An Autophagy-Related Prognostic Signature and Verification that ATIC Inhibits Autophagy Through the AMPK/mTOR Pathway in Hepatocellular Carcinoma

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Abstract

Background: Autophagy regulates many cell functions related to cancer, ranging from cell proliferation and angiogenesis to metabolism. Due to the close relationship between autophagy and tumors, we investigated the predictive value of autophagy-related genes.

Methods: Data from patients with hepatocellular carcinoma were obtained from the TCGA and ICGC databases. Regression analysis of differentially expressed genes was performed. Based on a prognostic model, patients were divided into a high-risk or low-risk group. Kaplan-Meier survival analyses of patients in the two groups were conducted. The immune landscapes, as shown by the single-sample gene set enrichment analysis (ssGSEA), had different patterns in two groups. The prognostic model was verified using the ICGC database and clinical data of patients collected from Zhongnan Hospital. In the results of multivariate COX regression analysis, ATIC has the largest hazard ratio, so we studied the effect of ATIC on autophagy and tumor progression through experiments in vitro and in vivo.

Results: Fifty-eight autophagy-related genes were differentially expressed ($FDR < 0.05$, $\log FC > 1$); 23 genes were related to the prognosis of patients. A prognostic model based on 12 genes (ATG10, ATIC, BIRC5, CAPN10, FKBP1A, GAPDH, HDAC1, PRKCD, RHEB, SPNS1, SQSTM1 and TMEM74) was constructed using the method of multivariate COX regression analysis. For the high-risk group and low-risk group distinguished by the model, there was a significant difference in survival between the two groups ($P < 0.001$). The model had good predictive power ($AUC > 0.7$). Risk-related genes were related to the terms type II IFN response, MHC class I ($P < 0.001$) and HLA ($P < 0.05$). ATIC was confirmed to inhibit autophagy and promote the proliferation, invasion and metastasis of liver cancer cells through the AMPK/mTOR signaling pathway.

Conclusions: The prediction model can effectively predict the survival time of liver cancer patients. The risk score reflects patient immune cell features and immune status. ATIC inhibits autophagy and promotes the progression of liver cancer through the AMPK/mTOR signaling pathway.

Introduction

Worldwide, liver cancer is responsible for the 4th highest number of cancer-related deaths, and the number of new liver cancer cases every year is the 6th highest among all cancers[1]. The 5-year survival rate of liver cancer is only 18%, and it is the second most lethal cancer after pancreatic cancer. Furthermore, the long-term survival rate of liver cancer varies greatly among countries and regions[2, 3]. The molecular characteristics of liver cancer cells and the clinical prognosis of liver cancer are very different from those of other cancers, which leads to challenges in predicting the prognosis of liver cancer patients[4, 5].

Autophagy can improve the viability of tumor cells and exacerbate tumor progression[6]. Numerous studies have reported that there is a connection between autophagy and the human immune system and immune-related functions. Recent studies have reported that autophagy can reduce cell surface inhibition

of antitumor immune responses and promote immune evasion in pancreatic cancer[7]. Autophagy can regulate the balance, activation and differentiation of a variety of immune cells, as well as the secretion of a variety of cytokines[8]. In view of the close relationship between autophagy and immunity, we analyzed autophagy and immune-related functions in this study. In short, autophagy plays an important role in tumorigenesis and tumor progression, and immunotherapy is an important strategy for tumor treatment in the future[9].

Due to the close relationship between autophagy and tumors, we designed this study to explore the relationship between autophagy and the prognosis of liver cancer. Here, we identify genes that are closely related to the prognosis of liver cancer patients through bioinformatic methods such as COX regression analysis and survival analysis, and provide ideas for subsequent mechanistic studies, and establish models for predicting survival.

In the results of multivariate COX regression analysis, 5-minoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate (IMP) cyclohydrolase (ATIC) has the largest hazard ratio, suggesting that ATIC is closely related to patient prognosis. Therefore, we chose ATIC for further verification. ATIC is considered to be related to autophagy, but there is no experimental evidence to prove the relationship between ATIC and autophagy[10]. In this study, we determined the relationship between ATIC and tumor cell autophagy through experiments in vivo and in vitro. We found that ATIC can inhibit autophagy and promote liver cancer progression by affecting the AMPK/mTOR pathway.

This article identified autophagy-related genes related to the prognosis of liver cancer, analyzed the relationship of prognosis-related autophagy genes with immune cells and immune-related functions, and constructed a model to predict the survival of patients with liver cancer in the clinic uses bioinformatic methods. The results provide future research directions. Furthermore, we have proved that ATIC can inhibit autophagy and promote liver cancer progression.

Materials And Methods

Data collection

The mRNA sequencing data of 50 normal samples and 374 liver cancer samples and clinical data of 374 liver cancer patients were downloaded from The Cancer Genome Atlas (TCGA, portal.gdc.cancer.gov). Then, mRNA sequencing data from 240 liver cancer patients were downloaded from the International Cancer Genome Consortium (ICGC, daco.icgc.org). Excluding patients with incomplete data, a total of 365 patients from the TCGA database and 231 patients from the ICGC database were included in the analysis. A list of 256 autophagy-related genes was obtained from the Human Autophagy Database (autophagy.lu). The expression data of autophagy-related genes were extracted from TCGA sequencing data.

Identification of differentially expressed genes and prognosis-related genes

The "limma" R package was used to perform differential analysis. The screening conditions were $\log_2$ fold-change ($\logFC$) > 1 and false discovery rate (FDR) < 0.05. The survival R package was used to identify prognosis-related genes. The genes that were both differentially expressed and related to prognosis were determined. A protein interaction network was generated for the overlapping genes. The "igraph" and "reshape2" R packages were used to calculate the correlations between the expression levels of overlapping genes and to determine the correlation network (correlation threshold: 0.4).

