Abstract
EMT is a pivotal mechanism involved in tumor metastasis, which is the leading cause of poor prognosis for hepatocellular carcinoma (HCC). Sirtuin family members function as NAD+-dependent deacetylases that are essential for tumor metastasis and epithelial–mesenchymal transition (EMT). However, no causal association has been established between Sirtuin6 (SIRT6) and HCC metastasis. SIRT6 expression pattern and its association with HCC metastasis were investigated by informatic analysis, and verified by qRT-PCR and immunofluorescence assay in HCC tissues. Transwell assay, qRT-PCR, and immunofluorescence assay were utilized to assess the effects of SIRT6 on metastasis and E-cadherin expression in vitro and in vivo. Immunoprecipitation assay was performed to observe whether SIRT6 deacetylated Beclin-1 in HCC cells. Immunofluorescence assay and inhibitor treatment rescue experiments were used to clarify the mechanism by which SIRT6 facilitated EMT and metastasis. SIRT6 upregulation was quite prevalent in HCC tissues and closely correlated with worse overall survival, disease-relapse free survival, and HCC metastasis. Furthermore, SIRT6 promoted HCC cell migration, invasion, and EMT. Mechanistically, we found that SIRT6 deacetylated Beclin-1 in HCC cells and this event led to the promotion of the autophagic degradation of E-cadherin. Noticeably, E-cadherin degradation and invasion induced by SIRT6 overexpression could be rescued by dual mutation of Beclin-1 (inhibition of acetylation), CQ (autophagy inhibitor), and knockdown of Atg7. In addition, SIRT6 promoted N-cadherin and Vimentin expression via deacetylating FOXO3a in HCC. These results established a relationship between SIRT6 and HCC EMT and further elucidated the mechanisms underlying HCC metastasis, helping provide a promising approach for the treatment of HCC.

Implications: Inhibiting SIRT6 represents a potential therapeutic approach to suppress HCC metastasis partially through reduction of autophagic degradation of E-cadherin.

Introduction
Hepatocellular carcinoma (HCC) is reported to be the fifth most common cancer and the second leading cause of cancer-related mortality worldwide (1). The highly metastatic biological features of HCC cells result in patients presenting with an advanced clinical stage and losing the opportunity for treatment with resection. Furthermore, frequent tumor recurrence after resection also results in poor prognosis (2). However, the mechanisms of HCC metastasis and recurrence need to be clarified. The epithelial–mesenchymal transition (EMT), which is characterized by the loss of epithelial features and the increase of mesenchymal features in cancer cells, is critical for the metastatic progression of multiple cancer types (3). E-cadherin is widely recognized as a pivotal receptor involved in cellular adhesion that can prevent tumor metastasis by inhibiting EMT.

Sirtuin6 (SIRT6) Promotes the EMT of Hepatocellular Carcinoma by Stimulating Autophagic Degradation of E-Cadherin
Li Li Han1, Lijun Jia1, Fei Wu2, and Chen Huang2

1Department of Oncology, The Second Affiliated Hospital, College of Medicine, Xi’an Jiaotong University, Xi’an, Shaanxi, P.R. China. 2Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education of China, Xi’an Jiaotong University, Xi’an, China.

Corresponding Author: Li Li Han, The Second Affiliated Hospital of Xi’an Jiaotong University, No. 157 Xiwu Road, Xi’an 710004, China. Phone: 298-767-9518; Fax: 298-767-9515; E-mail: 61439376@qq.com

Mol Cancer Res 2019;17:2267–80
doi: 10.1158/1541-7786.MCR-19-0321
©2019 American Association for Cancer Research.
cytoplasmic components in lysosomes, plays an essential role in the progression of cancers (21–23). In spite of emerging studies showing that autophagy could serve as an important mediator of EMT (24, 25), the molecular mechanisms of autophagy involved in the EMT of cancer have not been clearly described.

In the current study, we provide novel evidence that SIRT6 promoted the EMT of HCC by inhibiting E-cadherin expression, which was dependent on autophagic degradation resulting from the deacetylation of Beclin-1, strongly suggesting that SIRT6 functioned as an oncogene in HCC. Importantly, our findings provide further insight into the molecular mechanisms underlying HCC metastasis; thus, the data from this study may contribute to providing a new, effective therapeutic target for HCC metastasis.

Materials and Methods

Patients and tissue samples
A total of 120 HCC tissue and paired adjacent noncancer tissues were collected from patients with HCC between June 2011 and June 2012. These patients with HCC accepted an operation at The Second Affiliated Hospital of Xi’an Jiaotong University (Xi’an, China), but neither accepted prior chemotherapy nor radiotherapy. Supplementary Table S1 showed the clinicopathologic data of these patients. Another 30 samples intrahepatic metastasis were collected from patients with HCC who accepted reoperation between February 2010 and December 2013. All the tissues were frozen at once in liquid nitrogen after operation. They were stored in −80°C refrigerator or in paraffin.

IHC assay
The IHC assay was performed as reported previously (26). Antibodies including anti-SIRT6 antibody (bs-4174R; at a dilution of 1:100), anti-E-cadherin antibody (bs-1016R, at 1:200), anti-N-cadherin antibody (bs-20622R; at 1:100), anti-Ki67 antibody (bs-23103R; at 1:100) and anti-vimentin antibody (bs-8533R; at 1:100). All these antibodies were purchased from Beijing Bios Biotechnology.

The qRT-PCR assay
The primers used in the study were designed and synthesized by Takara. The sequences are provided in Supplementary Table S2.

