

Decreased expression of acetyl-CoA synthase 2 promotes metastasis and predicts poor prognosis in hepatocellular carcinoma

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Key words

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Hepatocellular carcinoma (HCC) is one of the most malignant cancers and has high morbidity and mortality rates in China.⁽¹⁾ Surgery is considered one of the best treatment options for patients with this disease,^(2,3) although metastasis greatly limits the number of patients who are surgical candidates. Not only do many patients diagnosed with HCC show metastasis, but many postoperative patients also suffer from metastasis after undergoing curative resection.⁽⁴⁾ Therefore, metastasis is a major issue in the treatment of HCC, and elucidating its underlying mechanism is a key research topic.

Hypoxia is a common phenomenon that occurs in solid tumors, especially after tumor growth.⁽⁵⁾ Hypoxia can result in many changes in the genomic expression of tumor cells, allowing cells to adapt to harsh environments that may be associated with metastasis and poor prognosis.^(6–9) According to many reports, a series of changes caused by hypoxia is regulated by hypoxia-inducible factors (HIFs), namely, HIF-1 α and HIF-2 α . Hypoxia-inducible factor-2 α is more common in chronic hypoxia than HIF-1 α and is more specifically expressed in endothelial cells and hepatocytes.⁽¹⁰⁾ It has been reported that both the expression level and acetylation status of HIF-2 α are precisely adjusted in hypoxia.⁽¹¹⁾ We previously reported that HIF-2 α can regulate

Metastasis is a serious risk that may occur during the treatment of hepatocellular carcinoma (HCC), preventing many patients from being surgical candidates and contributing to poor prognosis. Hypoxia has been proved an important factor of metastasis through the epithelial–mesenchymal transition (EMT) pathway. Acetyl-CoA synthase 2 (ACSS2) provides an acetyl group for the acetylation of hypoxia-inducible factor (HIF)-2 α , and this epigenetic modification affects the activity of HIF-2 α and the subsequent EMT process. Here, we showed that ACSS2 expression was negatively correlated with HCC malignancy. Knockdown of ACSS2 increased the invasion and migration ability of HCC cells and promoted EMT without increasing the total protein level of HIF-2 α , even in hypoxic conditions. The immunoprecipitation assay revealed downregulated acetylation levels of HIF-2 α after ACSS2 knockdown in hypoxic conditions, which resulted in enhanced HIF-2 α activity. Finally, decreased expression of ACSS2 was found to be related to advanced stage and poor overall survival and disease-free survival rates in a cohort of patients with HCC. In conclusion, ACSS2 plays an important role in the acetylation process of HIF-2 α , which effectively modifies the activity of HIF-2 α under hypoxic conditions and greatly impacts on the prognosis of patients with HCC.

CDCP1 (CUB domain containing protein 1) to promote PKC δ (protein kinase C δ)-mediated migration in HCC.⁽¹²⁾

Acetyl-CoA synthase 2 (ACSS2) is an enzyme that converts acetate to acetyl-CoA, which is an important intermediate metabolite.^(13,14) Acetyl-CoA synthase 2 also plays a key role in the acetylation of HIF-2 α , which can provide the acetyl group for acetylation.^(15,16) Recent studies on ACSS2 have been carried out for a variety of cancers, and there are is controversy regarding the effect of ACSS2 on HCC and other cancers.^(17–19)

Materials and Methods

Cell culture and transfection. The MHCC97H, MHCC97L, and HCCLM3 cell lines were obtained from the Liver Cancer Institute of Fudan University (Shanghai, China), and the other HCC cell lines were purchased from ATCC (American Type Culture Collection Manassas, VA, USA). The LO2 cell line was obtained from the Chinese Academy of Sciences, Shanghai, China. ACSS2 gene expression was silenced with ACSS2 siRNA, purchased from Suzhou GenePharma China (Suzhou, China) and synthesized using the following probes: ACSS2-

Homo-1791,5'-CAGGAUGGCUAAUACUGGATT-3' (sense) and 5'-UCCAGUAAUAGCCAUCUGTT-3' (antisense); and ACSS2-Homo-2176,5'-CAUCUGUCAUCAGUCACCU-3' (sense) and 5'-AGGUGACUGAUGACAGAUGTT-3' (antisense). The ACSS2 siRNA (50 pmol) was incorporated into MHCC97H cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