Construction of prognostic models

We used both LASSO regression and multivariate Cox regression analyses to establish prognostic models based on the TCGA data. Then, we used ICGC data to verify the established models. According to the model, ICGC samples were divided into a high-risk and low-risk group. The predictive power of the models was evaluated by survival analysis, ROC curve analysis and independent prognostic analysis. The "Rtsne" R package was used for principal components analysis (PCA), and t-stochastic neighbor embedding (t-SNE) was used for dimensionality reduction and data visualization. After the two models were tested and compared, the prognostic model established by the LASSO regression method had better predictive power. Therefore, we used the prognostic model established by LASSO regression for subsequent analyses.

Identification of risk-related genes, gene set enrichment analysis and immune infiltration analysis

The "limma" R package was used to analyze the differentially expressed genes in the high-risk and low-risk groups. Then, GO functional enrichment analysis and KEGG pathway enrichment analysis were performed with the risk-related genes obtained from the analysis of the TCGA and ICGC data. The gene set enrichment analysis used the "clusterProfiler" and "org.Hs.eg.db" R packages, and P value < 0.05 and q value < 0.05 were used as thresholds.

Studies have shown that tumor gene expression levels are related to local immune cell activity and immune-related functions[11]. As such, we used the single-sample gene set enrichment analysis (ssGSEA) function in the "GSVA" R package to calculate the immune-related scores of each sample and the relationship between the immune-related scores and risk scores. We used the same method to calculate the relationship between immune infiltration and the expression of the representative gene ATIC in the model.

Cell culture and transfection

Huh7, HepG2 and HCCLM3 liver cancer cells were obtained from Shanghai Cell Bank (Shanghai, China). The cell line has recently been authenticated and has recently been tested for mycoplasma. Huh7, HepG2 and HCCLM3 cells were grown in DMEM (Invitrogen, Carlsbad, CA). All media were supplemented with 10% fetal bovine serum (Invitrogen). Jtsbio (Guangzhou, China) constructed and synthesized the siRNA and shRNA lentivirus vector specific for ATIC. The sequence of shRNA is the same as siRNA 3:

ATIC-siRNA 1: forward, 5'-CCUGCAAUCUCUAUCCCUUTT – 3'; reverse, 5'-AAGGGAUAGAGAUUGCAGGTT – 3'.

ATIC-siRNA 3: forward, 5'-GGUGUCGUCGACAAGUCAUTT – 3'; reverse, 5'-AUGACUUGUCGACGACACCTT – 3'.

For overexpression of ATIC, full-length ATIC cDNA was amplified by PCR and cloned into the expression vector pcDNA3.1 (Invitrogen). The empty vector was used as a control. Transfection was performed using Lipofectamine 3000 reagent (Invitrogen). Forty-eight hours after transfection, cells were harvested for further analysis.

RNA extraction and RT-qPCR analysis

Total RNA was isolated from the prepared tissues and cells using TRIzol reagent (Invitrogen). Complementary DNA was synthesized using a PrimeScript RT kit (TaKaRa, Dalian, China). Then, SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was used to perform qPCR with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The data were calculated using the 2- $\Delta\Delta C_t$ method, and GAPDH was used as an endogenous control. The primer sequences are as follows:

ATIC-F: 5'- CACGCTCGAGTGACAGTG- 3';

ATIC-R: 5'- TCGGAGCTCTGCATCTCCG- 3';

GAPDH-F: 5'-GTCTCCTCTGACTTCAACAGCG – 3';

GAPDH-R: 5'- ACCACCCTGTTGCTGTAGCCAA-3'.

Transmission electron microscopy

Conventional electron microscopy was performed as follows. Cells were fixed with 2.5% glutaraldehyde and then postfixed with 1% osmium tetroxide. The samples were dehydrated in an ethanol series and embedded in Embed812 resin. Ultrathin sections were cut with a diamond knife. Next, the sections were mounted on copper grids and double-stained with uranyl acetate and lead citrate. The number of autophagic vacuoles was determined for a minimum of 100 cells.

Detection of cell viability and migration ability

Cell counting kit 8 (CCK8) was used to measure cell viability. An Infinite M200 spectrophotometer (Tecan) was used to detect the spectrophotometric absorbance of each sample at 450 nm. Transwell assays were used to assess cell migration. BioCoat Matrigel Invasion Chambers (BD Biosciences) were used to assess cell invasion. Three random areas were assessed to determine the cell counts for the cell migration and invasion experiments. An annexin V-FITC apoptosis detection kit (Invitrogen) was used for annexin V and propidium iodide staining of cells, and the percentage of apoptotic cells was determined by flow cytometry (Beckman, USA). To assess the cell cycle, 48 hours after transfection, the cells were stained with propidium iodide and assessed by fluorescence-activated cell sorting (FACS).

Colony formation assay

The treated liver cancer cells were cultured in a 6-well plate. After 14 days, the culture medium was discarded, and the cells were washed twice with PBS. Then, paraformaldehyde was used to fix the cells at room temperature for 20 minutes, 0.1 mM crystal violet (500 µl) was added, the cells were stained for 10 minutes, washed twice with PBS, and the results were analyzed under a microscope.

Western blot Analysis

Anti-AMPKα antibody (ab32047), anti-p-AMPKα antibody (ab92701), anti-mTOR antibody (ab134903), anti-p-mTOR antibody (ab109268), anti-LC3 antibody (ab192890) and anti-GAPDH antibody (ab8245) were purchased from Abcam. Compound C (C-C) was purchased from Sigma Chemical (St Louis, MO). Antibodies against caspase 3 (19677-1-AP), caspase 7 (27155-1-AP) and caspase 9 (10380-1-AP) were purchased from Proteintech. Cell samples were lysed in SDS (Beyotime, Shanghai, China). The protein concentration was determined with the Enhanced BCA Protein Assay Kit (Beyotime), and the proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5% skim milk for 1 hour, the membranes were incubated with the specific primary antibody overnight at 4°C and then incubated with the secondary antibody at room temperature for 2 hours. The protein bands were exposed using an enhanced chemiluminescence (ECL) system (Pierce Biotech, Rockford, Illinois, USA). Anti-GAPDH antibody (1:1,000, Sigma, St. Louis, Missouri, USA) was used to detect GAPDH as a loading control.