Cell culture and transfection
The immortalized normal human liver cell line LO2 was purchased from FuHeng Cell Center in 2018. The HCC cells Hep3B were bought from the Chinese Academy of Sciences Cell Bank in 2018. The HCC cells (HCCLM3, Bel-7402, MHCC-97L, and MHCC-97H) were purchased from FuHeng Cell Center in 2017. All the cells were cultivated in DMEM (Gibco), supplemented with 10% FBS (HyClone) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2. All cells were cultured not more than 20 passages each time after thawing. All cell lines were not contaminated by Mycoplasma as tested annually by the Mycoplasma PCR Detection Kit (Sigma). At the end of the study, all the cell lines were authenticated using short tandem repeat DNA (STR) analysis at FuHeng Cell Center. SIRT6, E-cadherin, FOXO3a, SIRT6 overexpression without fluorescence labeling and their control vector were bought from Addgene. SIRT6 and Agg siRNA were purchased from Jikai Gene Chemical Technology Co., Ltd. These vectors or siRNA were transfected into HCC cells using Lipofectamine 2000 (Thermo Fisher Scientific). To established stably knocked down SIRT6 HCC cells, RNAi lentivirus particles targeting SIRT6 (shSIRT6-1 and shSIRT6-2) and lentivirus negative control (shCtrl; Jikai Gene Chemical Technology Co., Ltd.) were transfected into HCC cells. The cells were incubated for a further 3 days and passaged for further experiments.

Western blotting assay
Cells were lysed using RIPA Buffer (Cell Signaling Technology). After that, the protein concentrations were assessed by bicinchoninic acid assays (7780, Cell Signaling Technology). Total proteins (25 μg) were resolved by 12% SDS-PAGE and then transferred to fluoride membrane. Fluoride membrane was put into primary antibodies after being blocked in 5% nonfat milk for 1 hour. The membranes were washed with PBS-T after being kept at 4°C overnight and subsequently incubated in secondary antibodies for purpose protein and an anti-mouse secondary antibody for GAPDH at 37°C for 2 hours. At last, DNR Bio-Imaging System was used to test the membranes. Anti-SIRT6 antibody (#12486; 1:200), anti-E-cadherin (#3195; 1:200), anti-vimentin (#574S; 1:200), and anti-Becin1 (#3945; 1:200) were purchased from Cell Signaling Technology, Inc. The anti-Agg (ab133528; 1:5000), anti-FOXO3a (ab12162; 1:1000) and GAPDH antibody (ab6922; 1:200) were bought from Abcam.

Cell migration and invasion assays
The 24-well Transwell plates (Corning Costar) were used to measure the migration and invasive ability. For the cell migration assays, 2 × 104 HCC cells in 200 μL FBS free media were implanted into the upper chamber uniformly, while 600 μL media with 10% FBS was placed into the bottom chambers. After 48-hour incubation, cells left on the membrane in the top chamber side were wiped off with a cotton swab, while cells migrated through the membrane were fixed with methanol. The migratory cells were stained with crystal violet. The number of migratory cells in 10 random fields was carefully counted under a microscope. For invasion assay, we performed the same procedures as describe above except we coated the membrane in the top chamber side with 200 mg/mL of Matrigel (BD Biosciences).

Immunoprecipitation assay
To perform acetylation immunoprecipitation of acetylated proteins, cells (MHCC-97H cells transfected with a control siRNA or two effective SIRT6-siRNAs for 48 hours; Hep3B cells transfected with a control vector or Flag-SIRT6 for 48 hours; MHCC-97H cells cotransfected with a Flag-FOXO3a and SIRT6-siRNAs for 48 hours) were lysed using E1A lysis buffer containing 2 mmol/L TSA and 10 mmol/L NAM. Total cell lysate was transferred to a new centrifugal tube and then added with prepared Protein A/G agarose. After being shaken for 10 minutes on ice, the supernatant and Protein A/G agarose mixture was centrifuged at 12,000 RPM (4°C, 15 minutes) and then the immunoprecipitated antibodies were added into the tube. After being kept overnight at 4°C, the mixture was added in 50 μL protein A/G agarose and then incubated at 4°C for 8 hours.
Luciferase reporter assay

The 3’UTR sequence was cloned and amplified into a dual-luciferase reporter vector. Cells were added in a 96-well plate and subsequently transfected with target genes and the luciferase reporter vector. Cells were added in a 96-well plate and subsequently transfected with target genes and the luciferase reporter vector. Cells were washed three times with cold PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cells were then incubated with blocking solution (0.2% powdered milk, 1% BSA, and 0.01% Triton X-100). After incubation for 30 minutes at room temperature, the cells were treated with rabbit anti-E-cadherin (Proteintech, no. 20874-1-AP, 1:100) and mouse anti-α-tubulin (Proteintech, no. 66184-1-lg, 1:100) overnight at 4°C. Cells were washed with PBS and staining with anti-rabbit Alexa Fluor 594–conjugated secondary antibody (Invitrogen, 1:1,000) and anti-mouse Alexa Fluor 488–conjugated secondary antibody (Invitrogen, 1:1,000) for 1 hour at 37°C. To visualize the nuclei, cells were stained with DAPI (4, 6-diamidino-2-phenylindole). The fluorescence images were examined using an ultrahigh resolution laser scanning confocal microscope (Leica TCS SP8 STED). Manders’ overlap coefficient (R), indicating an actual overlap of the signals, was used to analyze the degree of colocalization.

Immunofluorescence assay

HCC cells were seeded at a density of 7 × 10^4 per well in 24-well plates. Cells were transfected for 48 hours with SIRT6 overexpression vector or the control vector as previously described. Cells were washed three times with cold PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cells were then incubated with blocking solution (0.2% powdered milk, 1% BSA, and 0.01% Triton X-100). After incubation for 30 minutes at room temperature, the cells were treated with rabbit anti-E-cadherin (Proteintech, no. 20874-1-AP, 1:100) and mouse anti-α-tubulin (Proteintech, no. 66184-1-lg, 1:100) overnight at 4°C. Cells were washed with PBS and staining with anti-rabbit Alexa Fluor 594–conjugated secondary antibody (Invitrogen, 1:1,000) and anti-mouse Alexa Fluor 488–conjugated secondary antibody (Invitrogen, 1:1,000) for 1 hour at 37°C. To visualize the nuclei, cells were stained with DAPI (4, 6-diamidino-2-phenylindole). The fluorescence images were examined using an ultrahigh resolution laser scanning confocal microscope (Leica TCS SP8 STED). Manders’ overlap coefficient (R), indicating an actual overlap of the signals, was used to analyze the degree of colocalization.