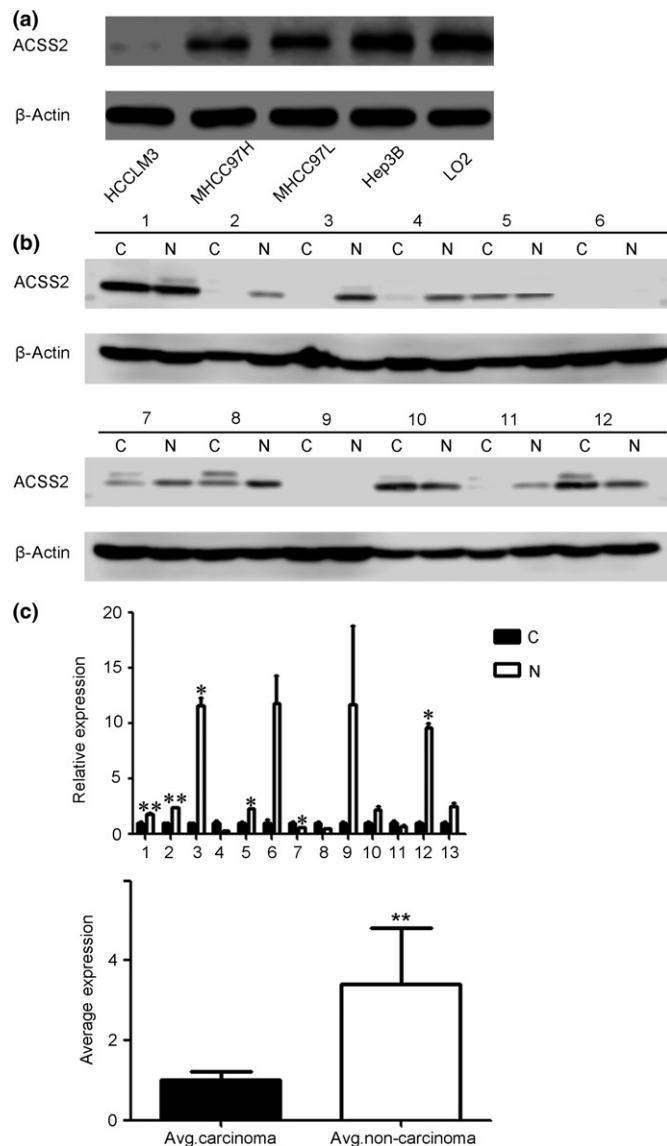


Fig. 1. Acetyl-CoA synthase 2 (ACSS2) expression in hepatocellular carcinoma (HCC) cell lines and patients. (a) Expression of ACSS2 in four HCC cell lines was detected by Western blot analysis. The data showed that a higher degree of malignancy was associated with lower ACSS2 protein expression in the HCC cell lines. The LO2 cell line, which was derived from normal liver tissue, was used as a control. The LM3 and MHCC97H cell lines are highly metastatic compared with the MHCC97L cell line, whereas the Hep3B cell line shows almost no metastatic ability. All cell lines were maintained in normoxic conditions. (b) ACSS2 expression was also detected in 12 pairs of human HCC carcinoma (C) and non-carcinoma (N) tissues by Western blot analysis. Non-carcinoma tissues showed higher expression. (c) Another 13 pairs of human HCC and non-carcinoma tissues were assessed by RT-PCR, and a similar result was obtained. Average (Avg.) expression of ACSS2 mRNA in the carcinoma and non-carcinoma tissues is shown. Data are shown as the mean \pm SD of three independent experiments. Error bars represent SD. * $P < 0.05$; ** $P < 0.01$.

Transfected cells were cultured for 48 h after siRNA treatment for further experiments. MHCC97H-NC and MHCC97H-siACSS2 cells were obtained by transfecting MHCC97H-wt cells with siRNA. All cells were maintained in DMEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS (Gibco) and cultured in an incubator with 5% CO₂ and 95% air. The hypoxic environment was created by adding CoCl₂ (400 μ M) to the DMEM.

Patients and specimens. The HCC tissue specimens for IHC were acquired from 110 patients who underwent surgery at Tianjin Medical University Cancer Institute and Hospital (Tianjin, China; 2010–2015). We chose paraffin blocks that were well fixed and embedded and that had complete clinical pathology and follow-up data. The clinical stage of the HCC patients was judged based on the TNM classification system of the International Union Against Cancer. Ethical approval was obtained from the Tianjin Medical University Cancer Institute and Hospital research ethics committee. The protocol for the research project conforms to the provisions of the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013). The other cases used for Western blot (WB) detection and RT-PCR were from patients who received surgery during the 2015. Carcinoma (C), non-carcinoma (N), precancerous (PR), and portal vein tumor thrombus (PVTT) tissues were collected from these patients to compare the ACSS2 expression levels.

Transwell invasion assays. Transwell chambers (Corning, Corning, NY, USA) with 8- μ m pores and Matrigel (Corning, Bedford, MA, USA) were used for the Transwell invasion experiments. Fifty microliters of Matrigel diluted with DMEM (1:4) was added to the Transwell chambers, and the chambers were then incubated at 37°C in an incubator with 5% CO₂ for 1 h. Next, the MHCC97H cells were diluted to a concentration of 1×10^5 cells/mL in DMEM only, and 200 μ L cell suspension was plated into the upper chamber. Then 600 μ L DMEM containing EGF (epidermal growth factor) (10 ng/mL) was added to the lower chamber. After 48 h, the cells that successfully invaded the lower membrane were fixed with 4% paraformaldehyde and stained. We used a light microscope to observe and photograph the cells at $\times 10$ magnification. We chose three random fields for counting and took the average of the three fields as the result of the Transwell invasion experiment.

Scratch test. Cells were plated at a concentration of 5×10^5 cells per well into 6-well plates. After transfection, we allowed the cells to become confluent and then replaced the culture

Table 1. Background characteristics of patients with hepatocellular carcinoma

Parameter	Yes	No
Age, ≥ 50 years	19	6
Gender, male	23	2
Tumor size, ≥ 5 cm	12	13
Tumor number, multiple	7	18
Satellite lesions	11	14
Portal vein invasion	10	15
Microvascular invasion	17	8
Cirrhosis	20	5
Clinical stage, advanced	11	14
Histological grade, poor	8	17
HBV infection	21	4
HCV infection	0	25

HBV, hepatitis B virus; HCV, hepatitis C virus.

medium with DMEM containing 0.5% serum to begin the experiment. A 10- μ L tip was used to induce scratches with equal widths. The cells were then maintained at 37°C in a 5% CO₂ air incubator, and the distances between both sides of the scratch at 0, 3, 6, 9, 12, and 24 h after scratching were recorded. We chose three random sites to calculate the mean and standard deviation. Images were collected at 0 and 24 h.

Western blot analysis and antibodies. Protein from human tissue samples and cells was quantified by the BCA method (Thermo Fisher Scientific, Waltham, MA, USA) and separated by SDS-PAGE. The protein was then transferred to membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk powder, the membrane was incubated with anti-ACSS2 rabbit polyclonal antibody (1:1000, ab66038; Abcam,

HongKong, China), anti-HIF-2 α rabbit polyclonal antibody (1:1000, NB100-122; Novus Biologicals, Littleton, Colorado, USA), anti-transforming growth factor- α (TGF α) rabbit mAb (1:1000, ab208156; Abcam), anti-cyclinD1 rabbit mAb (1:5000, ab134175; Abcam), anti-E-cadherin rabbit polyclonal antibody (1:1000, ab15148; Abcam), anti-N-cadherin rabbit polyclonal antibody (1:500, ab18203; Abcam), anti-Snail rabbit mAb (1:1000, #3876; Cell Signaling Technology, Danvers, MA, USA), anti-vimentin rabbit mAb (1:1000, ab92547; Abcam), and anti- β -actin mouse mAb (1:1000, sc-47778; Santa Cruz Biotechnology, CA, USA). After incubating overnight at 4°C, the membrane was further probed with anti-mouse (1:4000, sc2005; Santa Cruz Biotechnology) or anti-rabbit IgG (1:4000, sc2004; Santa Cruz Biotechnology).