Xenotransplantation and lung metastasis models

For these experiments, 6-week-old BALB/c nude mice were used. The treated liver cancer cells were washed and suspended, and 1×10^7 cells/0.1 ml in a single-cell suspension were inoculated into the armpits of nude mice. The long diameter (a) and short diameter (b) of subcutaneous xenograft tumors were measured every 5 days. The tumor volume was calculated according to the formula $V = ab^2/2$. After 5 weeks, the nude mice were sacrificed, and the tumors were extracted. The differently treated cells in the logarithmic growth phase were selected, and a cell suspension was made and injected through the tail vein. The nude mice were sacrificed after a month and the lung tissues were collected for hematoxylin-eosin (H&E) staining. Generally, $1-5 \times 10^6$ cells/200 µl were used. The animal studies were approved by the Animal Research Committee of Wuhan University.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, California, USA) and SPSS 24.0 software (SPSS Inc., Chicago, USA). Each experiment included at least three independent experiments. The data are expressed as the mean $\pm$ standard deviation (SD). The differences between the experimental groups were assessed by Student's t-test or one-way ANOVA. A two-sided P value was calculated, and a probability level of 0.05 was considered statistically significant.

Results

Identification of differentially expressed genes related to autophagy and prognosis

We first identified 58 genes that were abnormally expressed in tumors compared with normal tissues, of which 4 genes had abnormally low expression and 54 genes had abnormally high expression (Fig. 1A and B). A total of 43 prognosis-related genes were screened using univariate Cox regression analysis (Fig. 1C). The intersection of the two gene sets resulted in 23 overlapping genes (Fig. 1D and Table. S1).

The overlapping genes were all highly expressed in tumor tissues (Fig. 1E), and they were all unfavorable factors for prognosis (Fig. 1F). Among them, BIRC5, PEA15 and RAB24 were the most significant genes. CAPN10 and SPNS1 were the genes with the greatest impact on prognosis. To explore the interactions and coexpression relationships between the overlapping genes, we generated a protein interaction network. MAPK3, SQSTM1, CASP8 and HSP90AB1 were the core genes in the network and showed research potential (Fig. 1G). In the coexpression network, the lines between genes represent coexpression relationships, and red represents a positive correlation. IKBKE and PRKCD had the strongest coexpression relationship (Fig. 1H).

The established model has satisfactory predictive power

We used LASSO regression analysis and multivariate Cox regression analysis to construct prognostic models. Lasso regression analysis filtered out 12 genes(ATG10, ATIC, BIRC5, CAPN10, FKBP1A, GAPDH, HDAC1, PRKCD, RHEB, SPNS1, SQSTM1 and TMEM74) (Fig. 2A and B). These 12 genes are closely related to the patient's prognosis. Based on these 12 genes, a prognostic model can be constructed to predict the patient's survival. The risk score was calculated by multiplying the expression of each gene in the model by the corresponding coefficient and then adding the sum. The formula for calculating the risk score obtained by LASSO regression is as follows:

$$\text{Risk score} = \text{EX}_{\text{ATG10}} * 0.265 + \text{EX}_{\text{ATIC}} * 0.216 + \text{EX}_{\text{BIRC5}} * 0.065 + \text{EX}_{\text{CAPN10}} * 0.034 + \text{EX}_{\text{FKBP1A}} * 0.114 + \text{EX}_{\text{GAPDH}} * 0.019 + \text{EX}_{\text{HDAC1}} * 0.230 + \text{EX}_{\text{PRKCD}} * 0.018 + \text{EX}_{\text{RHEB}} * 0.185 + \text{EX}_{\text{SPNS1}} * 0.189 + \text{EX}_{\text{SQSTM1}} * 0.094 + \text{EX}_{\text{TMEM74}} * 0.147 \text{ (EX, expression).}$$

The prognostic model established by multivariate Cox regression analysis included 10 genes. The results showed that ATIC, HDAC1, HSP90AB1, MAPK3, RHEB, SPNS1 and SQSTM1 were independent factors affecting prognosis (Fig. 2C). The formula for calculating the risk score from this model is as follows:

$$\text{Risk score} = \text{EX}_{\text{ATIC}} * 0.535 + \text{EX}_{\text{CASP8}} * -0.400 + \text{EX}_{\text{FKBP1A}} * 0.391 + \text{EX}_{\text{HDAC1}} * 0.422 + \text{EX}_{\text{HSP90AB1}} * -0.321 + \text{EX}_{\text{MAPK3}} * -0.563 + \text{EX}_{\text{PRKCD}} * 0.216 + \text{EX}_{\text{RHEB}} * 0.415 + \text{EX}_{\text{SPNS1}} * 0.972 + \text{EX}_{\text{SQSTM1}} * 0.252.$$

We use data from ICGC database to verify the model established based on TCGA data. The patients in the TCGA group and the ICGC group were divided into a high-risk group and a low-risk group according to risk score. The results show that the risk score is related to the patient's disease stage and grade (Table. 1). Furthermore, the difference in the prognosis of the two groups was calculated. The results showed

that the survival difference between the high-risk group and the low-risk group was more obvious for the model established by LASSO regression analysis than for the model established by Cox regression analysis (Fig. 2D-G).

ROC risk curves were drawn and the area under the curve (AUC) values were calculated to evaluate the predictive performance of the model. For the LASSO regression model, the 1-, 2-, and 3-year AUC values for TCGA patients were 0.768, 0.714 and 0.696, respectively (Fig. 2H). The ICGC group was used to test the efficacy of the model, and the AUC values were 0.745, 0.761 and 0.739, respectively (Fig. 2I). The AUC values of the model established by multivariate Cox regression analysis were slightly lower than those of the model established by multivariate COX regression (Fig. 2J and K). Therefore, the model established by LASSO regression analysis had better predictive performance, and the model was further verified and analyzed. The distribution of risk scores among patients in the TCGA cohort was symmetrical (Fig. 2L). The distribution of patient survival statuses showed that the survival time of patients in the high-risk group was shorter than that of patients in the low-risk group ($P < 0.01$, Fig. 2M). When ICGC patient data were used for verification, the same distribution was observed (Fig. 2N and O). To show the clustering of risk scores more clearly, we conducted PCA and t-SNE analysis. The results showed that in both the TCGA and ICGC groups, patients in the high-risk and low-risk groups were clearly distributed in different clusters (Fig. 2P-S).