Luciferase reporter assay

The 3’UTR sequence was cloned and amplified into a dual-luciferase reporter vector. Cells were added in a 96-well plate and subsequently transfected with target genes and the luciferase reporter vectors. After 24 hours, Luciferase Assay Reagent II and Luciferase Assay Substrate mixture were added into the lysed cells. At last, the fluorescence activity was immediately assessed on standard instrument.

Animal models assay

BALB/c male mice (aged 5 weeks, weight 13–15 g) were purchased from SLAC laboratory animal company (SCXK-2007-004) and raised in the specific pathogen-free environment. The mice were also divided into four groups (Hep3B-NC group, Hep3B-SIRT6 group, MHCC-97H-shCtrl group, MHCC-97H-shSIRT6-1 group and MHCC-97H-shSIRT6-2 group; 6 mice in each group). We injected the indicated cells into the lateral tail vein of the nude mice. The mice were sacrificed after 4 weeks. The number of nodules in the lung was counted and then the tumor tissues were stained with eosin and hematoxylin.

Ethics approval

The Ethics Committee of the Medical School of Xi’an Jiaotong University reviewed and approved the study, and written informed consent was obtained from each participant at each examination phase. The study complied with the principles of the Helsinki Declaration. The animal experiments carried out in this study were approved by the Experimental Animal Ethical Committee of Xi’an Jiaotong University.

Statistical analysis

Statistical analyses were performed using SPSS, and the data are expressed as mean ± SE. Student t test was used to evaluate the difference between the 2 groups. The χ^2 test was used to assess the association between SIRT6 expression and HCC patient clinicopathologic parameters. The correlation protein expression in HCC tissues was assessed by Spearman correlation analysis. The Kaplan–Meier method (the log-rank test) was used to plot the survival curves. *, P < 0.05 with a two-sided test was expressed as statistically significant. All the experiments were repeated in triplicate.

Results

Aberrantly increased SIRT6 expression was associated with HCC metastasis

To confirm that SIRT6 was expressed and to evaluate its clinical significance in HCC patients, we performed assays using both datasets from The Cancer Genome Atlas (TCGA) and HCC clinical samples. We analyzed HCC RNA sequencing data collected from the TCGA public database and showed that SIRT6 expression was higher in HCC tissues (n = 375) than in normal tissues (n = 51; Fig. 1A, P < 0.01). To further examine the role of SIRT6 in HCC, we performed qRT-PCR and IHC assays using 120 HCC clinical samples. qRT-PCR results showed that SIRT6 expression was much higher in HCC tissues than in normal tissues (2.98 ± 0.19 vs. 4.57 ± 0.21, P < 0.05, Fig. 1B). In addition, SIRT6 expression in HCC tissues from HCC patients with metastasis was increased compared with that of patients without metastasis (3.59 ± 0.28 vs. 5.77 ± 0.23, P < 0.01, Fig. 1B). IHC assays also had similar results (Fig. 1C). Higher SIRT6 expression levels were significantly associated with microscopic vascular invasion (P < 0.001), larger tumor size (P = 0.010), and advanced TNM stage in HCC (P = 0.001, Supplementary Table S1). The Kaplan–Meier method (using the log-rank test) suggested that lower SIRT6 expression was associated with increased OS and disease-free survival (DFS) compared with those of patients with higher levels of SIRT6 (P < 0.01, Fig. 1D). To further evaluate whether SIRT6 contributed to HCC metastasis, we collected samples from another 30 patients with HCC who underwent reoperation due to postoperative intrahepatic metastasis and compared the SIRT6 expression of the metastatic tissue with that of the primary tumor tissue. qRT-PCR results showed SIRT6 expression in metastatic tissue was considerably higher than in primary HCC tissue (5.69 ± 0.19 vs. 4.57 ± 0.21, P = 0.01, Fig. 1E). IHC assays also supported the results (Fig. 1F).

The effects of SIRT6 on HCC migration and invasion in vitro and in vivo

We further examined SIRT6 expression and function in HCC cells. Consistent with our findings in HCC tissues, Western blot results confirmed that SIRT6 levels were markedly increased in HCC cells compared with those of the immortalized normal human liver cell line LO2. The expression of SIRT6 was the lowest in Hep3B cells and was the highest in MHCC-97H cells (Fig. 2A). We transfected either SIRT6 (SIRT6 group) or control vectors (NC group) into Hep3B and LO2 cells (Fig. 2B), while we stably knocked down SIRT6 in MHCC-97H cells with two different effective lentiviruses (shSIRT6-1 and shSIRT6-2 group). Transwell assay showed that the migration and invasion capability of Hep3B and LO2 cells overexpressing SIRT6 were enhanced considerably compared with those of controls. In contrast, knocked down SIRT6 in MHCC-97H cells led to opposite results (P < 0.01, Fig. 2C).
We further established an HCC xenograft model of pulmonary metastasis in nude mice to evaluate the effect of SIRT6 on HCC metastasis in vivo. We found that SIRT6 overexpression in Hep3B cells (SIRT6 group) resulted in significantly increased lung metastatic capability compared with those transfected with the control vectors cells (NC group), while SIRT6 knockdown (shSIRT6-1 group, shSIRT6-2 group) markedly decreased lung metastasis ($P < 0.05$, Fig. 3A) compared with that of controls (shCtrl group). We further performed an IHC assay to analyze SIRT6, Ki67, and E-cadherin expression levels in the HCC xenograft samples. Figure 3B shows that SIRT6 expression levels were increased in the tumor tissues of the SIRT6 group compared with the NC group, while SIRT6 expression levels were markedly decreased in these tumor tissues of the SIRT6 knockdown group compared with that in the shCtrl group. We also found that Ki67 expression in the tumor tissues of the xenograft model was increased or decreased following SIRT6 overexpression or knockdown, suggesting SIRT6 could promote HCC cells proliferation capacity, while E-cadherin expression was significantly decreased in the metastasis xenograft sample.
samples of the SIRT6 group. Compared with that in the shCtrl group, E-cadherin expression was increased in the shSIRT6-1 and shSIRT6-2 group (Fig. 3B), suggesting that SIRT6 could inhibit E-cadherin expression in HCC. In short, these data revealed that SIRT6 promoted HCC metastasis in vitro and in vivo.