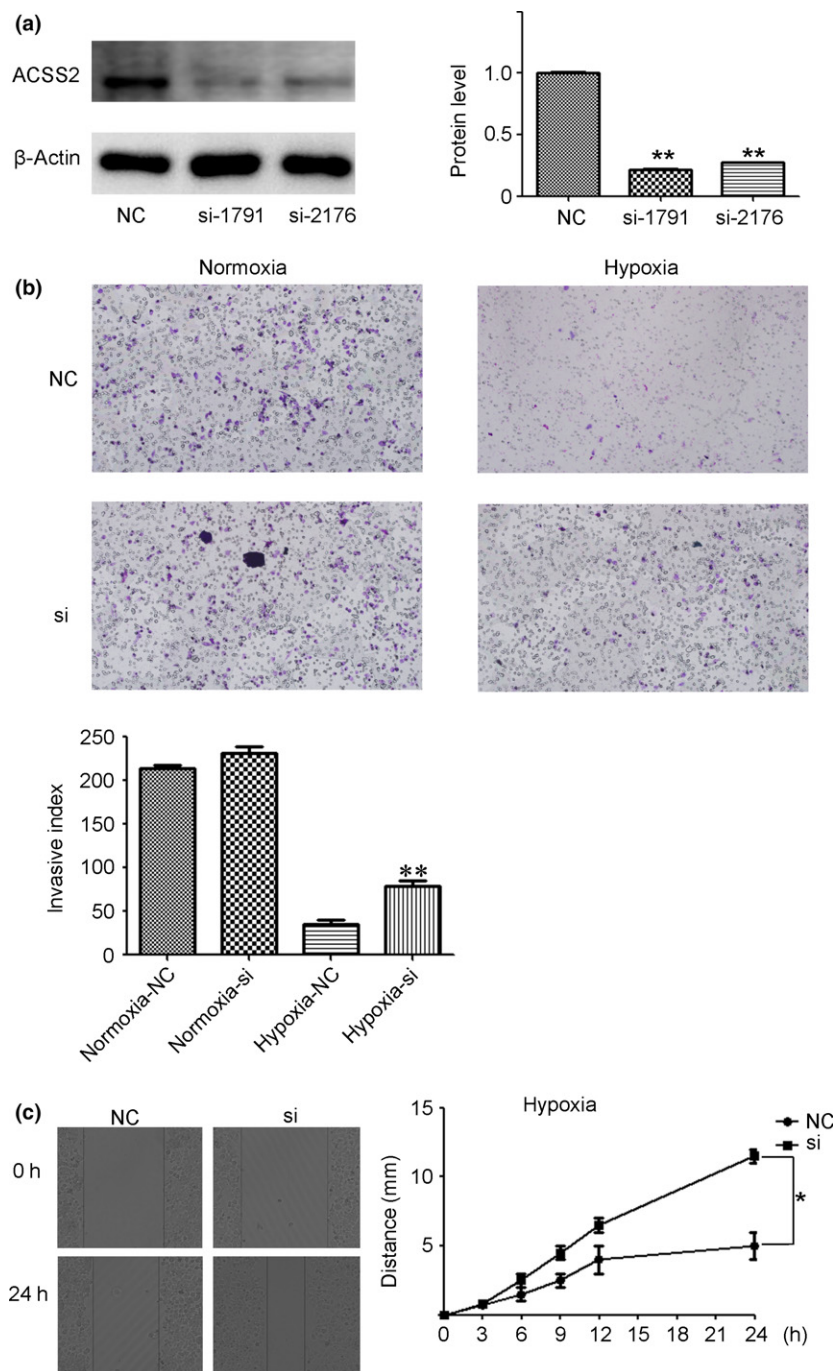


Fig. 2. Downregulation of acetyl-CoA synthase 2 (ACSS2) increased the invasion and migration of hepatocellular carcinoma (HCC) cells in hypoxic conditions. (a) siRNA knockdown was verified by Western blot analysis in MHCC97H cells. Grayscale analysis of the three replicates is shown as a histogram. ACSS2 expression was normalized to β -actin. (b,c) Transwell invasion experiment and scratch test showed that downregulation of ACSS2 resulted in increased invasiveness and migration of HCC cells in hypoxic conditions. Data are shown as mean \pm SD of three independent experiments. Error bars represent SD. * $P < 0.05$; ** $P < 0.01$. NC, normal control.

