A Novel Signature Based On Mtorc1 Pathway In Hepatocellular Carcinoma

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Abstract

Background

mTORC1 signal pathway play a role in the initiation and progression of hepatocellular carcinoma (HCC), but no relevant gene signature was developed. This research aimed to explore the potential correlation between mTORC1 signal pathway and HCC and establish the related genes signature.

Methods

HCC cases were retrieved from The Cancer Genome Atlas (TCGA), International Cancer Genome Consortium (ICGC) and Gene Expression Omnibus (GEO) databases. The genes to be included in mTORC1-associated signature were selected by performing univariate, multivariate Cox regression analysis and lasso regression analysis. Then, the signature was verified by survival analysis and multiple receiver operating characteristic (ROC) curve. Moreover, the correlation between signature and immune cells infiltration was investigated. Furthermore, a nomogram was established and evaluated by C-index and calibration plot.

Results

The signature was established with the six genes (ETF1, GSR, SKAP2, HSPD1, CACYBP and PNP). Under the grouping from signature, patients in the high-risk group showed worse survival than those in the low-risk group in both three datasets. The signature was found significantly associated with the infiltration of B cells, CD4+T-cells, CD8+T-cells, dendritic cells, macrophages and neutrophils. The univariate and multivariate Cox regression analysis indicated that mTORC1 related signature can be the potential independent prognostic factor in HCC. Finally, the nomogram involved age, gender, stage and signature have been established.

Conclusion

The mTORC1 associated gene signature established and validated in our research could be used as a potential prognostic factor in HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancer around the world, becoming the second leading causes of tumor related death [1]. Owing to the high rate of metastasis, HCC patients with advanced stage are usually with a poor prognosis [2]. Although the treatment and biomarkers of HCC have developed, the clinical outcomes of HCC patients are still unsatisfactory [3]. The occurrent and development of HCC involved interactions between genetics, epigenetics and transcriptomic alterations [4]. Many studies have verified that different biomarkers have certain prognostic value in HCC. For
example, Gu et al found that CCL14 was a potential prognostic biomarker that correlated with tumor immune cell infiltration in HCC [5]. Another study found the strong correlations between PRPF3 expression and prognosis in HCC[6]. However, as a biomarker, a single gene usually has a lower prognostic value than multigene prognostic signature. Therefore, many gene related signature have been developed for predicting prognosis of HCC. For instance, Zhang et al established a gene signature associated with HCC microenvironment and successfully verified it[7]. Other predictive signatures based on immune[8] and glycolysis[9] also play an important role in prognosis of HCC.

Generally, the gene researches usually focus on comparing the gene expression between two groups, or pay attention to the highly upregulated and downregulated genes. Nevertheless, some genes which showed no significant difference but had important biological function and characteristics were omitted. In view of this, a computational method, Gene Set Enrichment Analysis (GSEA) determined whether a prior defined set of genes shows statistically significant differences between two biological states[10]. The advantage of GSEA is that it can identify the genes which expression is based on the trend of overall level. Consequently, in this research, we identified the pathway and gene with GSEA. Then, we constructed the signature based on related genes and verified it, providing the more comprehensive and accurate prognostic model for clinic.

2. Materials And Methods

2.1 Data collection

The gene expression data with the type of level 3 RNA-seq FPKM dataset and the clinical messages in TCGA website (https://portal.gdc.cancer.gov/) were retrieved. A total of 377 HCC cases have been downloaded and analyzed, which included 50 normal samples and 327 tumor samples. As the cohorts of validation, GSE76427 datasets were retrieved from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), while ICGC-LIRI dataset was retrieved from International Cancer Genome Consortium (ICGC) database (https://icgc.org/).

2.2 Identification of pathways and related genes.

After data collection, we extracted the clinical details and generated the expression matrix of all genes in HCC of TCGA dataset. Then we divided the patients into two groups according to the survival status and employed GSEA to choose the most relevant pathway. The pathways were considered for further analyses if a normalized P-value < 0.01. After that, we collected the related genes of involved pathway from Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb/index.jsp).