SIRT6 promoted the EMT of HCC

To explore whether SIRT6 promoted HCC cell invasion and migration by modulating the EMT of HCC, we examined EMT-associated markers in HCC cells using Western blot analysis. As expected, significantly downregulated E-cadherin was observed in Hep3B and LO2 cells after SIRT6 was upregulated. An increased vimentin and N-cadherin expression could be observed after SIRT6 was upregulated in Hep3B and LO2 cells, but the degree of change of vimentin or N-cadherin expression was not as significant as that of E-cadherin (Fig. 4A). In MHCC-97H cells, downregulated SIRT6 caused opposite results (Fig. 4A). We further performed an IHC assay to compare the expression levels of E-cadherin, vimentin, and N-
SIRT6 promoted HCC cell growth and metastasis in vivo. A, Nude mouse tumor cell metastasis assay. Increased SIRT6 promoted HCC xenograft tumor growth (*, P < 0.01), while decreased SIRT6 inhibited the lung metastatic capability of HCC cells (**, P < 0.01). Photomicrograph of HE-stained lung tissues that showed a metastasized HCC cell mass (magnification, ×200). B, IHC assay showed SIRT6, Ki67, and E-cadherin expression in the xenograft tumor tissue in different groups (magnification, ×200). SIRT6 group: Hep3B cells transfected with SIRT6 overexpression vector; NC group: Hep3B cells transfected with control vector. shCtrl group: MHCC-97H cells transfected with lentivirus negative control; shSIRT6-1 and shSIRT6-2: MHCC-97H transfected with two different effective lentivirus particles targeting SIRT6.

SIRT6 promoted E-cadherin degradation in HCC via the lysosomal pathway

The reduction of E-cadherin is a pivotal process of EMT, and our results showed that SIRT6 overexpression reduced E-cadherin expression in HCC cells. Furthermore, Spearman correlation analysis showed that the association between SIRT6 and N-cadherin or vimentin was weak (r = 0.247 and r = 0.284, respectively), whereas the association between SIRT6 and E-cadherin was stronger (r = −0.777). Thus, we speculated that SIRT6 may mainly induce EMT by promoting the degradation of E-cadherin. A SIRT6 vector was transfected into Hep3B cells at concentrations ranging from 2.5 to 10.0 μg for 48 hours, and the expression levels of SIRT6 and E-cadherin were determined using both qRT-PCR and Western blot assays. The results showed that both mRNA and protein expression of SIRT6 increased as the concentration of the vector increased (P < 0.01, Fig. 5A and B). The qRT-PCR assay results suggested that E-cadherin mRNA expression did not change markedly following the increase of SIRT6 expression (Fig. 5B). However, Western blot assay showed that E-cadherin protein expression decreased as the SIRT6 vector concentration increased (P < 0.01, Fig. 5A).

To confirm these results, we tested the expression level of SIRT6 and E-cadherin at different time points from 0 to 72 hours after transfection with the SIRT6 vector at fixed concentrations (10 μg) into Hep3B cells (Fig. 5C–E). Both mRNA and protein expression of SIRT6 was observed to gradually increase with the extension of time following transfection with the SIRT6 vector (Fig. 5C and F). In contrast, there was an obvious trend of decreased E-cadherin protein expression with increasing SIRT6 (Fig. 5C). However, the E-cadherin mRNA expression was not markedly changed (Fig. 5F). These data suggested that SIRT6 influenced E-cadherin expression by regulating protein degradation in HCC.

The lysosomal and proteasome-based proteolytic pathways are two pathways that are mainly responsible for protein degradation. To further uncover the mechanisms of the SIRT6-mediated inhibition of E-cadherin protein expression, we treated CQ (chloroquine, lysosome function inhibitor) and MG132 (proteasome inhibitor) into Hep3B cells half an hour after transfection with SIRT6 vector. Western blot assay showed that CQ could markedly reduce the degradation of E-cadherin that was caused by rising SIRT6 expression in Hep3B cells (P < 0.01, Fig. 5D and G). However, MG132 did not influence the degradation of E-cadherin that was caused by rising SIRT6.
Figure 4. SIRT6 promoted the EMT of HCC cells. A, Western blot analysis of EMT related protein expression following the modulation of SIRT6 expression in HCC cells and the immortalized normal human liver cell line LO2. GAPDH was used as an internal control (**, P < 0.05). B, IHC of E-cadherin, N-cadherin, and vimentin comparing tissues with high SIRT6 levels and those with low SIRT6 levels. C, Left, SIRT6 expression negatively correlates with E-cadherin expression (Spearman r = -0.777, P < 0.001); middle, SIRT6 expression positively correlates with N-cadherin expression (Spearman r = 0.247, P = 0.007); right, SIRT6 expression positively correlates with vimentin expression (Spearman r = 0.284, P = 0.002). SIRT6 group: Cells transfected with SIRT6 overexpression vector; NC group: Cells transfected with control vector. shCtrl group: MHCC-97H cells transfected with lentivirus negative control; shSIRT6-1 and shSIRT6-2: MHCC-97H transfected with two different effective lentivirus particles targeting SIRT6.
by the rising expression of SIRT6 in HCC cells \( (P < 0.01, \text{Fig. } 5 \text{E and G}) \). Thus, the results indicated that SIRT6 was involved in the degradation of E-cadherin that occurred via the lysosomal pathway.

SIRT6 promoted the autophagic degradation of E-cadherin by deacetylating Beclin-1

Protein degradation that occurs through the lysosome mainly depends on the endocytic pathway or the autophagic pathway.
Beclin-1 is a well-known autophagy marker and regulator, and Beclin-1 acetylation inhibits autophagy by suppressing autophagosome maturation (27, 28). SIRT6 is an NAD⁺-dependent deacetylase that targets a variety of proteins to regulate cellular processes and activities. Therefore, we hypothesized that SIRT6 inhibited E-cadherin autophagic degradation by deacetylating Beclin-1.

