

Sirt6 suppresses hepatocellular carcinoma cell growth via inhibiting the extracellular signal-regulated kinase signaling pathway

ZHI-GAO ZHANG and CHENG-YONG QIN

Department of Gastroenterology, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, P.R. China

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Abstract. Sirt6, a member of the mammalian sirtuin family, is a protein that is located in the nucleus and is an NAD⁺-dependent deacetylase important in the control of metabolic activity and genome stability. Recently, several studies have demonstrated the potential role of Sirt6 in tumor biology; however, the role of Sirt6 in hepatocellular carcinoma (HCC) remains unclear. In the present study, Sirt6 protein expression was found to be downregulated in human HCC tissue compared with adjacent normal tissue. Knockdown of Sirt6 promoted growth of the HepG2 HCC cell line, whereas overexpression of Sirt6 inhibited the growth of HepG2 cells. Overexpression of Sirt6 induced apoptosis in HepG2 cells, which was demonstrated by a terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay and cleaved caspase-3 immunoblotting. Furthermore, overexpression of Sirt6 decreased intracellular reactive oxygen species and superoxide anion levels. Finally, overexpression of Sirt6 inhibited phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), and blocking the ERK1/2 pathway with chemical-specific inhibitor U0126, attenuated the tumor suppressive effect of overexpression of Sirt6. Collectively, these data suggest that Sirt6 is a tumor suppressor in HCC cells and may be a promising therapeutic target in HCC.

Introduction

Hepatocellular carcinoma (HCC) is a challenging malignancy of global importance associated with high prevalence and mortality (1). Previously, HCC was predominant in undeveloped or less-developed countries; however, incidence of HCC has increased recently in developed regions, including Western

Europe, the United States and Japan (2-3). HCC has several noteworthy epidemiological features, including oncogene mutation, ethnic groups and the presence of several well-documented environmental potentially preventable risk factors, such as hepatitis C virus infection (4,5). Although there has been a growing understanding of the molecular mechanisms underlying hepatocarcinogenesis in recent years (1), the causes and biology of HCC are not yet fully understood. Moreover, the efficacies of current treatment for HCC are unsatisfactory (6) resulting in poor prognosis with a median survival time of four months (2).

Sirtuins, mammalian homologs of the yeast protein silent information regulator 2 (Sir2), are a unique subclass of deacetylases and mono-ADP-ribosyltransferases that use NAD⁺ as a cosubstrate (7). There are seven members of the sirtuin family in mammals (Sirt1-Sirt7) (7). These proteins influence a wide array of pathophysiological processes, including DNA repair, cell survival, stress responses, metabolic homeostasis and aging (7). Sirt1, the most studied member of the family, has been implicated in carcinogenesis and cancer progression, and is considered to be a therapeutic target (8). Sirt1 induces histone deacetylation and methylation, promoter CpG island methylation, transcriptional repression and deacetylation of tumor suppressor proteins (8). Sirt1 was reported to have a bidirectional effect on tumor initiation, progression and drug resistance; it can operate as a tumor suppressor or as an oncogenic factor, depending on the context and the study conditions (8). Sirt3 is another member of the sirtuin family, which preferentially localizes to mitochondria and is involved in mitochondrial energy production and substrate oxidation (9-10). Unlike Sirt1, accumulating evidence suggests that Sirt3 is a tumor suppressor in breast cancer (11), oral cancer (12) and HCC (13).

Sirt6 is a less-studied nuclear sirtuin member. Thus far, Sirt6 has been found to regulate glucose homeostasis in the liver (14) and maintain genome stability (15-16). In 2012, Min *et al* (17) showed that Sirt6-dependent inhibition of survivin contributed to activator protein-1 binding site-induced tumor suppression. Another group confirmed that deletion of Sirt6 *in vivo* increased the number, size and aggressiveness of tumors (18). However, evidence from other studies indicates that Sirt6 may be tumorigenic in pancreatic cancer (19) and breast cancer (20). Consequently, the exact role of Sirt6 in cancer is still being analyzed. In the present study,

Correspondence to: Professor Cheng-Yong Qin, Department of Gastroenterology, Provincial Hospital Affiliated to Shandong University, 324 Jingwu Weiqi Road, Jinan, Shandong 250021, P.R. China
E-mail: cyqin01@126.com

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the expression of Sirt6 in human HCC tissues and the potential role of Sirt6 in HCC were investigated.

