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Sirt3 inhibits hepatocellular carcinoma cell growth through reducing Mdm2-mediated p53 degradation

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ABSTRACT

Sirt3 is a member of the mammalian sirtuin family that is localized to mitochondria and plays a role in the control of the metabolic activity. Recently, Sirt3 has been reported to be associated with the deregulating metabolism of cancer cells. However, the role of Sirt3 in hepatocellular carcinoma (HCC) has never been studied. In this study, we found that Sirt3 protein expression was downregulated in human HCC tissue. We also showed that overexpression of Sirt3 using adenovirus inhibited HCC cell growth (two cell lines: HepG2 and HuH-7 cells) and induced apoptosis, which was evidenced by the increase of LDH leakage, enhancement of TUNEL-positive cells number and promotion of AIF translocation to nuclei. Sirt3 overexpression reduced the intracellular NAD^+ level, repressed the ERK1/2 signaling pathway, and activated the Akt and JNK signaling pathways. Furthermore, Sirt3 overexpression upregulated p53 protein level through downregulating Mdm2 and thereby slowing p53 degradation. Collectively, our data suggests that Sirt3 may play an important role in HCC development and progression and may be a promising therapeutic target for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer death worldwide especially in developing countries, and the incidence rate is currently rising in the United States [1]. HCC displays great genomic heterogeneity and the major known causes of HCC include viral hepatitis infection (hepatitis B or C) and alcoholism-induced cirrhosis [2]. However, many other mechanisms remain unknown.

In cancer cells, the chronic and uncontrolled cell proliferation always involves not only deregulated control of cell growth and division but also adjustments of energy metabolism in order to support cancer cell proliferation. Abnormal glycolytic fueling has been shown to be associated with several oncogenes (e.g., *MYC* and *RAS*) and mutant tumor suppressors (e.g., *TP53*) [3,4]. In a recent important review, deregulating cellular energy metabolism has been thought to be an emerging hallmark of cancer [5]. In fact, mitochondrion, the energy factory and producer of cells, is very important in cancer [6], and is believed to be a target for cancer therapy [7].

Sirtuins are a conserved family of NAD^+-dependent deacetylases involved in stress response, metabolism, and longevity [8]. Sirtuins are widely expressed in different tissues and localized to different subcellular compartments [8]. Among the seven members (Sirt1–Sirt7), only three (Sirt3–Sirt5) are localized to the mitochondrion. Because the deacetylase activity of Sirt4 and Sirt5 is very weak, Sirt3 is believed to be the major protein deacetylase within the mitochondrion [9]. Sirt3 deacetylates complexs I and II to maintain basal ATP levels [10], modulates mitochondrial metabolic responses during fasting [11], and targets the mitochondrial enzymes MnSOD to maintain reactive oxygen species (ROS) homeostasis [12]. In 2006, increased level of Sirt3 transcription was found to be associated with breast cancer [13]. Later, Kim et al. demonstrated that knockout of Sirt3 might promote development of breast tumor in mice [14]. In the past 2 years, functions of Sirt3 in colon cancer cell line-induced xenografts [15], breast cancer [16] and oral cancer [17] also have been reported. However, the exact role of Sirt3 in cancer is far from being known. In this study, we investigated the expression of Sirt3 in human HCC tissues and explored the potential role of Sirt3 in HCC.

2. Materials and methods

2.1. Reagents

Antibodies against Sirt3, apoptosis-inducing factor (AIF), lamin A/C and MnSOD were purchased from Millipore Chemicon International (Temecula, CA, USA). Antibodies against p53, p21, Mdm2, phospho-JNK, total-JNK, phospho-p38, total-p38, phospho-ERK1/2, total-ERK1/2, phospho-Akt, total-Akt and acetyl-p53 (Lys-382) were

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purchased from Cell Signaling Biotechnology (Danvers, MA, USA). Antibody against Actin was from Sigma. Cell viability assay (Cell Counting Kit-8) was from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Cycloheximide was from Sigma (St. Louis, MO, USA). Fluorometric terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) kit was purchased from Promega (Madison, WI, USA). DAPI was purchased from Invitrogen (Carslbad, CA, USA). Nuclear and mitochondrial protein isolation kit was purchased from Qiagen (Hilden, Germany). Enhanced chemiluminescence and protease/phosphatase inhibitors were purchased from Pierce (Rockford, IL, USA).