To determine whether SIRT6 functions as a deacetylase of Beclin-1, we performed an immunoprecipitation assay and used an anti-acetylated lysine antibody to evaluate the effect of SIRT6 on Beclin-1 acetylation. We transfected two effective siRNAs targeting different sequences of SIRT6 to knock down SIRT6 in Huh7 cells and found that decreased SIRT6 markedly increased Beclin-1 acetylation (P < 0.05, Fig. 6A). Moreover, we confirmed these results in Hep3B cells. We also performed an immunoprecipitation assay to measure the acetylated Beclin-1 levels in Hep3B cells stably expressing NC vector (NC group) or Flag-SIRT6 (Flag-SIRT6 group) using an anti-acetylated lysine antibody. The results showed that increased SIRT6 significantly reduced Beclin-1 acetylation (P < 0.05, Fig. 6B). These data suggested that SIRT6 could deacetylate Beclin-1; thus, SIRT6 may stimulate autophagy in HCC cells by inducing deacetylation of Beclin-1.

To explore the role of SIRT6 in the regulation of E-cadherin degradation via autophagy, we assessed the expression of LC3-II/LC3-I (autophagy marker) and P62 (autophagy degradation marker) in HCC cells in which SIRT6 was increased or decreased. The Western blot results showed that increased SIRT6 in Hep3B cells markedly upregulated LC3-II/LC3-I and downregulated E-cadherin and P62 (P < 0.05, Fig. 6C). Compared with control cells (Ctrl group), SIRT6 was decreased in Huh7 cells (shSIRT6-1 and shSIRT6-2 group), which resulted in the significant upregulation of LC3-II/LC3-I and downregulation of E-cadherin and P62 (P < 0.05, Fig. 6C). These results demonstrated that the regulatory function of SIRT6 for E-cadherin depended on the autophagic degradation pathway. To further study whether autophagic degradation contributed to the inhibition of E-cadherin in HCC, we performed fluorescent assay to assess whether E-cadherin bound to P62 and lysosome-associated membrane protein type 2A (LAMP2A). To observe the colocalization of proteins and fluorescence, and transfected the SIRT6 overexpression vector (SIRT6 group) or control vector (NC group) into Hep3B cells. The Western blot assay showed SIRT6 overexpression markedly increased SIRT6 expression in Hep3B cells (P < 0.01, Supplementary Fig. S1A). As shown in Supplementary Fig. S1B, the degree of E-cadherin (red)/p62 (green) co-localization were dramatically increased in Hep3B cells overexpressing SIRT6 (SIRT6 group) compared with NC group (Supplementary Fig. S1B). LAMP2A was well known as the main characteristic of autophagy. Therefore, we also analyzed degree of E-cadherin (red)/LAMP2A (green) co-localization in HCC cells. As shown in Supplementary Fig. S1B, the colocalization rate of E-cadherin and LAMP2A was also increased upon the upregulation of SIRT6 in Hep3B cells. These results suggested that the autophagic degradation contributed to regulatory function of SIRT6 for E-cadherin.

Recent research has revealed acetylation sites on Beclin-1 at Lys430 and Lys437, and dual mutation of Beclin-1 (mutation of lysines 430 and 437 to arginines, 2KR) leads to the inhibition of acetylation (29). To more definitively uncover the role of SIRT6 in the regulation of autophagic degradation, wild-type (WT Beclin-1) or 2KR-mutant Beclin-1 (2KR Beclin-1) vectors were transfected into HCC cells. Decreased p62 expression would indicate that autophagic degradation was induced by Beclin-1 deacetylation in HCC cells. Our results showed that p62 decreased following transfection with the 2KR Beclin-1, further confirming that Beclin-1 deacetylation could induce autophagic degradation (Fig. 6D). Furthermore, our data showed that increased SIRT6 inhibited p62 expression in Hep3B cells transfected with WT Beclin-1, while p62 expression was not further downregulated in Hep3B cells transfected with 2KR Beclin-1, even when SIRT6 expression increased (Fig. 6D). Furthermore, the changes in E-cadherin and p62 expression were consistent (Fig. 6D). Compared with Hep3B cells that were transfected with WT Beclin-1, Hep3B cells that were transfected with 2KR Beclin-1 exhibited enhanced Hep3B cell migration and invasion capability (Fig. 6E). Upregulated SIRT6 could promote Hep3B migration and invasion even after transfection with the WT Beclin-1 vector but could not enhance Hep3B migration and invasion after transfection with the 2KR Beclin-1 vector (Fig. 6E). These findings indicated that SIRT6 promoted the autophagic degradation of E-cadherin and this process was dependent upon the deacetylation of Beclin-1.

SIRT6 promoted invasion, migration, and EMT of HCC via the autophagic degradation of E-cadherin.

To verify that SIRT6 promoted invasion and migration by regulating E-cadherin, we transfected an E-cadherin overexpression vector into Hep3B cells that were overexpressing SIRT6. Transwell assays showed that infection with pCMV-E-cadherin vector counteracted the promotional effect of SIRT6 expression on the migration and invasion capacity of Hep3B cells (P < 0.01, Supplementary Fig. S1C and S1D). Deletion of Atg7 has been shown to block autophagy. To confirm that autophagic degradation was necessary for the SIRT6-mediated inhibition of E-cadherin, we generated Hep3B cells with autophagic inhibition by knocking down Atg7 following SIRT6 overexpression. Transwell assays suggested that decreased Atg7 counteracted the promotional effect of SIRT6 expression over the migration and invasion capacity of Hep3B cells (P < 0.01, Fig. 7A). A Western blot assay showed that E-cadherin was upregulated following Atg7 downregulation (Fig. 7C). To help confirm the results, we transfected another effective siRNA targeting different sequences of Atg7 to knock down Atg7 in Hep3B cells with overexpressed SIRT6 and found similar results (P < 0.05, Supplementary Fig. S1C and S1D).

To confirm whether SIRT6 promoted EMT by regulating E-cadherin autophagic degradation, we treated Hep3B cells that were overexpressing SIRT6 with CQ. Transwell assays suggested that CQ counteracted the promotional effect of SIRT6 overexpression on the migration and invasion capacity of Hep3B cells (Fig. 7D). Our findings suggested that SIRT6 induced metastasis and EMT of HCC by promoting autophagic degradation.