Materials and Methods

Reagents. Antibodies against Sirt6 and β -actin were purchased from Sigma (St. Louis, MO, USA). Antibodies against cleaved caspase-3, phosphorylated extracellular signal-regulated kinase (ERK1/2), total-ERK1/2 and U0126 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). An immunofluorescence terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit was purchased from Promega Corporation (Madison, WI, USA). DAPI and dichlorofluorescein (DCF) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Enhanced chemiluminescence and protease/phosphatase inhibitors were purchased from Pierce (Rockford, IL, USA).

Human HCC tissue. Four pairs of HCC and matched normal adjacent tissue extracts were obtained from Chinese patients who underwent surgical resection for diagnosis and therapy in the Provincial Hospital Affiliated to Shandong University (Jinan, China). Samples were obtained after receiving informed consent according to an established protocol approved by the Ethics Committee of Shandong University (Jinan, China).

Cell culture. HepG2 human HCC cells and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) in 95% O₂ and 5% CO₂ (21).

Plasmid construction. The adenovirus expressing Sirt6 (Ad-Sirt6) and the control adenovirus expressing green fluorescent protein (Ad-GFP) were generated using the Adenoviral Expression system (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions as described previously (22). Mouse Sirt6 mRNA was extracted from mouse liver tissue using TRIzol (Invitrogen Life Technologies) and was reverse transcribed with AMV to obtain cDNA. The full length of the mouse Sirt6 cDNA was amplified using polymerase chain reaction (PCR) with specific primers. Full length PCR products of Sirt6 were then subcloned into the pacAd5 CMV-IRES vector (Cell Biolabs, Inc.). Next, pacAd5 CMV-IRES-Sirt6 and pacAd5 backbone vectors were linearized by *PacI* (New England Biolabs, Ipswich, MA, USA).

The short hairpin-RNA (shRNA)-induced RNA interference (RNAi) was achieved using the RAPAd[®] shRNA Adenoviral Expression system (Cell Biolabs, Inc.) according to the manufacturer's instructions. The nucleotide sequence for shRNA was designed using BLOCK-iT[™] RNAi Online Designer tool (Invitrogen Life Technologies). The following nucleotide sequence against human Sirt6 was used in this study: 5'-GGTCTGGCAGTCTTCCAGTGT-3'.

Generation of adenovirus. HEK293 cells were used to produce adenovirus. The purified linearized DNAs or plasmids were transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen Life Technologies). At 6 h after transfection,

the medium containing Lipofectamine 2000 was removed and novel medium was added. Adenovirus-containing HEK293 cells and media were harvested on the 6th day post-transfection. Viruses were released by three freeze/thaw cycles and stored at -80°C. For virus transfection, 30 μ l viral stock solution was added into culture medium (2 ml) of HepG2 cells for 6 h.

Cell proliferation assay. Cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used to detect cell proliferation as described previously (13,23). Ad-GFP and Ad-Sirt6-transfected cells (3×10^4) were seeded into 48-well plates and cultured overnight to allow attachment. On the second day, cells were serum-starved for 8 h, and FBS was added into medium. At 12, 24, 36 and 48 h, cells were incubated with 10 μ l CCK-8 solution for 2 h, and the optical density at 450 nm was analyzed (Tecan Ultra 384 reader; Tecan, Männedorf, Switzerland). Experiments were performed in duplicate.

TUNEL assay. TUNEL staining was performed as described previously (13,24). Cells were transfected with Ad-GFP or Ad-Sirt6 for 6 h. At 24 h after treatment, cells were incubated in TUNEL reaction buffer in a humidified chamber for 1 h at 37°C in the dark, then rinsed four times with phosphate-buffered saline (PBS) and incubated with DAPI (1 mg/ml) for 15 min. The stained cells were visualized using a fluorescence microscope (IX-71, Olympus, Tokyo, Japan). TUNEL-positive cells (green) were counted as apoptotic cells. Images were acquired digitally from a randomly selected pool of 10-15 fields under each condition (25).