2.3 Construction and verification of signature
First, we performed the differentially expressed analysis to select the related genes. The “limma” package under R studio software was employed and a P-value < 0.05 was considered statistically significant. Second, we employed the univariate and multivariate Cox regression analysis to choose the prognostic genes among the differentially expressed genes. The genes in this section were eligible for further selection if a P-value < 0.05. Third, the lasso regression analysis was executed for checking selected genes. In this analysis, a lasso penalty was applied, to simultaneously accounting for shrinkage and variable selection. The optimal value of the lambda penalty parameter was defined by performing 10 cross-validations. Using the “glmnet” package, the coefficient of each included genes and risk score of each case were calculated. The calculation formula of risk score was following: risk score = (coefficientmRNA1 × expression of mRNA1) + (coefficientmRNA2 × expression of mRNA2) +⋯+ (coefficientmRNAn × expression mRNA). The cases were divided into two groups (high risk or low risk), according to the risk score median. To explore the time-dependent prognostic value of our gene signature, the survival analysis was performed using the “survival” package in the R studio software. The relationship between signature and other clinical messages were also evaluated and visualized with a heatmap. Besides, the multiple receiver operating characteristic (ROC) curve was performed for checking the accuracy. In addition, we investigated the correlation between signature and six different immune cells (B cells, CD4+ T-cells, CD8+ T-cells, dendritic cells, macrophages and neutrophils). The infiltration data of six immune cells were retrieved from tumor immune estimation resource (https://cistrome.shinyapps.io/timer/). Moreover, the univariate and multivariate Cox regression analysis were performed to verify whether the risk score is an independent prognostic factor.

2.4 Predictive nomogram design

A predictive nomogram based on age, gender, stage and risk score was constructed using the “rms” package and Cox regression model to predict the overall survival (OS) at 3 years and 5 years of HCC patients. Then, we used the Harrell’s concordance index (C-index) and calibration plot to evaluate the nomogram.

3. Results

The clinical data details of the patients used in this study are shown in Table 1. Figure 1 show the screening process and validation of our study. The result of GSEA in Fig. 2 showed that a total of 6 pathways were eligible and the details of pathways were summarized in Table 2. Considering the highest normalized enrichment score of mTORC1 pathways, we chose the 199 relevant genes in this pathway for further analysis. The differentially expressed analysis showed that 160 genes were significantly different in HCC (see Additional file 1). After that, the univariate and multivariate Cox regression analysis demonstrated that 15 genes (ETF1, CTSC, GSR, HSPE1, SKAP2, HSPD1, TES, TFRC, ASNS, EPRS, CANX, CACYBP, UNG, TBK1 and PNP) were included with P < 0.05 (see Additional file 2 and file 3). As illustrated in Fig. 3a and 3b, the results of lasso regression analysis further confirmed the signature composed of 6
genes (ETF1, GSR, SKAP2, HSPD1, CACYBP and PNP). And the coefficients of ETF1, GSR, SKAP2, HSPD1, CACYBP and PNP were 0.03402, 0.00670 0.02556, 0.00181, 0.02034 and 0.00916, respectively.
| Clinical characteristics       | Number | percent (%) |
|-------------------------------|--------|-------------|
| **TCGA-LIHC (n = 377)**       |        |             |
| Survival status               |        |             |
| Survival                      | 249    | 66          |
| Death                         | 128    | 34          |
| Age (1 patient missing)       |        |             |
| ≤ 65 years                    | 235    | 62.5        |
| > 65 years                    | 141    | 37.5        |
| Gender                        |        |             |
| Female                        | 122    | 68          |
| Male                          | 255    | 32          |
| Stage (24 patients missing)   |        |             |
| I                             | 175    | 50          |
| II                            | 87     | 24.6        |
| III                           | 86     | 24.4        |
| IV                            | 5      | 1           |
| Grade (5 patients missing)    |        |             |
| G1                            | 55     | 14          |
| G2                            | 180    | 48          |
| G3                            | 124    | 33          |
| G4                            | 13     | 5           |
| T classification (3 patients missing) |        |             |
| T1                            | 185    | 49          |
| T2                            | 95     | 26          |
| T3                            | 81     | 22          |
| T4                            | 13     | 3           |
| **GSE76427 (n = 115)**        |        |             |
| Survival status               |        |             |
| Survival                      | 92     | 80          |
| Death                         | 23     | 20          |
| Age                           |        |             |
| ≤ 65 years                    | 65     | 56.5        |
| > 65 years                    | 50     | 43.5        |
| Gender                        |        |             |
| Female                        | 22     | 19.1        |
| Male                          | 93     | 80.9        |
## Clinical characteristics

| Stage | Number | percent (%) |
|-------|--------|-------------|
| I     | 55     | 47.8        |
| II    | 35     | 30.4        |
| III   | 21     | 18.3        |
| IV    | 4      | 3.5         |

ICGC-LIRI (n = 260)

| Survival status | Number | percent (%) |
|-----------------|--------|-------------|
| Survival        | 214    | 82.4        |
| Death           | 46     | 17.6        |

| Age              | Number | percent (%) |
|------------------|--------|-------------|
| ≤ 65 years       | 98     | 37.7        |
| > 65 years       | 162    | 62.3        |

| Stage | Number | percent (%) |
|-------|--------|-------------|
| I     | 40     | 15.4        |
| II    | 117    | 45          |
| III   | 80     | 30.8        |
| IV    | 23     | 8.8         |

Table 2
Details of signal pathways selected by GSEA.

| Name                  | Size | ES      | NES      | Normalized p-value |
|-----------------------|------|---------|----------|--------------------|
| MTORC1 SIGNALING       | 200  | 0.581694| 1.982214 | 0                  |
| UV_RESPONSE_UP         | 158  | 0.506378| 1.955258 | 0                  |
| GLYCOLYSIS             | 200  | 0.506672| 1.88337  | 0                  |
| G2M_CHECKPOINT        | 199  | 0.73077 | 1.881815 | 0.002088           |
| MYC_TARGETS_V1        | 199  | 0.685811| 1.860498 | 0.004141           |
| E2F_TARGETS           | 200  | 0.745006| 1.856894 | 0.002079           |

Note: ES = Enrichment Score, NES = Normalized Enrichment Score.