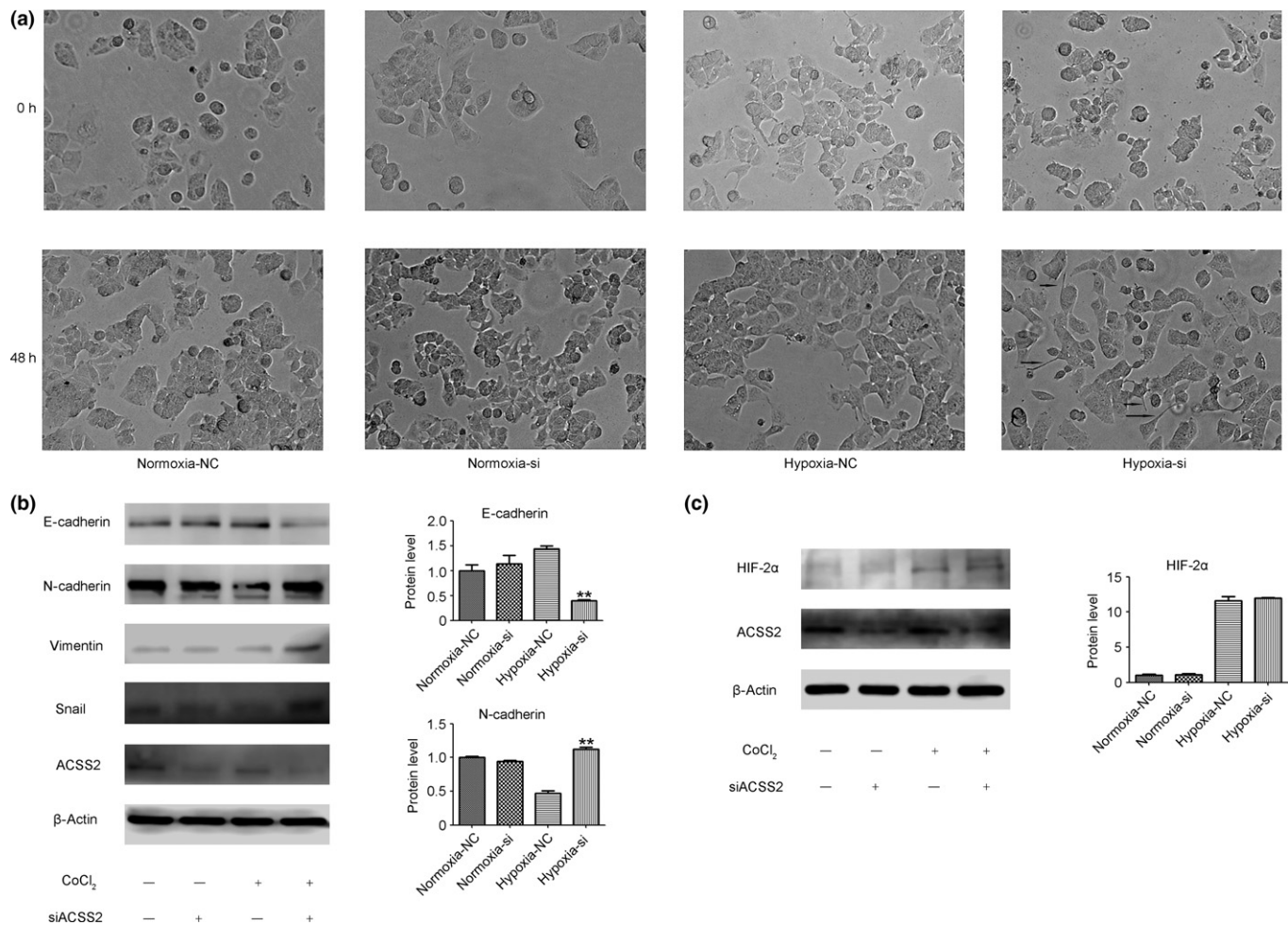


Fig. 3. Knockdown of acetyl-CoA synthase 2 (ACSS2) promoted epithelial-mesenchymal transition of hepatocellular carcinoma cells without increasing hypoxia-inducible factor-2 α (HIF-2 α). (a) Morphology of MHCC97H after siRNA interference in normoxic and hypoxic conditions. (b) Western blot images show that there were more N-cadherin, vimentin, and Snail, and less E-cadherin expression in the siACSS2 cells compared to the normal control (NC) cells in hypoxic conditions. Grayscale analysis of N-cadherin and E-cadherin is shown as histograms, which represent three replicates. Data in the histograms are normalized to β -actin. (c) There was no difference in HIF-2 α expression between the siACSS2 group and the NC group. Grayscale analysis of HIF-2 α is also shown. Error bars represent SD. * $P < 0.05$; ** $P < 0.01$.

Real-time PCR. RNA from human tissue samples and cells was extracted with TRIzol (Invitrogen). Then 2 μ g total RNA was reverse transcribed into cDNA using a transcriptase First Strand cDNA Synthesis Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Next, quantitative real-time PCR using a Bio-Rad (Hercules, CA, USA) Laboratories CFX96 system was carried out to analyze the amount of cDNA produced in the above transcription step. The Power SYBR Green Master Mix used in the RT-PCR was purchased from Roche (Basel, Switzerland). We determined the expression in a sample using three separate experiments with triplicate wells per experiment. The primer sequences used were as follows: ACSS2 forward, 5'-GGATTCCAGCTGCAGTCTTC-3'; ACSS2 reverse, 5'-CATGCCACCACAAGTCAATC-3'; phosphoglycerate kinase 1 PGK1 (Phosphoglycerate kinase 1) forward, 5'-GACTGTGTAGGCC-CAGAAGT-3'; PGK1 reverse, 5'-CTGGCTCGGCTTTAACCTTG-3'; vascular endothelial growth factor VEGF (Vascular endothelial growth factor) forward, 5'-GGCCAGCACATAG-GAGAGAT-3'; VEGF reverse, 5'-ACGCTCCAGGACTTA-TACCG-3'; glucose transporter 1 GLUT1 (Glucose transporter 1) forward, 5'-GGCCATCTTTTCTGTTGGGG-3'; GLUT1 reverse, 5'-CCAGCAGGTTTCATCATCAGC-3'; TGF α forward, 5'-CGCTCTGGGTATTGTGTTGG-3'; TGF α reverse, 5'-

TGGAATCTGGGCAGTCATT-3'; cyclinD1 forward, 5'-GCA TGTTTCGTGGCCTCTAAG-3'; and cyclinD1 reverse, 5'-TTCAATGAAATCGTGCGGG-3'.

Immunohistochemistry. All HCC patient paraffin blocks used for this assay were approved by the Tianjin Medical University Cancer Institute and Hospital and were confirmed histologically by HE staining. After blocking with 3% BSA, the tissues were incubated with anti-ACSS2 antibody (1:100, ab66038; Abcam), anti-HIF-2 α antibody (1:250, NB100-122; Novus Biologicals) and anti-TGF α antibody (1:1000, ab208156; Abcam) for 30 min and then transferred to 4°C overnight. The next day, after incubating with a goat anti-rabbit secondary antibody PV-6000 Kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at 37°C, the tissues were stained with DAB and hematoxylin and then dehydrated and dried before observing. Slides with the primary antibodies omitted were used as negative controls. The staining results were estimated by one researcher who was blind to the clinicopathologic staging and outcomes of all patients during observation. Five fields were selected for an average.