2.2. Human HCC tissue

Four pairs of HCC and matched peritumoral liver tissue extracts were obtained from Chinese patients who underwent surgical resection for diagnosis and therapy. Samples were obtained following informed consent according to an established protocol approved by the Ethic Committee of Sichuan University.

2.3. Cell culture

Two HCC cells lines were used: HepG2 and HuH-7 cells. HepG2 cell line was from ATCC (No. HB-8065). HuH-7 cell line was provided and licensed by Cell Bank of Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% (v/v) fetal bovine serum (FBS) in 95% O_2 and 5% CO_2 [18].

2.4. Construction of the adenovirus expressing Sirt3

The adenovirus expressing Sirt3 (Ad-Sirt3) or control adenovirus expressing GFP (Ad-GFP) were generated using the RAPAd® CMV Adenoviral Bicistronic Expression System (Cell Biolabs, San Diego, CA) according to the manufacturer's protocols. Briefly, human Sirt3 cDNA was cloned into pacAd5 CMV-IRES vector. Then, pacAd5 CMV-IRES-Sirt3 and pacAd5 9.2-100 backbone vectors were linearized by *PacI*. The purified linearized DNAs were cotransfect into 293 cells using Lipofectamine™ Plus (Invitrogen) [19]. On day 10, adenovirus-containing cells and media were harvested. Viruses were released by three freeze/thaw cycles and stored at −80 °C [20]. For virus transfection, 20 μl viral stock was added into culture medium (2 ml) for 6 h.

2.5. Cell viability assay

Cell viability was evaluated by a non-radioactive cell counting kit-8 (CCK-8) assay as described previously [21]. Ad-GFP or Ad-Sirt3 transfected HCC cells (5×10^3) were seeded into 48-well plates and cultured overnight to allow attachment. At four different time points (12, 24, 36 and 48 h), cells were incubated with 10 μL of CCK-8 solution for 3 h at 37 °C, and then the optical density at 450 nm was analyzed using a microplate reader (Tecan Infinite M100, Switzerland). Experiments were performed in duplicate.

2.6. Membrane integrity measurement assay

Cell membrane integrity was determined by LDH leakage assay using Promega CytoTox-ONE™ kit (Promega) [22]. In brief, 10^4 cells were seeded in 96-well plates and transfected with Ad-GFP or Ad-Sirt3. After 24 h, medium was transferred to a black fluorescence plate and incubated for 10 min with CytoTox-ONE™ reagent followed by stop solution. Fluorescence was measured at 560/590 nm.

2.7. TUNEL assay

TUNEL staining was done as described previously [23]. Cells were then transfected with Ad-GFP or Ad-Sirt3 for 6 h. H_2O_2 (100 μM) was used as positive control to induce apoptosis. At 1 day after treatment, cells were incubated in TUNEL reaction buffer in a 37 °C humidified chamber for 1 h in the dark, then rinsed twice with PBS and incubated with DAPI (1 mg/mL) for 10 min. The stained cells were visualized using a fluorescence microscope (IX-71, Olympus, Japan). TUNEL-positive cells (green) were counted as apoptotic cells.

2.8. Immunochemistry

Immunochemistry was performed as described previously [24]. Briefly, the cells were seeded onto coverslips, transfected with Ad-GFP or Ad-Sirt3 for 6 h. At 24 h after transfection, cells were washed twice with PBS and then fixed with buffer containing 4% paraformaldehyde and 0.1% Triton X-100 at room temperature for 20 min, followed by incubation with antibody against AIF at 37 °C for 2 h and incubation with Alexa Fluor 555-conjugated secondary antibody.

2.9. Immunoblotting and immunoprecipitation

Immunoblotting analyses of cell-extracts were performed as described previously [25,26]. Cells were lysed with RIPA buffer with protease inhibitor/protein phosphatase inhibitors. Samples were subjected to 10% SDS–PAGE, and transferred onto PVDF membranes at 100 V for 1 ∼ 2 h. After being blocked in blocking buffer with 5% (w/v) nonfat milk and 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) for 4 h, the membrane was incubated with specific primary antibody and then followed by HRP-labeled secondary antibody. The membranes were then detected using the enhanced chemiluminescence system.