SIRT6 promoted N-cadherin and Vimentin expression via deacetylating FOXO3a.

Many Sirtuin family members, such as SIRT1, SIRT2, and SIRT3, are involved in the regulation of Forkhead Box Class O 3a (FOXO3a), which plays an important role to inhibit metastasis and EMT in human cancers. Thus, we performed further assays to evaluate whether SIRT6 contributed to regulate FOXO3a like other SIRTs family members. Western blot assay results showed
Figure 6.
SIRT6 promoted autophagic degradation of E-cadherin by deacetylating Beclin-1. A, MHCC-97H cells were transfected with control vector or two effective SIRT6-siRNAs for 48 hours. The protein was extracted and immunoprecipitated using an anti-acetylated lysine antibody. AC, acetylated lysine. B, Hep3B cells were transfected with a control vector or Flag-SIRT6 for 48 hours. The protein was extracted and immunoprecipitated using an anti-acetylated lysine antibody. C, p62, E-cadherin and LC3-II/I protein expression was assessed by Western blotting in Hep3B cells transfected with a SIRT6 vector (SIRT6 group) or an NC vector (NC group) and in MHCC-97H cells treated with two different SIRT6-shRNA (shSIRT6-1 and shSIRT6-2 group) or control sh-RNA (Ctrl group). D, Hep3B cells overexpressing SIRT6 or expressing an NC vector were transfected with WT-Beclin-1 or 2KR-Beclin-1 for 48 hours, and protein expression was measured by Western blotting. E, Transwell assay showed the migration and invasion capability of Hep3B cells stably expressing a SIRT6 vector or an NC vector were transfected with WT-Beclin-1 or 2KR-Beclin-1. SIRT6 group: Hep3B cells transfected with SIRT6 overexpression vector; NC group: Hep3B Cells transfected with control vector; shCtrl group: MHCC-97H cells transfected with lentivirus negative control; shSIRT6-1 and shSIRT6-2: MHCC-97H transfected with two different effective lentivirus particles targeting SIRT6. si-control group: MHCC-97H cells transfected with control siRNA; siRNA-1 and siRNA-2: MHCC-97H transfected with two different effective SIRT6-siRNAs.
that downregulation of SIRT6 in HCC cells resulted in the upregulation of FOXO3a expression (Supplementary Fig. S2A). As SIRT6 is an NAD-dependant deacetylase, we carried out an immunoprecipitation experiment to test whether SIRT6 regulated the acetylation level of FOXO3a. The Flag-FOXO3a was cotransfected with si-SIRT6s into MHCC-97H cells. FOXO3a proteins were pulled down from cell lysates with anti-Flag beads, and the acetylation levels were detected by an anti-acetylated lysine antibody (Supplementary Fig. S2B). The relative acetylation rate of FOXO3a was statistically analyzed compared with the intensity of total FOXO3a. As shown in Supplementary Fig. S2B, the acetylation level of FOXO3a in the downregulated SIRT6 HCC cells was significantly increased, suggesting that SIRT6 contributed to the deacetylation of FOXO3a. In addition, luciferase assay revealed that SIRT6 suppressed FOXO3a promoter activity (Supplementary Fig. S2C). We next wanted to know whether FOXO3a is essential for function of SIRT6 on N-cadherin and Vimentin. We transfected Hep3B cells overexpressing SIRT6 with a FOXO3a overexpression vector (SIRT6-FOXO3 cells), and the results showed that compared with Hep3B cells co-transfected with SIRT6 control vector and FOXO3a control vector (NC cells), FOXO3a overexpression rescued the promotion function of SIRT6 on N-cadherin and Vimentin even if SIRT6 was overexpressed (Supplementary Fig. S2D). To further test whether the deacetylation activity of SIRT6 is required for its function of FOXO3a, N-cadherin and Vimentin regulation. We transfected two SIRT6 mutants (SIRT6S56Y, SIRT6H133Y) lacking deacetylation activity into HCC cells. The results showed FOXO3a, N-cadherin and Vimentin expression were not influenced by the SIRT6 mutants (Supplementary Fig. S2E). In a word, these data suggested SIRT6 promoted N-cadherin and Vimentin via deacetylating FOXO3a.

**Discussion**

Sirtuin family members play a crucial role in human cancer metastasis, which is the leading cause of death for patients with malignant tumors. However, no causal association has been established between SIRT6 and metastasis in HCC. Furthermore, the role that SIRT6 plays in cancer is unclear, especially in HCC.
Here, we used data from the TCGA database and HCC clinical samples, a xenograft model of pulmonary metastasis and in vitro experiments to perform a comprehensive analysis. For the first time, we showed that SIRT6 promoted the EMT of HCC by enhancing E-cadherin autophagic degradation via Beclin-1 deacetylation. These data provide valuable information regarding the role of SIRT6 in tumorigenesis, which could enhance the understanding of the role of SIRT6 in HCC and the molecular mechanisms underlying HCC metastasis.

Recent reports focused on SIRT6 in HCC tumorigenesis presented conflicting results. Our analysis from both the TCGA public datasets and HCC clinical samples consistently suggested that there were abnormal increases in the expression of SIRT6 in HCC. Increased SIRT6 was considerably associated with poor clinical prognosis and shorter DFS after surgery. Elevated SIRT6 was shown to promote HCC metastasis both in vivo and in vitro, strongly suggesting that SIRT6 acts as oncogene in HCC. Our results support several previous studies showing that SIRT6 functions as an oncogene during HCC development. Importantly, we further showed that elevated SIRT6 expression had a positive relationship with HCC metastasis. However, certain studies have suggested that SIRT6 acted as a tumor suppressor in HCC. Min L and colleagues reported that dysplastic liver nodules were specifically characterized by decreased c-Fos-SIRT6 levels (30). SIRT6 was also suggested to inhibit HCC cell proliferation by deacetylating nuclear pyruvate kinase M2 (31). We speculate these conflicting results might be due to the diversity of cellular signaling pathways and experimental conditions.