Quantitative PCR analysis. qPCR analysis was performed on an OpticonDNA engine (MJ Research, Inc., St. Bruno, QC, Canada) using PrimerScript[®] RT Reagent kit (Takara Bio, Inc., Shiga, Japan) and normalized as described previously (23,26). The total RNA was extracted from human tissue using TRIzol (Invitrogen Life Technologies) and 10 μ g RNA was used for first strand cDNA synthesis. cDNA (100 ng) was amplified using primers as follows: Sense: 5'-CGCAGTACGTCCGAGACACAGT-3' and antisense: 5'-TTGGTAGCCAGCGGCAGGTT-3' for Sirt6; and sense: 5'-GCACTCTTCCAGCCTTCCTTCC-3' and antisense: 5'-CCGCCAGACAGCACTGTGT-3' for β -actin. The mRNA level of housekeeping gene β -actin served as a control.

Immunoblotting. Immunoblotting analyses of cell-extracts were performed as described previously (27-28). Human tissues or cells were lysed in 50 mM Tris-HCl (pH 7.5) and 1% SDS with protease/phosphatase inhibitor cocktail (Pierce), and then heated at 95°C for 10 min. Samples were subjected to 10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes (Millipore, Milford, MA, USA) at 100V for 90 min. Following blocking in 5% skimmed milk and PBS containing 0.1% Tween-20, membranes were incubated with primary antibodies (cleaved caspase-3 and Sirt6) followed by horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membranes were then detected using an enhanced chemiluminescence kit (Pierce).

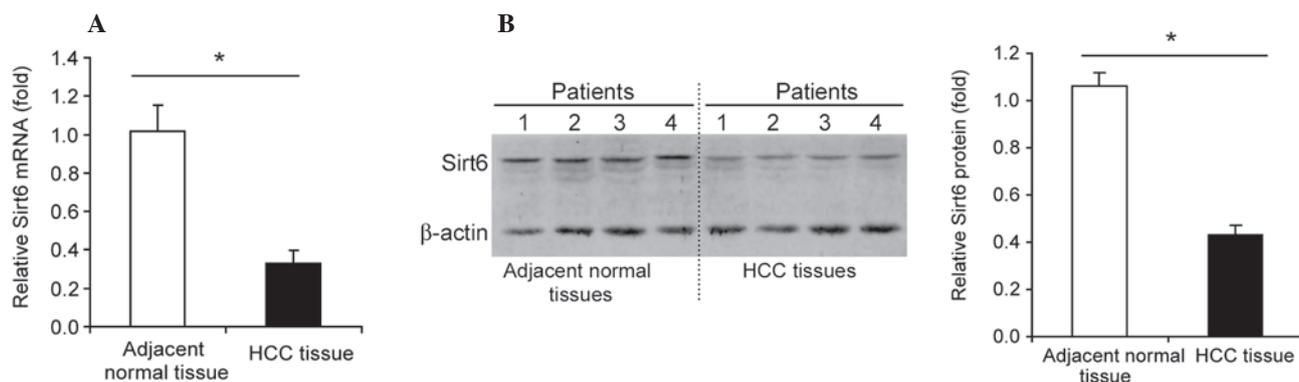


Figure 1. Downregulation of Sirt6 in human hepatocellular carcinoma (HCC) tissue. (A) The mRNA level of Sirt6 in human HCC tissue was measured by quantitative polymerase chain reaction analysis. (B) Immunoblotting and quantitative analysis of the expression of Sirt6 in human HCC tissue and normal adjacent tissue. * $P < 0.05$ versus normal tissue. Number of tissues=4.