The risk score is an independent prognostic factor and is related to immune cells and immune function

We used univariate and multivariate Cox regression analyses to determine whether the risk score was an independent prognostic indicator. The results showed that in the TCGA and ICGC groups, disease stage and risk score were independent factors affecting prognosis ($P < 0.001$, Fig. 3A-D). We further performed an enrichment analysis of genes with differential expression ($\log FC > 1$, adjusted $P < 0.05$). The results of the GO analysis showed that the genes were mainly involved in cell cycle-related functions, including nuclear division and chromatic segregation (Fig. 3E and F). The results of the KEGG pathway enrichment analysis showed that the autophagy-related genes were closely related to the cell cycle (Fig. 3G and H). In addition, we also found that the autophagy-related genes were also related to immune function in the GO analysis and KEGG analysis. Related GO terms included humoral immune response, chemokine production, IL-17 signaling pathway and cytokine activity.

The differences in immune cells and functions were quantified and analyzed. Between the high-risk group and the low-risk group in the TCGA cohort, there were significant differences in the activation degree of immune cells related to cellular immunity, including macrophages, natural killer (NK) cells, T helper 1 (Th1) cells, Th2 cells and regulatory T (Treg) cells. In terms of immune-related functions, CCR and the type II IFN response, which are also related to cellular immunity, were significantly differentially activated between the high-risk and low-risk group. In addition, immune terms related to antigen presentation, such as APC costimulation and MHC class I, were also significantly differentially enriched between the two groups (Fig. 3I and J). In the ICGC cohort, the degree of activation of macrophages and Th2 cells was obviously different between the high-risk and low-risk group, which was the same as the result in the

TCGA group. The changes in immune function in the ICGC group were the same as those in the TCGA group, and significant enrichment of MHC class I and the type II IFN response was found in both cohorts (Fig. 3K and L).

For the key gene ATIC in the model, we divided patients into a high expression group and a low expression group according to the expression level of ATIC. In the TCGA cohort, the expression of ATIC was related to the infiltration of CD8 + T cells, macrophages and mast cells (Fig. 3M). ATIC was related to immune checkpoints and type I and type II IFN response immune functions (Fig. 3N). In the ICGC cohort, ATIC was still associated with CD8 + T cells and NK cells but not with macrophages (Fig. 3O). In the ICGC cohort, ATIC was related to immune checkpoints and IFN response, which is consistent with the results in TCGA (Fig. 3P).

Based on the differential genes between the high-risk group and the low-risk group, we generated a connectivity map to identify small molecules with therapeutic potential using online tool cMAP (Table S2). Among them, meclofenamic acid, UNC-0321, fananserin and icariin showed great therapeutic potential. Doxorubicin and erlotinib are used in the treatment of liver cancer. For high-risk patients, these two drugs may have a better effect (Fig. 3Q and R).

ATIC is highly expressed in liver cancer tissues and associated with a poor prognosis

The results of the multivariate Cox regression analysis showed that ATG10, ATIC, HDAC1, RHEB, SPNS1 and TMEM74 had a significant effect on prognosis. Among them, the abnormally high expression of ATIC and HDAC1 was the most obvious harmful factor affecting prognosis ($P < 0.05$, hazard ratio > 1). The immunohistochemical results in The Human Protein Atlas database (<https://www.proteinatlas.org>) verified that the expression of ATIC, HDAC1, RHEB, SPNS1 and TMEM74 in liver cancer tissues was significantly higher than that in normal liver tissues (Fig. 4A and B).

Since the hazard ratio of ATIC was greater than 1 and it had a large coefficient in the established model, we decided to functionally verify the effects of ATIC. According to follow-up and gene expression data from TCGA, high expression of ATIC was closely related to poor prognosis ($P < 0.001$, Fig. 4C). Compared with the normal liver cell line LO2, the HepG2 and Huh7 cell lines showed significantly higher expression of ATIC (Fig. 4D). We collected tumor samples and paracancerous samples of 52 liver cancer patients from the the Biological Repositories, Zhongnan Hospital of Wuhan University and verified that ATIC was significantly high expressed in liver cancer tissue samples ($P < 0.001$, Fig. 4E).

We analyzed the association between ATIC and the clinicopathological characteristics of liver cancer patients. As shown in Table 2, it was observed that ATIC expression was associated with lymph node invasion ($P = 0.048$) and prognosis (Fig. S1). However, the expression of ATIC was independent of age ($P = 0.393$), gender ($P = 0.760$) and portal vein tumor thrombosis (PVTT, $P = 0.405$) (Table 2). We also analyzed the relative risk of ATIC in the prognosis of liver cancer. The results of Cox regression analysis showed that, compared with low-expressing liver cancer patients, high ATIC expression was closely

related to the poor prognosis of liver cancer patients ($P = 0.002$). Multivariate Cox regression analysis further confirmed that the expression of ATIC was significantly correlated with the prognosis of liver cancer ($P = 0.006$) (Table 3).