EMT has been proposed to be a pivotal mechanism for cancer cell invasion and metastasis (32, 33). In the current study, our results showed that SIRT6 promotes HCC EMT. Downregulation of E-cadherin expression is frequently the first step of cancer cell metastasis and is a fundamental molecular mechanism of EMT. Deletion or mutation of genes, transcriptional inhibition, or autophagic degradation can contribute to repression of E-cadherin (34, 35). Downregulated E-cadherin and upregulated vimentin or N-cadherin induced by increased SIRT6 was observed in our data. However, Spearman correlation analysis suggested that the connections between SIRT6 and N-cadherin or vimentin were weak, whereas the connection between SIRT6 and E-cadherin was stronger. Infection with the E-cadherin overexpression vector counteracted the promotional effect of SIRT6 on the migration and invasion capacity of HCC cells. Thus, we focused on SIRT6-induced E-cadherin degradation, which provided a logical explanation and further evidence for a mechanism in which SIRT6 overexpression is positively correlated with HCC metastasis and poor patient prognosis.

In the current study, we found that SIRT6 inhibited E-cadherin in a manner dependent on autophagic degradation. This result is another interesting part of our results that supports recent research that helped confirm the promotional effect of autophagy on HCC EMT. Moreover, we found that autophagy inhibitor or deletion of Atg7 (which causes autophagy inhibition at the genetic level) could rescue the E-cadherin expression that was inhibited by SIRT6. These results suggest that autophagy inhibitors might serve as potential therapies for HCC metastasis. Autophagy, which acts as a vital regulator of the maintenance of cellular homeostasis by degrading damaged proteins and cytoplasmic components in the lysosomes, is involved in tumor invasion and EMT (24, 25). However, the relationship between autophagy and cancer metastasis, as well as EMT, has not full supported (36). Autophagy was reported to promote cancer metastasis because of its vital role in survival, which protected cancer cells against diverse cellular damage (37). Li J and colleagues also reported that autophagy promoted HCC cell invasion via TGFβ/Smad3 (38). On the other hand, Zhu and colleagues reported that suppression of autophagy promoted nasopharyngeal carcinoma EMT (39). We speculate that whether autophagy serves as a promotional factor or an inhibitory factor in metastasis and cancer EMT may be dependent on the tumor and tissue type.

Although the autophagic process is particularly complex, including autophagosome formation, autophagosome maturation, and degradation (40), the posttranslational modification of Beclin-1 can intervene at each vital autophagy step. Therefore, modifications of Beclin-1 are crucial for the autophagic pathway and may result in the promotion or inhibition of the autophagic response. We performed an immunoprecipitation assay in MHCC-97H cells transfected with two effective siRNAs against SIRT6 and Hep3B cells treated with the SIRT6 vector, and the results suggested that SIRT6 deacetylated Beclin-1. This result is plausible. Similar to other SirTuin family members, SIRT6 works as an NAD+-dependent deacetylase that targets a variety of proteins to regulate cellular processes and activities (41). Although a number of studies have focused on the phosphorylation of Beclin-1 (42), the acetylation and deacetylation of Beclin-1 has attracted widespread attention in recent years. Lee, I H and colleagues uncovered that Arg5, Arg7, Arg8, and Arg12 acetylation induced autophagy inhibition (43). Robert T and colleagues confirmed these conclusions by treating cells with deacetylase inhibitors and observing the promotion of autophagy (44). More importantly, another SirTuin family member, SIRT1, was recently confirmed to promote autophagy by deacetylating Beclin-1 (45). Here, we showed that SIRT6 has a similar function in the deacetylation of Beclin-1. These data help to explain the mechanisms underlying the regulatory effects of autophagy on EMT.

However, it is necessary to discuss the mechanisms that need to be further investigated in the future. Beclin-1 phosphorylation can regulate autophagy by directly inhibiting Beclin-1 complex formation (22). Whether dephosphorylation of Beclin-1 by interacting factors aids or is necessary for deacetylation of Beclin-1 by SIRT6 needs to be examined. However, our data showed that increased SIRT6 did not influence E-cadherin expression or EMT in HCC cells transfected with 2KR Beclin-1, which led to the inhibition of autophagy. Furthermore, an autophagy inhibitor counteracted the enhancement of invasion, migration, EMT capacity and the inhibition of E-cadherin expression that was induced by SIRT6 overexpression in HCC cells. These results provide conclusive evidence that SIRT6 promotes the autophagic degradation E-cadherin via deacetylation of Beclin-1 and promotes HCC cell invasion, migration and EMT. Thus, this study has certain significance. In addition, although the correlation between SIRT6 and N-cadherin or Vimentin are not as obvious as that of E-cadherin, we still tried to explore the mechanism underlying the function of SIRT6 on N-cadherin and Vimentin. Our results suggested SIRT6 contributed to regulate FOXO3a like other SirTuin family members, which plays an important role to inhibit metastasis and EMT in human cancers, and the regulation is essential for function of SIRT6 on N-cadherin and Vimentin. However, the mechanism is still to be further studied in the future research work.
In summary, the current study showed that SIRT6 was increased in HCC and was significantly associated with poor prognosis. SIRT6 functioned as an oncogene in HCC by promoting HCC cell invasion, migration and EMT. These effects were dependent on the induction of the autophagic degradation of E-cadherin. To the best of our knowledge, this is the first study to establish the relationship between SIRT6 and HCC metastasis and the first study to demonstrate that SIRT6 induced the autophagic degradation of E-cadherin via deacetylation of Beclin-1. Our findings suggested that SIRT6 could serve as a promising target for the treatment of HCC metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: L.L. Han
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Jia, F. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Jia
Writing, review, and/or revision of the manuscript: L.L. Han, C. Huang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.L. Han, C. Huang
Other (helped complete animal experiments and some statistical analysis): I. Jia
Other (helped in the revision of the manuscript and provided some material support): C. Huang