Immunoprecipitation. Hypoxia-inducible factor-2 α protein was immunoprecipitated from a 35-mm plate of HCC cells.

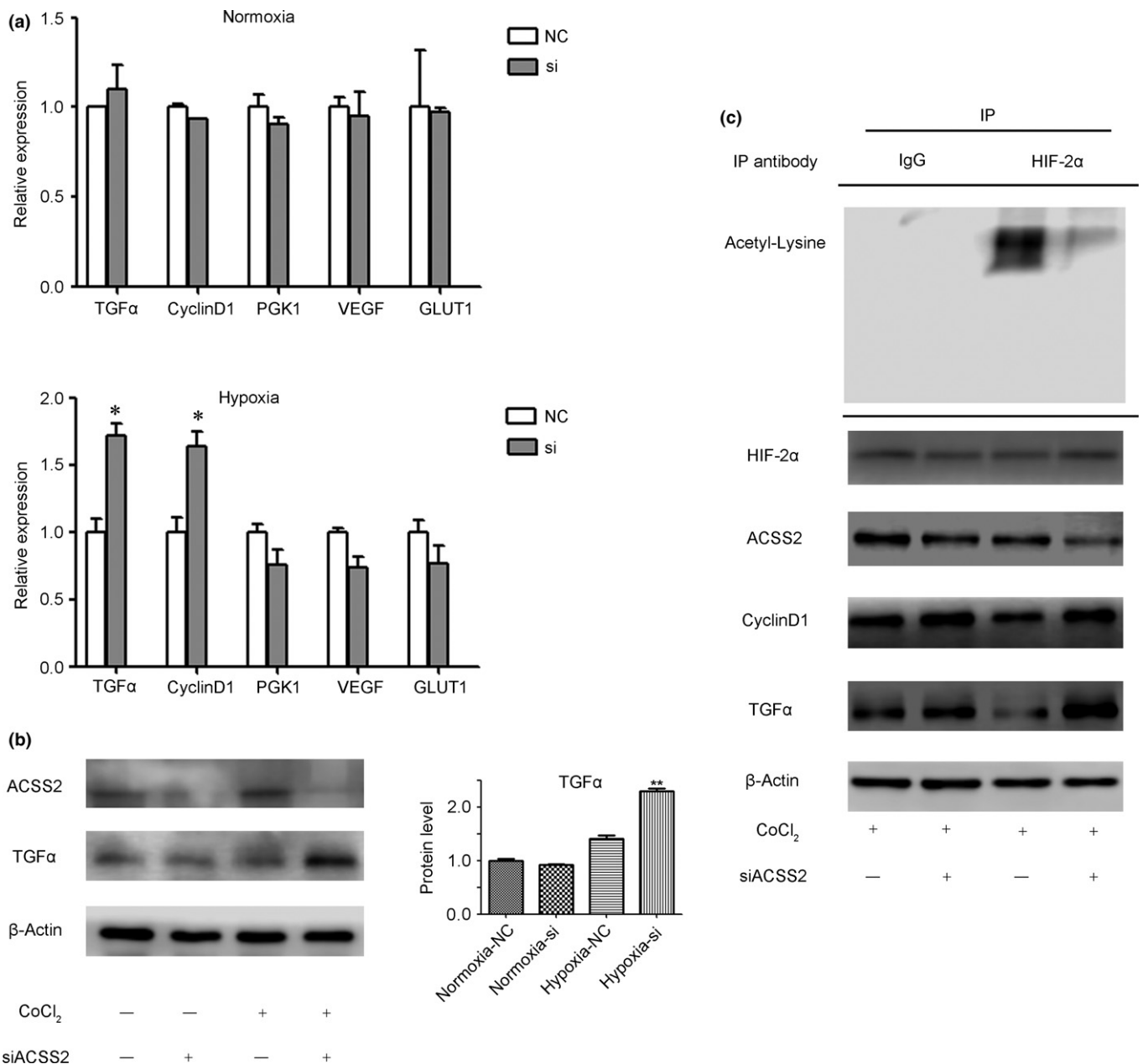


Fig. 4. Knockdown of acetyl-CoA synthase 2 (ACSS2) induced deacetylation of hypoxia-inducible factor-2 α (HIF-2 α) and enhanced the activity of HIF-2 α in hypoxic conditions. (a,b) RT-PCR and Western blot results revealed that ACSS2 knockdown enhanced the expression of some HIF-2 α downstream molecules in hypoxic conditions. Grayscale analysis of transforming growth factor α (TGF α) and ACSS2 is shown as histograms. Data are normalized to β -actin and are shown as mean \pm SD of three independent experiments. (c) Immunoprecipitation (IP) showed that ACSS2 knockdown led to higher deacetylated HIF-2 α expression under hypoxic conditions. Error bars represent SD. * $P < 0.05$; ** $P < 0.01$. GLUT1, glucose transporter 1; PGK1, phosphoglycerate kinase 1; VEGF, vascular endothelial growth factor.

Whole-cell extracts were incubated with protein A beads (16-156; Millipore) and normal rabbit IgG (2729S; Cell Signaling Technology) to clear non-specific binding. We then incubated the supernatants with 2 μ L anti-HIF-2 α (NB100-122; Novus Biologicals) antibody or normal rabbit IgG (2729S; Cell Signaling Technology) for 3 h before mixing with protein A beads. After binding overnight, the supernatant was removed by centrifugation, and the immunoprecipitated proteins were eluted and detected by WB.