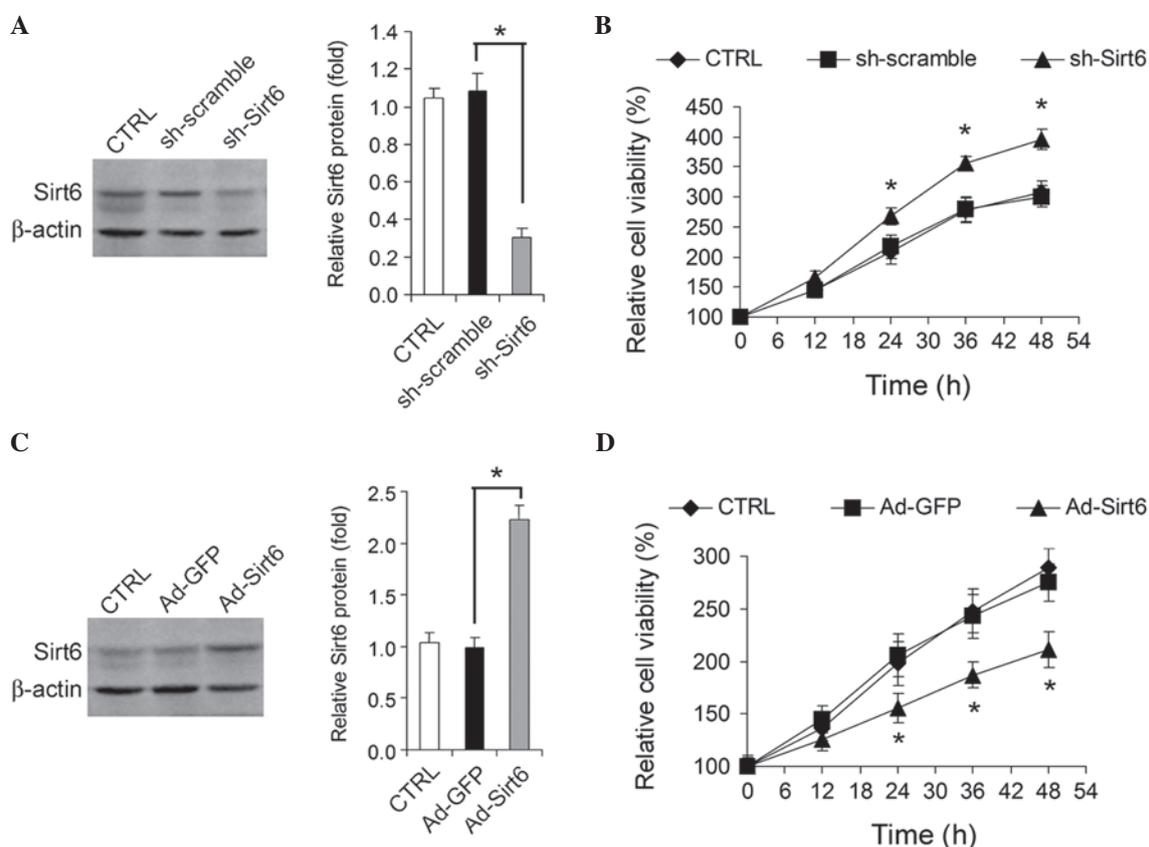


Figure 2. Modulation of Sirt6 affects hepatocellular carcinoma (HCC) cell growth. (A) Immunoblotting showing the short hairpin (sh)RNA-mediated knock-down of Sirt6 in HepG2 HCC cells and (B) cell growth curve of HepG2 HCC cells transfected with sh-scramble or sh-Sirt6. * $P < 0.05$ versus sh-scramble (vector control). Number of tissues=8. (C) Immunoblotting showing the adenovirus-mediated overexpression of Sirt6 in HepG2 HCC cells and (D) cell growth curve of HepG2 HCC cells transfected with adenovirus expressing-green fluorescent protein (Ad-GFP) or Ad-Sirt6. * $P < 0.05$ versus Ad-GFP (vector control). Number of tissues=8.

ROS measurement. A DCF assay was used for the quantification of intracellular ROS as previously described (29). Ad-GFP and Ad-Sirt6 transfected cells were plated onto 96-well plates (5×10^4 cells/well) and loaded with $100 \mu\text{M}$ DCF (Invitrogen Life Technologies) for 1 h at 37°C . Cells were subsequently washed using PBS buffer. Fluorescence was measured using a fluorescence microplate reader (Tecan) with an excitation filter of 485 nm and an emission filter at 530 nm.

Superoxide anion measurement. Ad-GFP- and Ad-Sirt6-transfected cells were seeded in 96-well plates and grown overnight. Following removal of the cell culture medium, the cells were washed with PBS, and $200 \mu\text{l}$ of $25 \mu\text{M}$ dihydroethidium (DHE) dissolved in PBS was added to each well for 1 h. The fluorescence (DHE ex/em: 530/620 nm) was measured using a multiwell plate reader (Tecan) for 20 min at 37°C .