Immunoprecipitation was performed as described previously [19,27]. In brief, cells were homogenated with protease inhibitor cocktail. To reduce nonspecific binding, 50 μL normal rabbit serum was added to 1 mL homogenate and incubated for 1 h on ice. Protein bead A/G was then added to the homogenate and incubated for 1 h with gentle agitation. The supernatant was incubated with primary antibody at 4 °C overnight. Antigen–antibody complex was harvested and boiled. The samples were separated on 10% SDS–PAGE pages for immunoblotting.

2.10. Statistical analysis

Data are expressed as mean ± SEM. Differences were evaluated by two-tailed Student's *t* test or ANOVA followed by Tukey's post hoc test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Sirt3 is downregulated in human HCC tissue and overexpression of Sirt3 inhibits HCC cell growth

As shown in Fig. 1A, Sirt3 was downregulated significantly (∼40–50%) in human HCC tissue compared with peritumoral normal tissue, suggesting the loss of Sirt3 may participate in the genesis and development of HCC. Therefore, we tested whether overexpression of Sirt3 can influence HCC cell growth *in vitro*. As expected, overexpression of Sirt3 (Fig. 1B) successfully inhibited growth of HepG2 (Fig. 1C) and HuH-7 (Fig. 1D) cells at different time points.

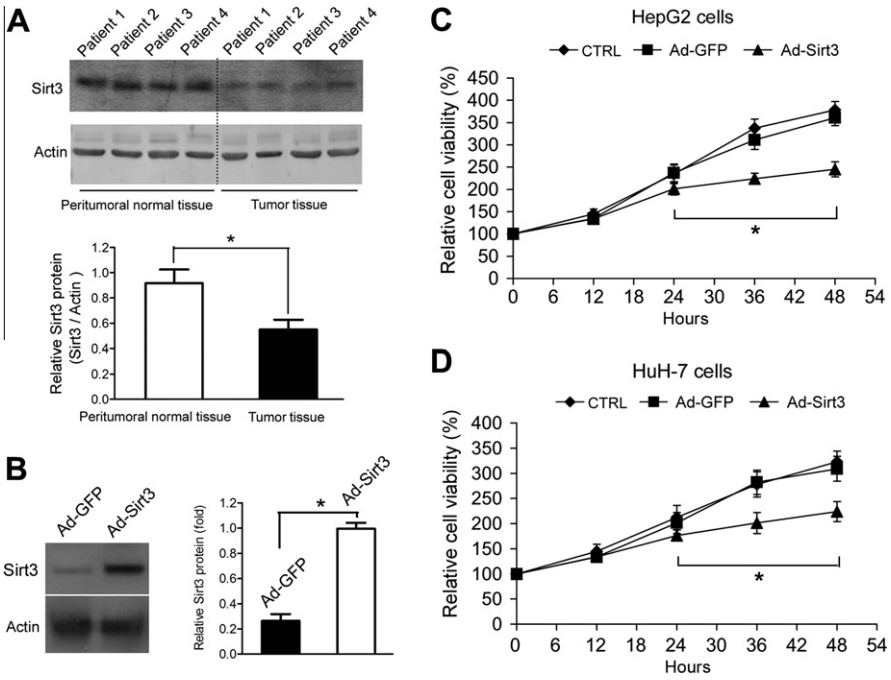

Fig. 1. Downregulation of Sirt3 in human primary HCC tissue and Sirt3 overexpression inhibits HCC cell growth. (A) Immunoblotting showing the expression of Sirt3 in human HCC tissue and peritumoral normal tissue. $^{*}P < 0.05$ versus normal tissue. $N = 4$. (B) Immunoblotting showing the overexpression of Sirt3 using adenovirus in HepG2 cells. $^{*}P < 0.05$ versus Ad-GFP (vector control). $N = 4$. (C and D) Cell growth curve of HepG2 and HuH-7 cells transfected with Ad-GFP or Ad-Sirt3. $^{*}P < 0.05$ versus Ad-GFP (control). $N = 8$.