Table 1
Correlation between risk score and clinicopathologic characteristics of patients from TCGA and ICGC database.

characteristics	TCGA cohort			ICGC cohort		
	High risk	Low risk	χ^2	High risk	Low risk	χ^2
No. of patients	182	183		115	116	
Age			0.120			0.061
≥ 65 y	67	82		81	68	
≤ 65y	115	101		34	48	
gender			0.882			0.480
Male	122	124		87	83	
Female	60	59		28	33	
Stage			0.010			0.006
I	69	101		13	23	
II	48	36		45	60	
III	51	32		43	28	
IV	1	3		14	5	
Unknown	13	11		0	0	
T			0.004			
T1	74	106		NA	NA	
T2	54	37		NA	NA	
T3	45	33		NA	NA	
T4	9	4		NA	NA	
Unknown	0	3		NA	NA	
N			0.615			
N0	128	120		NA	NA	
N1	2	2		NA	NA	
Unknown	52	61		NA	NA	
M			0.712			
M0	134	129		NA	NA	

characteristics	TCGA cohort		ICGC cohort	
M1	1	2	NA	NA
Unknown	47	52	NA	NA
Grade	< 0.001			
Grade 1	17	38	NA	NA
Grade 2	77	98	NA	NA
Grade 3	76	42	NA	NA
Grade 4	10	2	NA	NA
Unknown	2	3	NA	NA

Table 2
Correlation between ATIC expression and clinicopathologic characteristics of liver cancer patients

Characteristics	ATIC		P/ χ^2 value
	Low	High	
Age(y)	62.12 $\pm$ 10.667	59.77 $\pm$ 8.887	0.393
Gender			0.760
Male	19	18	
Female	7	8	
HBV infection			0.734
Yes	21	20	
No	5	6	
AFP			0.780
≥ 400	15	14	
< 400	11	12	
Tumor diameter (cm)	3.819 $\pm$ 1.828	4.735 $\pm$ 1.617	0.062
TNM classification			0.061
I	2	0	
II	13	7	
III	8	13	
IV	3	6	
PVTT			0.405
Yes	12	15	
No	14	11	
Lymphatic invasion			0.048
Yes	7	14	
No	19	12	
PVTT, Portal vein tumor thrombosis;HBV, hepatitis B virus; AFP, Alpha-fetoprotein.			

Table 3
Univariate and multivariate Cox-regression analysis of various prognostic parameters in patients with liver cancer

	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	P	HR	95% CI
TNM classification	< 0.001	7.021	3.686–13.372	0.001	4.407	1.870-10.383
Tumor diameter	0.003	1.386	1.115–1.721			
PVTT	0.001	4.286	1.798–10.217	0.006	5.969	1.686–21.128
AFP	0.001	5.048	1.990-12.807	0.029	4.297	1.157–15.967
ATIC	0.002	1.593	1.186–2.139	0.006	2.099	1.243–3.544
HR, Hazard ratio; CI, confidence interval.						

We used HepG2 and Huh7 cell lines for the next experiment. After transfection, the mRNA and protein expression levels of ATIC were significantly decreased (Fig. 4F). The results of westernblot are consistent with the results of PCR, showing that ATIC is highly expressed in liver cancer tissues (Fig. 4G) and liver cancer cell lines(Fig. 4H). siRNA not only reduced the mRNA level of ATIC, but also reduced the protein level (Fig. 4I).

ATIC inhibits autophagy and apoptosis of tumor cells and promotes malignant tumor behavior

siRNA negative control (siRNA NC) and siRNA 3 were used to transfect the liver cancer cell lines Huh7 and HepG2, and CCK8 was used to detect cell proliferation. After ATIC knockdown, the proliferation ability of the cells decreased significantly ($P < 0.05$, Fig. 5A). The results of the colony formation experiment showed that after 2 weeks of culture, the number of colonies formed by the cells with ATIC knockdown was significantly lower than that formed by control cells ($P < 0.05$, Fig. 5B). The transwell and wound healing experiments showed that knockdown of ATIC expression reduced the invasion and migration ability of cells (Fig. 5C and D). Consistent with the enrichment analysis results, knockdown of ATIC affected the cell cycle of tumor cells (Fig. 5E). The levels of LC3 I and LC3 II reflect cell autophagy. An increase in the proportion of LC3 II represents an increase in the level of autophagy. Apoptosis and autophagy are closely related. Knockdown of ATIC induced cell apoptosis (Fig. 5F). After transfection of pcDNA/ATIC, the level of ATIC protein increased significantly (Fig. 5G). Correspondingly, after ATIC knockdown, the levels of cleaved caspase 3 in cells increased, and at the same time, the proportion of the autophagy-related protein LC3 II increased (Fig. 5H). The levels of cleaved caspase 7 and 9 did not change, indicating that ATIC can not only affect the level of autophagy but also affect the caspase 3-related apoptosis pathway.

ATIC inhibits autophagy through the AMPK/mTOR pathway

After knocking down ATIC in Huh7 cells, intracellular autophagosomes (shown by the red arrow) were observed under a transmission electron microscope. The number of intracellular autophagosomes after siRNA treatment was significantly increased, which was consistent with the changes in LC3 levels (Fig. 6A). In the transplanted tumor mouse model, the growth rate of tumor cells with ATIC knockdown was significantly slower than that of control cells, and the tumor volume was significantly smaller than that of the control group (Fig. 6B). The results of immunohistochemistry showed that the expression of ATIC protein was lower in tumors treated with shRNA than in control tumors (Fig. 6C). The lung metastasis ability of tumor cells was significantly reduced after ATIC knockdown (Fig. 6D). After transfection of pcDNA/ATIC, the proliferation and migration of tumor cells were significantly enhanced (Fig. 6E and F). The mTOR pathway is closely related to autophagy, so we tested the AMPK/mTOR pathway after knocking down ATIC. After knocking down ATIC, the phosphorylation of AMPK α increased, while the phosphorylation of mTOR decreased (Fig. 6G). The AMPK/mTOR pathway inhibitor compound C (C-C) was used for rescue experiments. After adding C-C, the increase in p-AMPK α levels and the decrease in p-mTOR levels caused by siRNA treatment were reversed. Similarly, the use of AMPK blockers reversed the changes in LC3 protein levels caused by ATIC knockdown (Fig. 6H). Furthermore, C-C also prevented the decrease in cell proliferation and migration caused by knocking down ATIC (Fig. 6I-K). When siRNA and C-C were used to treat cells at the same time, the number of autophagosomes observed by transmission electron microscopy was significantly reduced compared with that seen for siRNA-treated cells (Fig. 6L). The apoptosis induced by ATIC knockdown was also reversed by C-C (Fig. 6M).