Besides, the heatmap of risk score and clinical parameters were shown in Fig. 3c. All the genes included in the signature were highly expressed in the high-risk group and lowly expressed in the low-risk group.
The correlation between gene expression of final included genes and clinical parameters were showed in Table 3. Meanwhile, significant difference was found between risk score and stage, grade, T and M, respectively (Table 3). The survival analysis of TCGA (Fig. 4a), GSE76427 (Fig. 4b) and ICGC (Fig. 4c) both indicated that significant difference was found between two groups (P < 0.05). And the multiple ROC curve plot (Fig. 4d-4f) demonstrated that the risk score got the highest predictability among analyzed factors in 1 year (AUC = 0.802), 3 years (AUC = 0.743) and 5 years (AUC = 0.719). In terms of immune cells infiltration, significant difference was found between risk score and B cell, CD4^+ T cell, CD8^+ T cell, Dendritic, Macrophage and Neutrophil (Fig. 5). Furthermore, we performed the univariate and multivariate Cox regression analysis, and the results in Table 4 revealed that the signature can be the independent prognostic factor in HCC (P < 0.01).

Table 3
The correlation between included genes (signature) and clinical parameters.

| id    | Age  | Gender | Grade | Stage | T    | M    | N    |
|-------|------|--------|-------|-------|------|------|------|
| ETF1  | 0.191| 0.209  | -1.899| -1.797| -1.298| 0.547| -1.291|
| GSR   | 0.782| -1.263 | -2.216| -1.886| -2.008| 2.607| 2.35  |
| SKAP2 | -0.721| 1.488  | 1.274 | -2.457| -2.009| 1.576| -0.815|
| HSPD1 | 0.988| -0.442 | -2.609| -1.418| -1.17 | 3.016| -1.426|
| CACYBP| 2.79 | -0.636 | -2.635| -1.547| -1.71 | 0.38 | -0.015|
| PNP   | 1.614| 1.766  | -1.139| -2.781| -2.819| 1.759| -0.813|
| Risk score | 0.94 | -0.45  | -2.687| -2.603| -2.428| 4.473| -1.171|

Note: the results in table represent correlation coefficient and P-value, the bold represent significantly different.
Finally, we built the nomogram based on established signature to predict 1-year, 3-years and 5-years OS for HCC patients (Fig. 6a). The C-index of nomogram was 0.73, and the calibration plot for the probability of survival at 1 year (Fig. 6b), 3 years (Fig. 6c) and 5 years (Fig. 6d) showed good agreement between the prediction by nomogram and real observation.

4. Discussion

In the initiation and progression of hepatocellular carcinoma, genetic factors usually play an important role. Meanwhile, mRNA gene signature based on a certain characteristic like glycolysis[11] and immune[12] have been developed for prognosis of cancers. In this research, we explored specific function to identify genes by GSEA that could predict the survival of HCC patients. According to our results, six
signal pathways was found to be highly related to survival and we established the gene signature with mTORC1 signal pathway. As we all known, mTOR pathway is a serine/threonine protein kinase belonging to the PI3K-related kinase family[13], which comprised of two distinct complexes (mTORC1 and mTORC2). With Raptor as its unique and key protein component, mTORC1 plays an important role in cell survival, autophagy, and metabolism[14]. Concerning for the mTOR signal pathway in HCC, it has been found that aberrant mTOR signaling was present in half of the HCC cases[15]. Meanwhile, an intact mTORC1 axis [16] and mTORC2-Akt1 cascade [17] were required for c-Myc-driven hepatocarcinogenesis. Moreover, some researches [15, 18] provided the theoretical basis of mTOR signaling pathway-oriented targeting treatment for HCC in clinic. Overall, these above-mentioned evidences demonstrated that mTOR signal pathway plays an important role in the development of HCC.