Statistical analysis. The results and data were analyzed by spss 18.0 software (SPSS, Chicago, IL, USA) and expressed as mean \pm SD. Differences between two groups were evaluated

with *t*-tests, and $P < 0.05$ was considered statistically significant. Some of the WB images were used for grayscale analysis, and the resulting histograms represent the average of three replicates. The χ^2 -test was used to explore the relationship between two variables. Survival curves (overall survival [OS] and disease-free survival [DFS]) of 110 patients were calculated by Kaplan–Meier analysis.

Results

Acetyl-CoA synthase 2 expression in HCC cell lines and patients. To elucidate the effect of ACSS2 on HCC, four HCC

Table 2. Clinical characteristics and laboratory parameters of patients with hepatocellular carcinoma according to ACSS2 expression level

Parameter	<i>n</i>	ACSS2 low expression, <i>n</i> = 56	ACSS2 high expression, <i>n</i> = 54	<i>P</i> -value
Age, years				
<50	35	21	14	0.193
≥50	75	35	40	
Gender				
Male	98	48	50	0.247
Female	12	8	4	
Tumor size, cm				
<5	52	25	27	0.574
≥5	58	31	27	
Tumor number				
Single	76	35	41	0.128
Multiple	34	21	13	
Satellite lesions				
No	59	21	38	0.001*
Yes	51	35	16	
Portal vein invasion				
No	58	21	37	0.001*
Yes	52	35	17	
Microvascular invasion				
No	35	10	25	0.001*
Yes	75	46	29	
Cirrhosis				
No	22	5	17	0.003*
Yes	88	51	37	
Clinical stage				
Stage I and II	63	22	41	<0.001*
Stage III and IV	47	34	13	
Histological grade				
Well	82	38	44	0.101
Poor	28	18	10	
HIF-2 α expression				
Low expression	60	27	33	0.174
High expression	50	29	21	
TGF α expression				
Low expression	65	28	37	0.048*
High expression	45	28	17	

HIF-2 α , hypoxia-inducible factor-2 α ; TGF α , transforming growth factor α . **P* ≤ 0.05.

cell lines and the LO2 cell line were selected for the detection of ACSS2 expression. We initiated the culture of the five cell lines at the same time and harvested the cells when they were in the logarithmic growth phase. Western blot results revealed that the MHCC97L and Hep3B cells showed higher ACSS2 expression than the more malignant MHCC97H and LM3 cell lines (Fig. S1).^(20–24) The LO2 cell line, which was derived from normal liver tissue,⁽²⁵⁾ also showed high ACSS2 expression (Fig. 1a). We then detected ACSS2 expression in C and NC tissues of 25 patients by WB and RT-PCR; patient background characteristics are shown in Table 1. The WB data showed that NC tissues exhibited higher ACSS2 expression

(Fig. 1b). Similar results were found in the RT-PCR data of another 13 pairs of human HCC samples (Fig. 1c). These results indicate that ACSS2 may be negatively correlated with the malignancy of HCC.

Acetyl-CoA synthase 2 knockdown promoted the invasion and migration ability of HCC cells under hypoxic conditions. We used siRNA technology to knock down ACSS2 gene expression and explored its influence on the invasion and migration ability of MHCC97H cells. Knockdown was verified (Fig. 2a), and Transwell experiments were then carried out to detect the invasion ability of the cells. A hypoxic environment was induced by adding CoCl₂ (400 μ mol/mL) to the medium.⁽²⁶⁾ The results revealed that the siACSS2 group showed higher invasion ability in the hypoxic environment than the control group (Fig. 2b). The migration test showed that knockdown of ACSS2 enhanced the migration ability of MHCC97H cells under hypoxic conditions (Fig. 2c). Taken together, these results suggest that downregulation of ACSS2 expression may result in increased metastases under hypoxia.

Knockdown of ACSS2 promoted epithelial–mesenchymal transition of HCC cells without HIF-2 α elevation under hypoxia. Epithelial–mesenchymal transition (EMT) is described as the process by which epithelial cells convert into individual migratory cells. The EMT phenomenon is also considered an important step in cancer metastasis.^(27,28) Our siRNA experiments found that more spindle-shaped cells were recognized in the hypoxia-si group under hypoxic conditions than in other groups (Fig. 3a). We then examined the relevant indicators of EMT in siACSS2 cells and negative control (NC) cells. The results showed that there were more N-cadherin, vimentin, and Snail and less E-cadherin expression in siACSS2 cells compared to NC cells during hypoxia, which indicated that EMT was promoted in siACSS2 cells under hypoxic conditions (Fig. 3b). Notably, this phenomenon did not occur in normoxic conditions (Fig. 3b). Due to the different results obtained between the hypoxic and normoxic conditions, we speculated that some hypoxia-induced molecules might lead to these differences. We next detected HIF-2 α expression in siACSS2 cells and NC cells under hypoxia and normoxia. The data showed that HIF-2 α expression was markedly higher under hypoxic conditions. However, there was no difference in HIF-2 α protein expression levels between the siACSS2 group and the NC group (Fig. 3c), which indicated that the total amount of HIF-2 α protein was not the driving factor affecting the EMT process after ACSS2 knockdown.

Acetylation of HIF-2 α affected the EMT process. Epigenetic modifications, including methylation and acetylation, have great impact on protein function.^(29,30) Hypoxia-inducible factor-2 α also undergoes these post-translational modifications. Acetylation and deacetylation modifications of HIF-2 α even surpass the role of expression regulation, which in turn affects the expression of the downstream molecules of HIF-2 α activity. We examined the downstream molecules of HIF-2 α by RT-PCR. The result revealed increased expression levels of some molecules downstream of HIF-2 α , including TGF α and cyclinD1, after ACSS2 knockdown, during hypoxia (Fig. 4a). However, these changes did not occur under normoxia. We also detected some molecules that were regulated by HIF-1 α , or both HIF-1 α and HIF-2 α , and there were no differences between the hypoxic and normoxic conditions after ACSS2 knockdown (Fig. 4a). Next, we selected TGF α for further verification by WB, and the data revealed a similar result (Fig. 4b). Then we used immunohistochemistry (IHC) to investigate the relationship between ACSS2 and TGF α and HIF-2 α