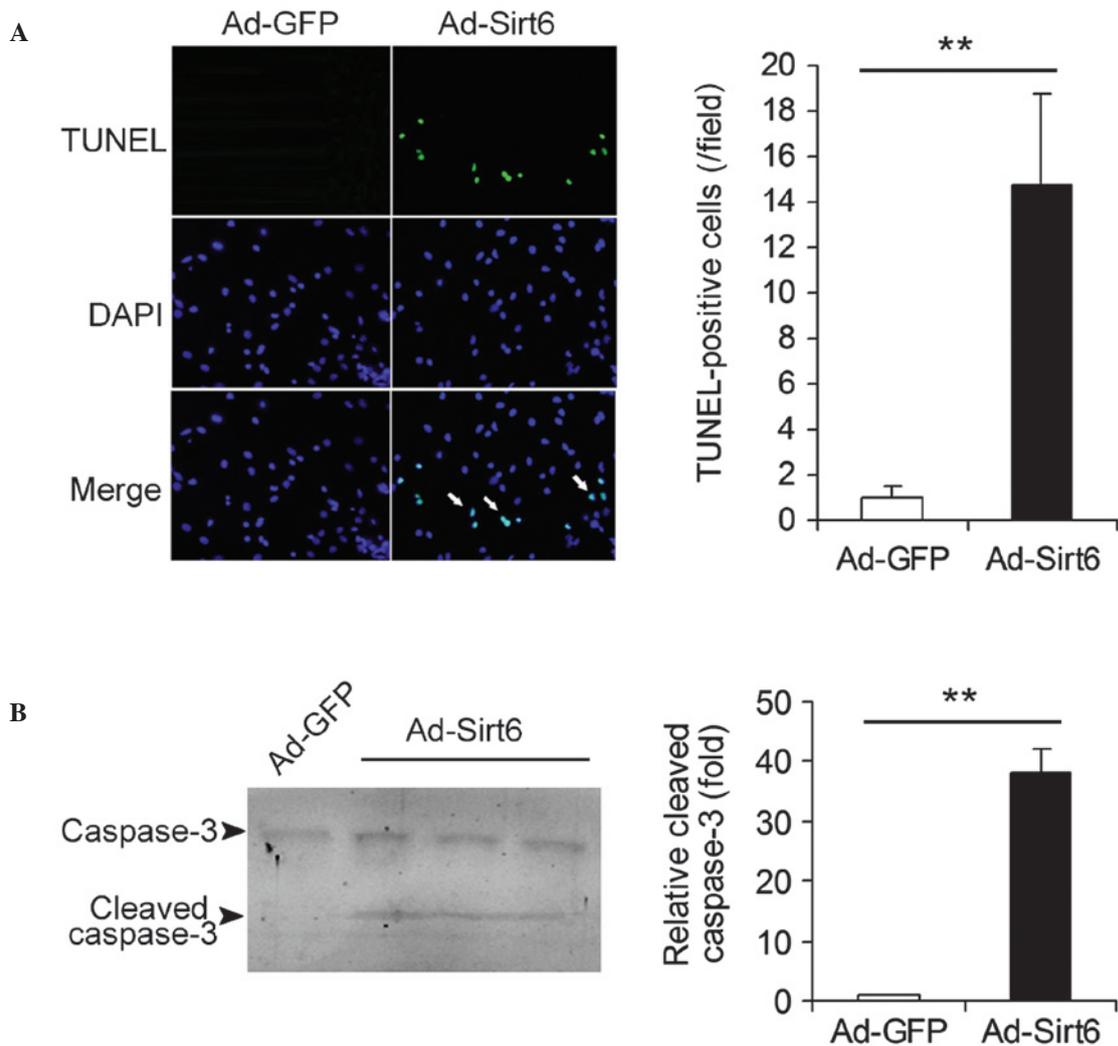


Figure 3. Apoptosis of hepatocellular carcinoma (HCC) cells induced by Sirt6 overexpression. (A) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay showed the apoptosis in HepG2 HCC cells overexpressing Sirt6. DAPI was used to stain the nucleus. Arrows indicate TUNEL-positive cells. (B) Immunoblotting showing the cleaved caspase-3 in Sirt6 overexpressed HepG2 HCC cells. ** $P < 0.01$ versus Ad-GFP (control). Number of tissues=8.