Discussion

Given the heterogeneous nature of HCC, it is difficult to carry out satisfactory risk assessment and clinical management for patients with liver cancer[12, 13]. Therefore, we want to establish a prognostic model to evaluate the risk and survival of patients to provide guidance for clinical treatment.

Autophagy is regulated by autophagy-related genes, and the abnormal expression of autophagy-related genes affects the occurrence and development of tumors by modulating autophagy[14]. Due to the relationship between autophagy-related gene expression and tumor progression, establishing a liver cancer prognostic model based on autophagy-related genes is reasonable. The prognostic model constructed in this study can predict whether a patient is at high-risk of poor prognosis and the long-term survival rate of the patient. In the GO analysis and KEGG analysis results, autophagy-related genes were closely related to mitosis and the cell cycle. Consistent with this finding, ATIC is known to affect the cell cycle and promote cell proliferation.

It has been reported that autophagy-related genes can inhibit tumor growth by affecting immune cell infiltration, but there are still few studies on the relationship between autophagy and immune infiltration[15]. The immune infiltration analysis results suggest that the autophagy-related genes in the model are related to the tumor immune microenvironment, the activity of immune cells, and immune function, but the mechanisms underlying these relationships are not yet fully clear. HDAC1 has been reported to affect antigen presentation and immune activation. Therefore, the relationship between

autophagy-related genes such as ATIC, PRKCD and SPNS1 and immune function deserves further study[16]. We grouped patients according to the expression of ATIC and analyzed the difference in immune cell infiltration between the two groups. It was found that CD8 + T cells, macrophages, immune checkpoints and IFN immune responses were different between the two groups. The main immune cell related to immunotherapy is CD8 + T cells[17, 18]; thus, we speculate that ATIC is related to the tumor immune microenvironment and immunotherapy. There are many mechanisms by which autophagy acts on immunity, including abnormal autophagy interfering with the survival and activity of T cells or autophagic activation promoting and inhibiting the secretion of cytokines[19, 20]. These findings suggest that the induction or suppression of autophagy combined with immunotherapy may be a prospective treatment strategy[21].

We screened small molecule drugs with therapeutic potential based on changes in the gene levels of patients in the high-risk group, but in vivo and in vitro experimental verification was not performed. The changes in the gene profile of cells treated with these small molecule drugs were exactly the opposite of the gene profile of patients in the high-risk group. This suggested that these small molecule drugs have a potential therapeutic effect on liver cancer. Among them, doxorubicin had been widely used in the clinical treatment of liver cancer[22]. Icariin was reported to inhibit the proliferation of tumors, but it had not been studied in liver cancer[23, 24]. As for the other molecules, we will conduct research on drug treatment effects and drug resistance in the future.

ATIC is related to tumor cell proliferation, rheumatoid arthritis and the efficacy of radiotherapy[25, 26]. There has been no previous report on the correlation between ATIC and autophagy and tumor immune microenvironment. In this study, we have proved through in vivo and in vitro experiments that ATIC can promote the progression of liver cancer through the AMPK/mTOR pathway. And, the study found that our model is related to the patient's prognosis and immune cell infiltration, and it has been verified by external databases, which further proves the reliable prognostic ability of our model. Previous studies have shown that autophagy is related to immune cell infiltration and tumor immune tolerance[27, 28]. We found that ATIC, as an autophagy-related gene, is related to immune cell infiltration through bioinformatics analysis, but the specific mechanism is still unclear. We selected ATIC for verification in this study, but in subsequent studies, we will also verify the relationship between several other genes and the immune microenvironment and conduct in-depth research on the mechanism of ATIC affecting immune responses.

In the analysis of correlation between ATIC expression and clinicopathologic characteristics of liver cancer patients, we found that the expression of ATIC is related to lymph node invasion and patient prognosis. The TNM stage of the ATIC high expression group was higher, but there was no statistical difference. We think it may be because our sample size is too small, and we will include more patients in the future.

The prognostic model we have established can effectively predict the prognostic risk of patients and provide guidance for clinical decision-making. For example, after a patient has undergone liver cancer

resection, are adjuvant treatments such as transcatheter arterial chemoembolization (TACE) necessary[29]? If the patient is a high-risk patient based on the model score, other adjuvant treatments are also recommended even if the tumor is a well-differentiated liver cancer.

At the same time, we have screened out many genes with research value through bioinformatics analysis. For example, SPNS1 is closely related to the prognosis of liver cancer patients according to the result of multivariate COX regression analysis, but there has not been any report on its mechanism to promote tumor occurrence and tumor development. Therefore, we hope that our analysis results can provide ideas for future research on the role of autophagy in tumors.

The constructed prognostic model can be used to predict the survival rate of patients, and the predictive performance has been verified in an external ICGC dataset to support its validity. That the key gene ATIC affects tumor progression by modulating autophagy has been verified in vitro and in vivo, and the effect of ATIC on autophagy in liver cancer has been clarified here for the first time.

Abbreviations

ATIC	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate (IMP) cyclohydrolase
TCGA	The Cancer Genome Atlas
ICGC	International Cancer Genome Consortium
logFC	log ₂ fold-change
FDR	False discovery rate
PCA	Principal components analysis
t-SNE	t-stochastic neighbor embedding
CCK8	Cell counting kit 8
AUC	Area under the curve
siRNA NC	siRNA negative control
C-C	Compound C

Declarations

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics approval and consent to participate

The experimental protocol was approved by the Medical Ethics Committee of Zhongnan Hospital of Wuhan University (Scientific Ethical Approval No.2020079). All the patients had signed informed consent before the experiments. The animal studies were approved by the Animal Research Committee of Wuhan University.