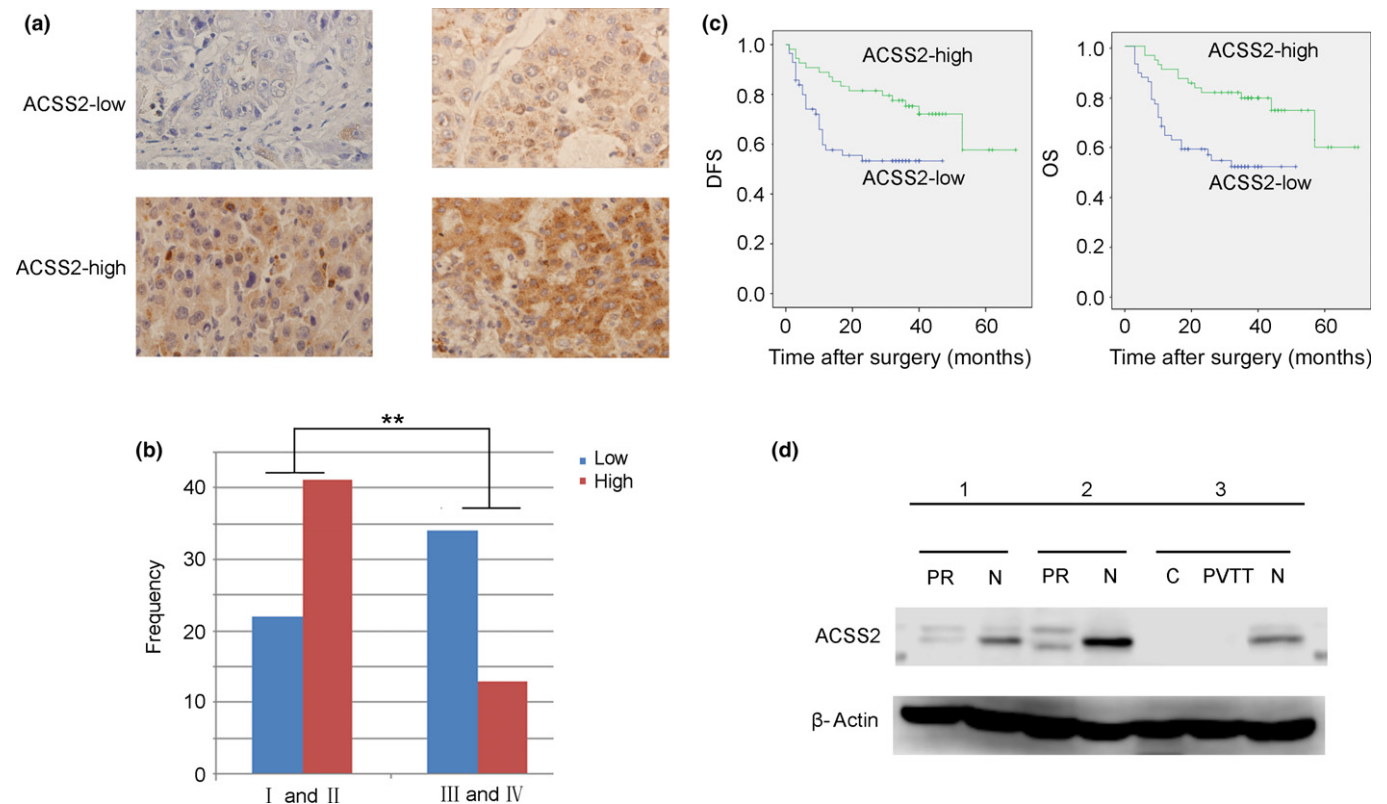


Fig. 5. Low acetyl-CoA synthase 2 (ACSS2) expression was associated with poor prognosis and progression in hepatocellular carcinoma (HCC) patients. (a) Representative images of ACSS2 expression in HCC tumors were obtained by immunohistochemical staining. (b) The relationship between ACSS2 immunohistochemical staining intensity and clinical stage is presented as histograms. Data indicate that more advanced HCC tumors (stage III and IV) show much less intense staining of ACSS2 protein than early stage tumors (stage I and II). The Y-axis indicates the frequency of patients in different groups. (c) Decreased expression of ACSS2 was related to poor prognosis ($P < 0.05$). DFS, disease-free survival; OS, overall survival. (d) Precancerous tissue (PR) also showed less ACSS2 expression than corresponding non-carcinoma tissue (N). Furthermore, vascular invasion tissue (portal vein tumor thrombus, PVTT) showed lower ACSS2 expression than non-carcinoma tissue (N), which was similar to its primary tumor (C).

in HCC samples, which indicated that the correlation between ACSS2 expression and TGF α expression had statistical significance (Table 2, Fig. S2). However, there was no similar statistical significance between the expression of ACSS2 and HIF-2 α (Table 2, Fig. S2). Moreover, to ensure the acetylation status of HIF-2 α , we undertook immunoprecipitation on the NC and ACSS2-knockdown groups. The data indicated significant downregulation of the acetylation levels of HIF-2 α protein in the ACSS2-knockdown group (Fig. 4c), which confirmed the influence of ACSS2 on HIF-2 α acetylation in other studies.^(15,16) Taken together, these results suggest that deacetylation of HIF-2 α occurred under hypoxia after ACSS2 knockdown, which enhanced the activity of HIF-2 α and promoted the EMT process.