In this study, we identified six genes in signature by performing the differentially expressed analysis, univariate Cox regression analysis and lasso regression analysis. Among our included genes, five genes have been found to be related to HCC from previous studies. Singh et al found that ETF1, CNOT6 and XRN1 gene in HepG2 cell led to significant alteration in stability of specific mRNAs and this mechanism may hold novel cancer therapeutic targets[19]. In another research, McLoughlin concluded that GSR, TRXR1, NRF2 and oxidative stress determined hepatocellular carcinoma malignancy[20]. Lee's study[21] found that HSPD1 was down-regulated during early apoptosis of the hepatoma cell mediated by Paeoniae Radix. In terms of CACYBP, it have been verified that CACYBP can promote hepatocellular carcinoma progression in the absence of RNF41 mediated degradation[22]. Moreover, a study[23] found that PNP/fludarabine suicide gene system induced HCC cell apoptosis and inhibited the growth of HCC cells. Although we found no evidence supporting the correlation between SKAP2 and HCC, it has been verified that SKAP2 promotes podosome formation to facilitates tumor-associated macrophage infiltration and metastatic progression[24].

Recently, further investigations have been performed to explore how the mTOR signal transduction mechanisms modulate sensitivity of targeted therapies, angiogenesis and tumor immunity[25]. The interest in mTOR targeting may improve immune response against cancer and develop new therapeutic strategy. It has been verified that an inflammatory-CCRK circuitry drove mTORC1-dependent metabolic and immunosuppressive reprogramming in obesity-related hepatocellular carcinoma[26]. In another study[27], Tan concluded that Tim-3-mediated PI3K/mTORC1 interference lead to the dysfunction of both tumor-infiltrating conventional natural killer cells and liver-resident natural killer cells. In our research, the results showed that mTORC1 signature significantly associated with B cell, CD4+ T cell, CD8+ T cell, Dendritic, Macrophage and Neutrophil, which indicated that the patients in high risk group may benefitted from immune targeted therapies and provided a new strategy for immune checkpoint-based targeting.

Being different from the previous prognostic studies in HCC, our predictive model firstly concentrated on mTORC1 signal pathway. More importantly, mTORC1 signal pathway was identified by GSEA, which indicated the underlying mechanism between survival of HCC and mTORC1 pathway. Moreover, the validation from four different datasets and a rigorous screening process enabled the identification of a reliable signature. However, our study has some limitations. First, prognostic signature showed a
relatively low diagnostic performance in predicting 5-years OS. It may be attributed to that only 200 associated genes were defined and evaluated for the initiation of screening process. Second, the using a single characteristic (mTORC1 signal pathway) to establish the predictive model is an intrinsic weakness. Indeed, many other mechanisms, such as metabolism\cite{28} and immune\cite{8}, have an influence on the development and progression of HCC. Furthermore, our signature explored the underlying effect between mTOR signal pathway and HCC, but it’s necessary to perform more independent trials and functional experiments to shed light on the mechanism linking them.

5. Conclusion

Our study is the first to identify a novel gene signature related to mTORC1 signal pathway that could be used as a potential prognostic factor in hepatocellular carcinoma.

Abbreviations

HCC
Hepatocellular carcinoma
TCGA
The Cancer Genome Atlas
ICGC
International Cancer Genome Consortium
GEO
Gene Expression Omnibus
ROC
Receiver operating characteristic
GSEA
Gene set enrichment analysis
OS
overall survival
C-index
Concordance index

Declarations

Ethics approval and consent to participate:
Not applicable.

Consent for publication:
Not applicable.

Availability of data and materials:
The datasets generated and/or analyzed during the current study are available in the TCGA (https://portal.gdc.cancer.gov/), GEO (https://www.ncbi.nlm.nih.gov/geo/), and ICGC (https://icgc.org/).

Competing interests:
The authors declare that they have no competing interests.

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Authors' contributions:
Zhuomao Mo and Shijun Zhang designed the manuscript. Zhuomao Mo and Shuqiao Zhang wrote and completed the manuscript. Zhuomao Mo and Shijun Zhang completed the data download and analysis. All the authors approved the final manuscript.

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Figures
Figure 1

The flowchart of this study
Figure 2

Enrichment plots of signal pathways which importantly differentiated between survival and death group
Figure 3

Lasso regression analysis results and heatmap. Notes: Panels (a) show the partial likelihood deviance for the lasso regression, panels (b) show the lasso regression analysis, panels (c) show the heatmap of signature and clinical parameters.
Figure 4

Survival analysis results and multiple ROC results. Notes: Panels (a) (b) (c) show the survival analysis of TCGA-LIHC cohort, GSE76427 cohort and ICGC-LIRI cohort, respectively. Panels (d) (e) (f) show multiple ROC results in 1 year, 3 years and 5 years, respectively.
Figure 5

Correlation plot between risk score and immune cells infiltration
Figure 6

Construction and validation of Nomogram. Notes: Panels (a) show the nomogram based on age, gender, stage and gene signature, panels (b) (c) (d) show the calibration plot of 1-year, 3-years and 5-years OS, respectively.

Supplementary Files

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