Statistical analysis. Data are expressed as the mean \pm standard error of the mean. Differences were evaluated by two-tailed Student's t-test or analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Downregulation of Sirt6 in human HCC tissue. Sirt6 mRNA levels were significantly downregulated (35-40%, $P < 0.05$) in human HCC tissue compared with normal adjacent tissue (Fig. 1A). A marked downregulation of Sirt6 protein levels was also observed in human HCC tissue compared with normal adjacent tissue (Fig. 1B).

Sirt6 regulates HepG2 cell growth. The effect of knockdown or overexpression of Sirt6 on HepG2 HCC cell growth *in vitro* was analyzed. Adenovirus-mediated knockdown of Sirt6 (Fig. 2A) by shRNA promoted HepG2 cell growth (Fig. 2B), whereas adenovirus-mediated overexpression of

Sirt6 (Fig. 2C) significantly inhibited HepG2 cell growth (Fig. 2D).

Overexpression of Sirt6 induces apoptosis in HepG2 HCC cells. The effects of overexpression of Sirt6 on the apoptosis of HCC cells were studied. Ad-GFP- and Ad-Sirt6-transfected cells were analyzed using the TUNEL assay. The apoptotic cells (TUNEL-positive cells, green) were detected in Ad-Sirt6 cells but not in control Ad-GFP cells (Fig. 3A). The protein levels of cleaved caspase-3, a key mediator and marker protein of apoptosis were also analyzed. Cleaved caspase-3 expression was detected in cells overexpressing Sirt6 but not in control cells (Fig. 3B). These results indicate that overexpression of Sirt6 induces apoptosis in HepG2 HCC cells.

Overexpression of Sirt6 decreases oxidative stress in HCC cells. The influence of Sirt6 overexpression on oxidative stress in HepG2 HCC cells was investigated. A DCF assay showed that overexpression of Sirt6 significantly decreased the total ROS level in HepG2 cells (Fig. 4A). Moreover, overexpression

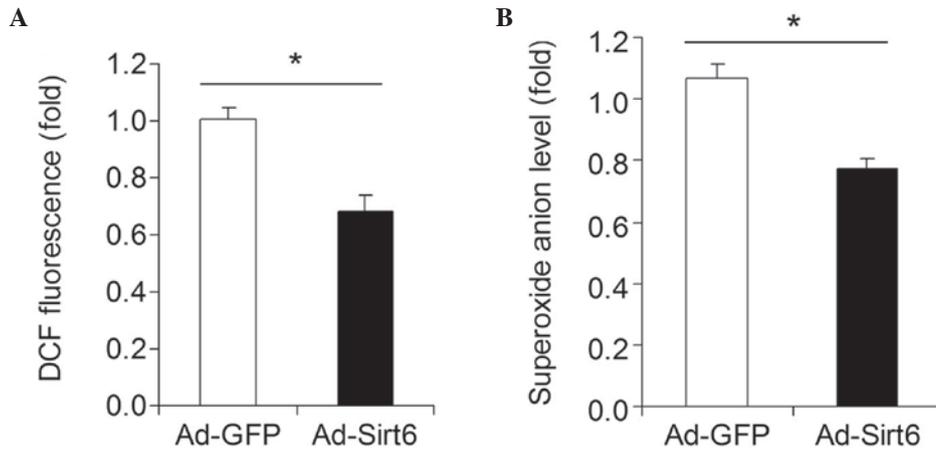


Figure 4. Sirt6 overexpression decreases reactive oxygen species (ROS) stress in hepatocellular carcinoma (HCC) cells. (A) Dichlorofluorescein fluorescence assay showed that Sirt6 overexpression decreased ROS level in HCC cells. (B) Sirt6 overexpression decreased superoxide anion levels in HCC cells. *P<0.05 versus adenovirus expressing green fluorescent protein (Ad-GFP). Number of tissues=6.