Low expression of ACSS2 is associated with poor prognosis and progression in HCC patients. The cell biology experiments above showed that the metastatic potential of HCC cells increased following knockdown of ACSS2 expression. Next, we undertook IHC assays using a cohort of 110 HCC patients (2010–2015). Staining intensity was divided into four levels, as shown in Figure 5(a): negative, weak, moderate, and strong staining. We defined the first two images as the low-expression group and the other two images as the high-expression group (Fig. 5a). We analyzed the clinical pathology characteristics of 110 patients by ACSS2 expression level (Table 2) and found that ACSS2 expression was related to satellite lesions,

cirrhosis, vascular invasion, and poor clinical stage. The correlation of ACSS2 expression and clinical stage was shown intuitively with a histogram (Fig. 5b). We then undertook survival analysis of the cases. Kaplan–Meier analysis revealed that low ACSS2 expression was associated with poor prognosis, which was reflected in both OS and DFS (Fig. 5c). The difference between high expression and low expression groups was statistically significant (Table 3). We recently obtained three pairs of special specimens: two specimens of PR and their corresponding N; the other specimen included C, PVTT, and N tissue. We detected ACSS2 expression in these specimens and found that the PR tissue showed lower ACSS2 expression than the corresponding non-carcinoma tissue (Fig. 5d). Moreover, the PVTT showed low ACSS2 expression, which was similar to the level in the primary tumor (Fig. 5d). All of the results above suggested that low expression of ACSS2 is not only an important feature in the development of HCC, but also a significant factor that impacts HCC metastasis.

Discussion

Acetyl-CoA synthase 2 is one isoform of the acetyl-CoA synthetase family, which consists of ACSS1, ACSS2, and ACSS3. Acetyl-CoA synthase 2 is localized in cytoplasm and nuclei, whereas the other two isoforms are located in mitochondria.⁽³¹⁾ Acetyl-CoA synthase 2 has been a key research topic in recent

Table 3. Analysis of prognostic factors influencing overall survival (OS) and disease-free survival (DFS) in patients with hepatocellular carcinoma

	OS			DFS		
	<i>n</i>	χ^2 -test	<i>P</i> -value	<i>n</i>	χ^2 -test	<i>P</i> -value
Age, years						
<50	35	3.494	0.062	35	1.502	0.220
≥50	75			75		
Gender						
Male	98	0.660	0.416	98	0.637	0.425
Female	12			12		
Tumor size, cm						
<5	52	4.621	0.032*	52	1.304	0.253
≥5	58			58		
Tumor number						
Single	76	11.437	0.001*	76	11.948	0.001*
Multiple	34			34		
Satellite lesions						
No	59	20.775	<0.001*	59	17.669	<0.001*
Yes	51			51		
Portal vein invasion						
No	58	12.616	<0.001*	58	14.445	<0.001*
Yes	52			52		
Microvascular invasion						
No	35	13.368	<0.001*	35	10.825	0.010*
Yes	75			75		
Cirrhosis						
No	22	2.578	0.108	22	6.321	0.012*
Yes	88			88		
Clinal stage						
Stage I and II	63	41.424	<0.001*	63	26.147	<0.001*
Stage III and IV	47			47		
Histological grade						
Well	82	0.535	0.465	82	0.062	0.804
Poor	28			28		
HIF-2 α expression						
Low expression	60	7.063	0.008*	60	1.998	0.158
High expression	50			50		
TGF α expression						
Low expression	65	4.294	0.038*	65	0.841	0.359
High expression	45			45		
ACSS2 expression						
Low expression	56	9.980	0.002*	56	7.048	0.008*
High expression	54			54		

HIF-2 α , hypoxia-inducible factor-2 α ; TGF α , transforming growth factor α . **P* ≤ 0.05.

years with emphases on two areas. The first area is the relationship between ACSS2 and energy metabolism. It has been reported that acetate is an important source of energy for many tumors, and ACSS2 plays a key role in this energy supply process.^(13,14,31,32) In addition, ACSS2 is indispensable for the

acetylation of certain molecules,⁽³³⁾ such as HIF-2 α .^(15,16) However, there are some controversies surrounding the effect of ACSS2 in tumors. Some scientists have recognized ACSS2 as a cancer-promoting factor,^(13,14,32) whereas others have reported opposing results, in that the loss of ACSS2 expression predicts poor prognosis in patients,^(34,35) especially in cancer of the digestive system. The theory of ACSS2 promoting tumors is mainly found in breast cancer and glioma, which emphasizes the role of ACSS2 in producing acetyl-CoA; the opposite opinion on ACSS2 in digestive system cancer is mainly from the IHC of clinical samples. Our data indicate that decreased expression of ACSS2 may promote HCC metastasis and predict poor prognosis. In contrast to mammary glands, our data indicate that normal liver tissue and normal liver cells show high ACSS2 expression, and the gradual loss of ACSS2 indicates the occurrence and progression of HCC.

Our invasion and migration experiments showed that there were significant changes after ACSS2 knockdown under hypoxia. We thus speculated that the impact of ACSS2 on invasion and metastasis might be executed through the HIF family, which is considered to play an important role in inducing EMT and metastasis.^(36–38) Previous studies have reported that the acetate/ACSS2 switch could regulate HIF-2 α stress signaling⁽¹⁵⁾ and that HIF-2 α is more common in chronic hypoxia.⁽¹⁰⁾ Therefore, we focused on both the amount and the activity of HIF-2 α . We found that ACSS2 knockdown reduced the acetylation levels of HIF-2 α and enhanced the expression levels of its downstream molecules. However, ACSS2 knockdown had no effect on the amount of HIF-2 α protein under hypoxia. We then identified changes in EMT status after ACSS2 knockdown and HIF-2 α deacetylation. All the results reported above indicate that ACSS2 knockdown deacetylates HIF-2 α , which enhances the activity of HIF-2 α and induces EMT. Our IHC experiment of clinical specimens also proved that low expression of ACSS2 was related to many metastasis indicators, such as satellite lesions and vascular invasion.

Taken together, our study revealed that ACSS2 plays an important role in the acetylation process of HIF-2 α protein and therefore effectively modulates the activity of HIF-2 α under hypoxic conditions, which has great impacts on the prognosis of HCC patients.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Expression of N-cadherin and E-cadherin in hepatocellular carcinoma cell lines.

Fig. S2. Representative images of hypoxia-inducible factor-2 α (HIF-2 α) and transforming growth factor α (TGF α) expression in a cohort of 110 human hepatocellular carcinoma samples.