Author contributions

HZ and PX designed the study, conducted the experiments and literature search, and generated the figures. WM and JL wrote and edited the manuscript. YY helped perform the analysis with constructive discussions. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The original data presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Consent for publication

Not applicable.

References

1. Villanueva A. Hepatocellular Carcinoma. *N Engl J Med*. 2019;380(15):1450–62.
2. Jemal A, Ward EM, Johnson CJ, Cronin KA, Ma J, Ryerson B, Mariotto A, Lake AJ, Wilson R, Sherman RL, et al: Annual Report to the Nation on the Status of Cancer, 1975–2014, Featuring Survival. *J Natl Cancer Inst* 2017, 109(9).

3. Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. *Nat Rev Gastroenterol Hepatol*. 2019;16(10):589–604.
4. Cancer Genome Atlas Research Network. Electronic address wbe, Cancer Genome Atlas Research N: Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell*. 2017;169(7):1327–41. e1323.
5. Schulze K, Imbeaud S, Letouze E, Alexandrov LB, Calderaro J, Rebouissou S, Couchy G, Meiller C, Shinde J, Soysouvanh F, et al. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet*. 2015;47(5):505–11.
6. Honscheid P, Datta K, Muders MH. Autophagy: detection, regulation and its role in cancer and therapy response. *Int J Radiat Biol*. 2014;90(8):628–35.
7. Yamamoto K, Venida A, Yano J, Biancur DE, Kakiuchi M, Gupta S, Sohn ASW, Mukhopadhyay S, Lin EY, Parker SJ, et al. Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. *Nature*. 2020;581(7806):100–5.
8. Harris J. Autophagy and cytokines. *Cytokine*. 2011;56(2):140–4.
9. Zhong Z, Sanchez-Lopez E, Karin M. Autophagy, Inflammation, and Immunity: A Troika Governing Cancer and Its Treatment. *Cell*. 2016;166(2):288–98.
10. Huo X, Qi J, Huang K, Bu S, Yao W, Chen Y, Nie J. Identification of an autophagy-related gene signature that can improve prognosis of hepatocellular carcinoma patients. *BMC Cancer*. 2020;20(1):771.
11. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell*. 2015;160(1–2):48–61.
12. Chan LK, Tsui YM, Ho DW, Ng IO. Cellular heterogeneity and plasticity in liver cancer. *Semin Cancer Biol* 2021.
13. Lu LC, Hsu CH, Hsu C, Cheng AL. Tumor Heterogeneity in Hepatocellular Carcinoma: Facing the Challenges. *Liver Cancer*. 2016;5(2):128–38.
14. Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. *Nat Rev Cancer*. 2017;17(9):528–42.
15. Mgrditchian T, Arakelian T, Paggetti J, Noman MZ, Viry E, Moussay E, Van Moer K, Kreis S, Guerin C, Buart S, et al. Targeting autophagy inhibits melanoma growth by enhancing NK cells infiltration in a CCL5-dependent manner. *Proc Natl Acad Sci U S A*. 2017;114(44):E9271–9.
16. Wijdeven RH, van Luijn MM, Wierenga-Wolf AF, Akkermans JJ, van den Elsen PJ, Hintzen RQ, Neefjes J. Chemical and genetic control of IFN γ -induced MHCII expression. *EMBO Rep* 2018, 19(9).
17. Bradley CA. Immunotherapy: CD8(+) T cells - burn fat, get fit. *Nat Rev Cancer*. 2017;17(11):635.
18. Li B, Severson E, Pignon JC, Zhao H, Li T, Novak J, Jiang P, Shen H, Aster JC, Rodig S, et al. Comprehensive analyses of tumor immunity: implications for cancer immunotherapy. *Genome biology*. 2016;17(1):174.

19. Sun K, Xu L, Jing Y, Han Z, Chen X, Cai C, Zhao P, Zhao X, Yang L, Wei L. Autophagy-deficient Kupffer cells promote tumorigenesis by enhancing mtROS-NF-kappaB-IL1alpha/beta-dependent inflammation and fibrosis during the preneoplastic stage of hepatocarcinogenesis. *Cancer Lett.* 2017;388:198–207.
20. Ullio C, Brunk UT, Urani C, Melchiorretto P, Bonelli G, Baccino FM, Autelli R. Autophagy of metallothioneins prevents TNF-induced oxidative stress and toxicity in hepatoma cells. *Autophagy.* 2015;11(12):2184–98.
21. Jiang GM, Tan Y, Wang H, Peng L, Chen HT, Meng XJ, Li LL, Liu Y, Li WF, Shan H. The relationship between autophagy and the immune system and its applications for tumor immunotherapy. *Mol Cancer.* 2019;18(1):17.
22. Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, Ayuso C, Sala M, Muchart J, Sola R, et al. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet.* 2002;359(9319):1734–9.
23. Alhakamy NA, Badr-Eldin UAF, Ahmed SM, Asfour OAA, Aldawsari HZ, Algandaby HM, Eid MM, Abdel-Naim BG, Awan AB. ZA et al: Optimized Icaria Phytosomes Exhibit Enhanced Cytotoxicity and Apoptosis-Inducing Activities in Ovarian Cancer Cells. *Pharmaceutics* 2020, 12(4).
24. Zhang DC, Liu JL, Ding YB, Xia JG, Chen GY. Icaria potentiates the antitumor activity of gemcitabine in gallbladder cancer by suppressing NF-kappaB. *Acta Pharmacol Sin.* 2013;34(2):301–8.
25. Liu X, Paila UD, Teraoka SN, Wright JA, Huang X, Quinlan AR, Gatti RA, Concannon P. Identification of ATIC as a Novel Target for Chemoradiosensitization. *Int J Radiat Oncol Biol Phys.* 2018;100(1):162–73.
26. Lv S, Fan H, Li J, Yang H, Huang J, Shu X, Zhang L, Xu Y, Li X, Zuo J, et al. Genetic Polymorphisms of TYMS, MTHFR, ATIC, MTR, and MTRR Are Related to the Outcome of Methotrexate Therapy for Rheumatoid Arthritis in a Chinese Population. *Front Pharmacol.* 2018;9:1390.
27. Cunha LD, Yang M, Carter R, Guy C, Harris L, Crawford JC, Quarato G, Boada-Romero E, Kalkavan H, Johnson MDL, et al. LC3-Associated Phagocytosis in Myeloid Cells Promotes Tumor Immune Tolerance. *Cell.* 2018;175(2):429–41. e416.
28. Wang X, Wu WKK, Gao J, Li Z, Dong B, Lin X, Li Y, Li Y, Gong J, Qi C, et al. Autophagy inhibition enhances PD-L1 expression in gastric cancer. *J Exp Clin Cancer Res.* 2019;38(1):140.
29. Raoul JL, Forner A, Bolondi L, Cheung TT, Kloeckner R, de Baere T. Updated use of TACE for hepatocellular carcinoma treatment: How and when to use it based on clinical evidence. *Cancer Treat Rev.* 2019;72:28–36.