References
1. Lafaro KI, Demirjian AN, Pawlik TM. Epidemiology of hepatocellular carcinoma. Surg Oncol Clin N Am 2015;24:1–17.
2. Siegel R, Miller K, Jemal A. Cancer statistics, 2017. CA Cancer J Clin 2017; 67:7–30.
3. Nieto MA, Huang RY, Jackson RA, Thiery JP. EMT: 2016. Cell 2016;166: 21–45.
4. Creighton CJ, Gibbons DL, Kurie JM. The role of epithelial-mesenchymal transition programming in invasion and metastasis: a clinical perspective. Cancer Manag Res 2013;5:167–95.
5. Wang Y, He J, Liao M, Hu M, Li W, Ouyang H, et al. An overview of Sirtuins effects as potential therapeutic target: structure, function and modulators. Crit Rev Biochem Mol Biol 2015;50:242–55.
6. Byles V, Zhu L, Lovaas JD, Chmilewski LK, Wang J, Faller DV, et al. SIRT1 induces EMT by cooperating with EMT transcription factors and enhances prostate cancer cell migration and metastasis. Oncogene 2012;31: 4619–29.
7. Gonzalez Herrera KN, Lee J, Haigis MC. Intersections between mitochondrial sirtuin signaling and tumor cell metabolism. Crit Rev Biochem Mol Biol 2015;50:242–55.
8. Li H, Feng Z, Wu W, Li J, Zhang J, Xia Y, et al. SIRT3 regulates cell proliferation and apoptosis related to energy metabolism in non-small cell lung cancer cells through deacetylation of NMNAT2. Int J Oncol 2013;43:1240–30.
9. Sun H, Huang D, Liu G, Jian F, Zhu J, Zhang L. SIRT4 acts as a tumor suppressor in gastric cancer by inhibiting cell proliferation, migration, and invasion. Oncos Targets Ther 2010;11:3959–68.
10. Li W, Zhu D, Qin S. SIRT7 suppresses the epithelial-to-mesenchymal transition in oral squamous cell carcinomas by promoting SmAD4 deacetylation. J Exp Clin Cancer Res 2018;37:148.
11. Huang Z, Zhao J, Deng W, Chen Y, Wang J, Song K, et al. Indentification of a cellularly active SIRT6 allosteric activator. Nat Chem Biol 2018;14: 1118–26.
12. Cea M, Cagnetta A, Adami S. Evidence for a role of the histone deacetylase SIRT6 in DNA damage response of multiple myeloma cells. Blood 2016; 127:1138–50.
13. Gong J, Wang H, Lou W. Associations of sirtuins with clinicopathological parameters and prognosis in non-small cell lung cancer. Cancer Manag Res 2018;10:3341–56.
14. Kugel S, Sebastian C, Fitalmant I, Ross KN, Saha SK, Jain E, et al. SIRT6 suppresses pancreatic cancer cell invasion through control of Lin28b. Cell 2016;165: 1401–15.
15. Ming M, Han W, Zhao B, Sundaresan NR, Deng CX, Gupta MP, et al. SIRT6 promotes COX-2 expression and acts as an oncogene in skin cancer. Cancer Res 2014;74:5925–33.
30. Min L, Ji Y, Bakiri L, Qiu Z, Cen J, Chen X, et al. Liver cancer initiation is controlled by AP-1 through SIRT6-dependent inhibition of survivin. Nat Cell Biol 2012;14:1203–11.

31. Bhardwaj A, Das S. SIRT6 deacetylates PKM2 to suppress its nuclear localization and oncogenic functions. Proc Natl Acad Sci U S A 2016;113:538–47.

32. Oka H, Shiozaki H, Kobayashi K, Inoue M, Tahara H, Kobayashi T, et al. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer Res 1993;53:1696–701.

33. Schipper JH, Frixen UH, Behrens J, Unger A, Jahnke K, Birchmeier W. E-Cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Res 1991;51:6328–37.

34. Serrano-Gomez SJ, Maziveyi M, Alahari SK. Regulation of epithelial-mesenchymal transition through epigenetic and post-translational modifications. Mol Cancer 2016;15:18.

35. Liu H, Ma Y, He HW, Zhao WJ, Shao RC. SphK1 (sphingosine kinase 1) induces epithelial-mesenchymal transition by promoting the autophagy-linked lysosomal degradation of CDH1/E-cadherin in hepatoma cells. Autophagy 2017;13:900–13.

36. Kneifel CM, Thorburn A, Debnath J. Autophagy and metastasis: another double-edged sword. Curr Opin Cell Biol 2010;22:241–5.

37. Tan YQ, Zhang J, Zhou G. Autophagy and its implication in human oral diseases. Autophagy 2017;13:225–36.

38. Li J, Yang R, Zhou Q, Wu Y, Shang D, Guo Y, et al. Autophagy promotes hepatocellular carcinoma cell invasion through activation of epithelial-mesenchymal transition. Carcinogenesis 2013;34:1343–51.

39. Zhu JF, Huang W, Yi HM, Xiao T, Li JY, Feng J, et al. Annexin A1-suppressed autophagy promotes nasopharyngeal carcinoma cell invasion and metastasis by PI3K/AKT signaling activation. Cell Death Dis 2018;9:1154.

40. Amaravadi RK, Yu D, Lu M, Bui T, Christophorou MA, Evan GI, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 2007;117:326–36.

41. Kardi Y, Naiman S, Amir G, Pestov V, Zinman G, Nahum L, et al. The sirtuin SIRT6 regulates lifespan in male mice. Nature 2012;483:218–21.

42. Maroni P, Bendinelli P, Rensi M, Matteucci E, Milan E, Desiderio MA. The autophagic process occurs in human bone metastasis and implicates molecular mechanisms differently affected by Rab5a in the early and late stages. Int J Mol Sci 2016;17:443.

43. Lee BH, Cao L, Mostoslavsky R, Lombard DB, Liu J, Bruns NE, et al. A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. Proc Natl Acad Sci U S A 2008;105:3374–9.

44. Robert T, Vanol F, Chioio I, Shubassi G, Bernstein KA, Rothstein R, et al. HDACs link the DNA damage response, processing of doublestrand breaks and autophagy. Nature 2011;471:74–9.

45. Sun T, Jiao L, Wang Y, Yu Y, Ming L. SIRT1 induces epithelial-mesenchymal transition by Promoting autophagic degradation of E-cadherin in melanoma cells. Cell Death Dis 2018;9:136.