of patients
Figure 9

Verify the universal applicability of the prognostic model in the ICGC cohort (a-b) Univariate and multivariate regression analysis of the relation between the RS and clinicopathological characteristics regarding overall survival in the ICGC cohort (green represents univariate analysis, and red represents multivariate analysis) (c) Subgroup survival analysis depending on different clinical features
Figure 10

The landscape of immune infiltration in high- and low-risk HCC patients (a-b) Relationships between the risk score and infiltration abundances of six types of immune cells (TIMER method, red represent high-risk group, blue represent low-risk group) (c) Cor-heatmap of absolute abundance of 22 immune infiltration cell proportions estimated by CIBERSORT-ABS method (d) Violplot of absolute abundance of 22 immune infiltration cell estimated by CIBERSORT-ABS method (red represent high-risk group, blue represent low-risk group) (e) Analysis of coexpression of 8 genes and immune checkpoints (f) The comparison of
expression level of CD274(PDL1), PDCD1, CTLA4 between different risk groups and correlation analysis between riskscore and the expression level of CD274(PDL1), PDCD1, CTLA4