Figures

Figure 1

Differentially expressed and prognosis-related genes. (A) Heat map of differentially expressed genes (DEGs). (B) Volcano plot. (C) Prognosis-related genes. (D) Venn diagram. (E) Heat map of overlapping genes. (F) Hazard ratios of overlapping genes. (G) Protein interaction network. (H) Protein coexpression network. Red, high expression; green, low expression.

Figure 2

Construction and verification of the prognostic models. (A) Cross validation plot for the penalty term in LASSO regression. (B) LASSO regression coefficients for different values of the penalty parameter. (C) Hazard ratios of genes in the multivariate Cox regression analysis. Survival curves between the high-risk group and the low-risk group of patients in the TCGA cohort (D) and the ICGC cohort (E) based on the LASSO regression model. Survival curves of the TCGA cohort (F) and the ICGC cohort (G) based on the

multivariate Cox regression model. ROC curves of the TCGA cohort (H) and ICGC cohort (I) categorized with the LASSO regression model. ROC curves of the TCGA cohort (J) and ICGC cohort (K) categorized with the multivariate Cox regression model. Distribution of risk scores (L and N) and survival statuses (M and O) in the TCGA cohort (L and M) and ICGC cohort (N and O). PCA (P) and t-SNE analysis (Q) of patients in the TCGA cohort. PCA (R) and t-SNE analysis (S) of patients in the ICGC cohort.

Figure 3

Analysis of the independent prognostic value of the risk score and its association with immune infiltration. (A) Univariate Cox regression analysis with the TCGA cohort. (B) Multivariate Cox regression analysis with the TCGA cohort. (C) Univariate Cox regression analysis with the ICGC cohort. (D)

Multivariate Cox regression analysis with the ICGC cohort. GO functional enrichment analysis with the TCGA cohort (E) and ICGC cohort. (F) KEGG pathway enrichment analysis with the TCGA cohort (G) and ICGC cohort (H). (I) The relationship between immune cell infiltration and risk score in the TCGA cohort. (J) The relationship between immune function and risk score in the TCGA cohort. (K) The relationship between immune cell infiltration and risk score in the ICGC cohort. (L) The relationship between immune function and risk score in the ICGC cohort. (M) The relationship between immune cell infiltration and expression of ATIC in the TCGA cohort. (N) The relationship between immune function and expression of ATIC in the TCGA cohort. (O) The relationship between immune cell infiltration and expression of ATIC in the ICGC cohort. (P) The relationship between immune function and expression of ATIC in the ICGC cohort. (Q) Candidate small molecule compounds with therapeutic potential. (R) Drugs for which high-risk patients may show high resistance.

Figure 4

Verification of differential expression of risk-related genes in tissues and cells. (A) Multivariate Cox regression analysis of genes in the LASSO regression model. (B) Expression of risk-related genes in liver cancer tissues and adjacent tissues (HPA database). (C) Survival curve of patients with high and

ATIC expression (TCGA database). (D) ATIC expression in different cell lines. (E) ATIC mRNA levels in liver tissues and adjacent tissues (patients in Zhongnan Hospital). (F) ATIC mRNA expression in cells treated with siRNA. (G) ATIC protein expression level in cancer tissues and adjacent tissues. (H) ATIC protein expression in different cell lines. (I) Protein expression level after ATIC knockdown. NC, negative control.

Figure 5

Knockdown of ATIC can inhibit the malignant behavior of tumor cells. (A) Cell viability detected by CCK8 assays. (B) Representative micrographs and colony numbers from the colony formation assays. (C) Representative micrographs and cell numbers from the transwell assays. (D) Wound healing assay results showing the differences in migratory capacities (left); statistical analysis of the wound healing

assay results (right). (E) Cell cycle assays conducted by flow cytometry. (F) Apoptosis assays conducted by flow cytometry. (G) ATIC protein expression after transfection of pcDNA/ATIC. (H) Apoptosis and autophagy-related protein expression after transfection of siRNA. FL caspase, full-length caspase; CL caspase, cleaved caspase.

Figure 6

ATIC inhibits autophagy through the AMPK/mTOR pathway and affects the malignant behavior of tumors in vivo and in vitro. (A) Autophagosomes observed by electron microscopy. (B) Tumor volume statistics in the two groups. (C) H&E analyses of xenograft tumors. (D) Representative views of lung metastases observed in the two groups of nude mice. (E and F) ATIC overexpression promoted tumor proliferation (E) and migration (F), as assessed by CCK8 and wound healing assays, respectively. (G) Western blot analysis of the AMPK/mTOR pathway after ATIC knockdown. (H) Western blot analysis of the AMPK/mTOR pathway and LC3 levels in different treated cells. (I) Cell viability assays after siRNA and C-C treatment. (J) Colony formation assays with the indicated cells. (K) Transwell results. (L) Electron microscopy images of cells treated with siRNA 3 and C-C. (M) Apoptosis assay conducted by flow cytometry.

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