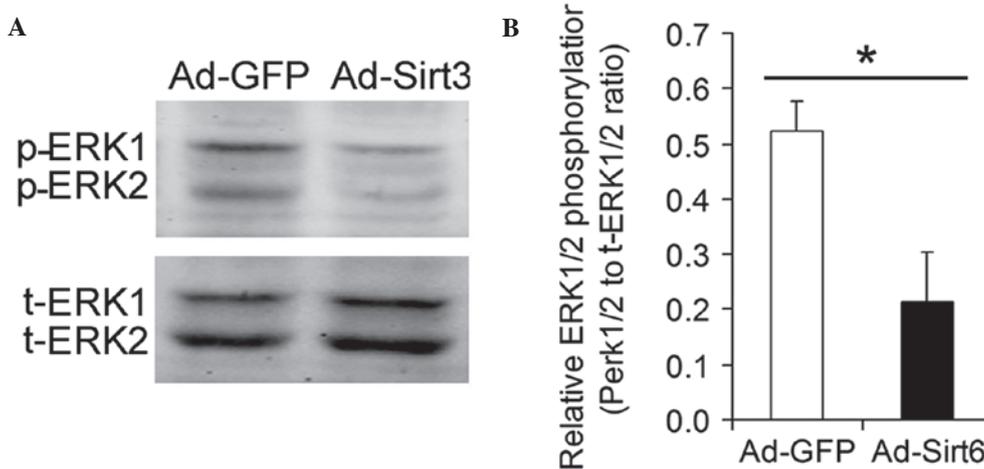


Figure 5. Influence of Sirt6 overexpression on activation of extracellular signal-regulated kinases 1/2 (ERK1/2) signaling pathway in hepatocellular carcinoma cells. Representative immunoblotting images and quantitative analysis of phosphorylation of ERK1/2 [ratio of phosphorylated ERK1/2 (p-ERK1/2) to total-ERK1/2 (t-ERK1/2)]. *P<0.05 versus Ad-GFP. Number of tissues=6.

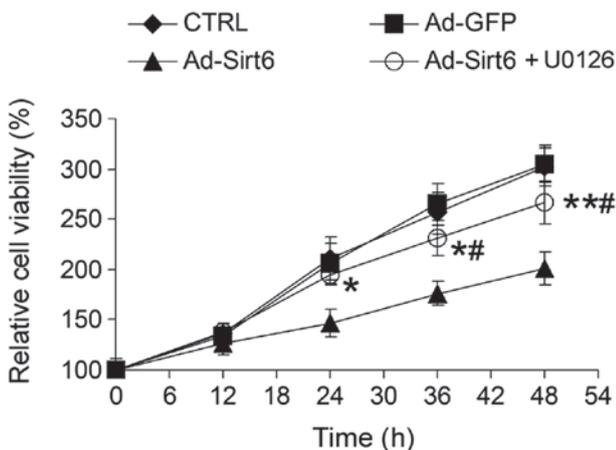


Figure 6. Sirt6 overexpression suppresses hepatocellular carcinoma (HCC) cell growth through regulating extracellular signal-regulated kinases 1/2 (ERK1/2) signaling pathway. Blocking the ERK1/2 signaling pathway with U0126 (10 μm) attenuated the inhibitory effect of Sirt6 overexpression on HCC cell growth. *P<0.05 versus Ad-Sirt6. #P<0.05 versus adenovirus expressing-green fluorescent protein (Ad-GFP). Number of tissues=6.

of Sirt6 downregulated the superoxide anion level in HepG2 cells (Fig. 4B).

Overexpression of Sirt6 inhibits ERK1/2 phosphorylation in HCC cells. ERK1/2 is a major transducer of extracellular mitogenic signals that promote cell proliferation. The influence of the overexpression of Sirt6 on the phosphorylation of ERK1/2 was analyzed. Overexpression of Sirt6 significantly inhibited ERK1/2 phosphorylation in HCC cells (Fig. 5).

Sirt6 suppresses HCC cell growth via regulation of the ERK1/2 signaling pathway. To examine whether the altered ERK1/2 signaling pathway induced by Sirt6 overexpression was important for Sirt6-mediated tumor suppression, HepG2 cells were treated with U0126, a chemical inhibitor of the ERK1/2 pathway. U0126 markedly attenuated the inhibitory effect of Sirt6 on HCC cell growth (Fig. 6), suggesting that Sirt6 suppresses HCC cell growth via inhibition of the ERK1/2 signaling pathway.

Discussion

In the present study, Sirt6 was markedly downregulated in HCC tissue compared with normal adjacent tissue. Using adenovirus-mediated knockdown and overexpression, modulation of Sirt6 affected the growth of HepG2 HCC cells. Further analyses, including a TUNEL assay and cleaved caspase-3 immunoblotting, revealed that Sirt6 overexpression promoted apoptosis in HepG2 HCC cells. In addition, Sirt6 overexpression decreased ROS/superoxide anion levels in HepG2 HCC cells. Finally, Sirt6 overexpression was found to inhibit phosphorylation of ERK1/2 in HepG2 HCC cells. Blocking the ERK1/2 pathway with U0126 attenuated the inhibitory effect of Sirt6 overexpression on HepG2 cell growth. These results suggest that Sirt6 is a tumor suppressor in HCC cells.

Several previous studies have demonstrated a change in Sirt6 expression in tumors. In a gene expression screening of endometrial carcinoma samples, Colas *et al* (30) revealed that Sirt6 was marginally upregulated. By contrast, in a recent study, Sebastián *et al* (18) reported that the Sirt6 locus was deleted in 35% of ~1,000 cancer cell lines and in 62.5% and 29% of pancreatic and colorectal cancer cell lines respectively. Moreover, this group demonstrated that Sirt6 expression was downregulated in 36 pancreatic ductal adenocarcinomas and in 55 colorectal carcinomas. In head and neck squamous cell carcinomas, Lai *et al* (31) confirmed that Sirt6 was downregulated in cancerous tissues when compared with noncancerous tissues. To the best of our knowledge, the present study provides the first evidence of Sirt6 downregulation in human HCC tissue. A report showed impaired Sirt6 expression in c-Jun-deficient livers during tumor initiation in mice and Sirt6 expression in human HCC tissue (17); however, whether Sirt6 expression was different in human HCC tissues compared to normal tissues was not investigated in this study.

In the present study, shRNA-mediated knockdown of Sirt6 was identified to promote HepG2 cell growth, which was consistent with a previous report (18). The tumor suppressive effect of Sirt6 has also been demonstrated in cervical carcinoma, fibrosarcoma, primary breast tumor and metastatic breast tumor cell lines (32). In concordance with these results, the results of the present study provide evidence for the tumor suppressive effect of Sirt6 in HCC cells. Notably, Sirt6 overexpression appeared to induce apoptosis in a variety of cancer cell lines but not in normal, non-transformed cells (32). Additionally, mono-ADP-ribosyltransferase, but not deacetylase activity, was required for the tumor suppressive effect of Sirt6 (32).

As a member of mitogen-activated protein kinases, ERK1/2 mediates intracellular signaling pathways involved in proliferative functions, including meiosis, mitosis and postmitotic differentiation in cells (33). A number of different stimuli, including cytokines, growth factors, transforming agents and carcinogens, activate the ERK1/2 pathway to promote cell proliferation (33). Activated ERK1/2 is an important feature in HCC and multiple anticancer agents inhibit HCC cell growth via inhibition of ERK1/2 signaling (34). Previous studies revealed that Sirt1 activated the ERK1/2 pathway (35-36), whereas Sirt3 repressed the ERK1/2 signaling pathway (13). To the best of our knowledge, there has been no report on the association between ERK1/2 and Sirt6. Using immunoblotting, Sirt6 overexpression was found to inhibit the phosphorylation of ERK1/2 in HCC

cells. In addition, blocking the ERK1/2 signaling pathway with the specific chemical inhibitor U0126, markedly attenuated the tumor suppressive effect of Sirt6. As ERK1/2 localizes to the cytoplasm and is translocated to the nucleus following phosphorylation (33), it is not known how Sirt6, a nuclear protein, regulates ERK1/2 phosphorylation. This is an important question that needs to be addressed in future studies.

In conclusion, the present study demonstrated that the expression of Sirt6 was decreased in human HCC tissue. Overexpression of Sirt6 in the HepG2 HCC cell line exhibited antitumor effects through the induction of apoptosis and the inhibition of the ERK1/2 signaling pathway. These findings on the regulation of HCC cell growth by Sirt6 may provide an improved understanding of HCC and aid in the possible development of therapeutic interventions.

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