3.2. Overexpression of Sirt3 induces apoptosis in HCC cells

We next studied the effects of overexpression of Sirt3 on apoptosis of HCC cells. First, we measured the membrane integrity in Sirt3-overexpressed cells. Overexpression of Sirt3 markedly increased the LDH concentration in culture medium of HepG2 (Fig. 2A) and HuH-7 (Fig. 2B) cells, indicating that overexpression of Sirt3 caused HCC cell membrane damage. Then, we performed TUNEL assay to directly examine apoptosis. As shown in Fig. 2C and D, TUNEL-positive cells were very obvious in positive control group (H_2O_2-treated group). There was no TUNEL-positive cell in Ad-GFP transfected cells, while small quantities of TUNEL-positive cells were detected in Sirt3-overexpressed cells.

Because the phenotypes of Sirt3 overexpression on HepG2 and HuH-7 cells were very similar, we studied the mechanisms underlying the effects of Sirt3 only in HepG2 cells in the following experiments. We measured the influence of Sirt3 overexpression on AIF. In Sirt3-overexpressed cells, nuclear AIF was increased, whereas mitochondrial AIF was reduced (Fig. 2E). Moreover, immunofluorescence analysis (Fig. 2F) showed that Sirt3 overexpression induced AIF nuclear translocation.

3.3. Overexpression of Sirt3 reduces intracellular NAD^+ level, represses ERK1/2 signaling pathway, and activates Akt and JNK signaling pathway

Because SIRT3 is a NAD^+-dependent deacetylase and consumes NAD^+ [28], we measured intracellular NAD^+ level in HepG2 cells. Ad-Sirt3 treated cells displayed lower NAD^+ level compared with Ad-GFP control cells (Fig. 3A). Moreover, we studied the effects of Sirt3 overexpression on several survival-related signaling pathways in HepG2 cells. As shown in Fig. 3B, under FBS condition, phosphorylation of ERK1/2 was remarkably suppressed, whereas phosphorylation of JNK was increased in Ad-Sirt3 transfected HepG2 cells. Activation of Akt, another important pro-survival signaling, was partly blocked by Sirt3 overexpression (Fig. 3C).

3.4. Overexpression of Sirt3 upregulates p53 protein level through attenuating Mdm2-mediated p53 degradation

Next, we studied the influence of Sirt3 overexpression on p53 signaling in HepG2 cells. Indeed, Sirt3 overexpression increased p53 protein level (Fig. 4A) and p53-downstream p21 protein level (Fig. 4B). However, we did not detect any difference of p53 mRNA between control cells and Sirt3 overexpressed cells (Fig. 4C). Thus, we turned to examine the effects of Sirt3 overexpression on p53 protein stability. As shown in Fig. 4D, the degradation of p53 in Sirt3 overexpressed cells was slowed down compared with that in control cell. These data suggested that Sirt3 modulated p53 protein level post-transcriptionally. Acetylation of p53 was reported to increase p53 stability [29]. Thus, we measured the acetylation of p53 and found there was no influence of Sirt3 overexpression on acetyl-p53 level (Fig. 4E). Ubiquitination of p53 by Mdm2 has been well-studied as a canonical ubiquitin-mediated proteasomal degradation pathway. Interestingly, we found that Sirt3 overexpression significantly downregulated Mdm2 protein level (Fig. 4F), suggesting that Sirt3 overexpression might increase p53 protein stability through attenuating Mdm2-mediated p53 degradation. Immunoprecipitation assay confirmed that the interaction between endogenous Mdm2 and p53 was significantly reduced by overexpression of Sirt3 (Fig. 4G).

4. Discussion

In the present study, we demonstrated that Sirt3 was markedly downregulated in HCC tumor tissue. Using adenovirus-mediated overexpression, we further showed that Sirt3 overexpression inhibited growth of two HCC cell lines (HepG2 and HuH-7 cells) and induced obvious apoptosis, which was evidenced by LDH leakage, TUNEL and AIF translocation to nuclei. In HepG2 cells, Sirt3 overexpression attenuated the intracellular NAD^+ level, repressed ERK1/2 signaling pathway, and activated Akt and JNK signaling pathway. Furthermore, Sirt3 overexpression upregulated p53

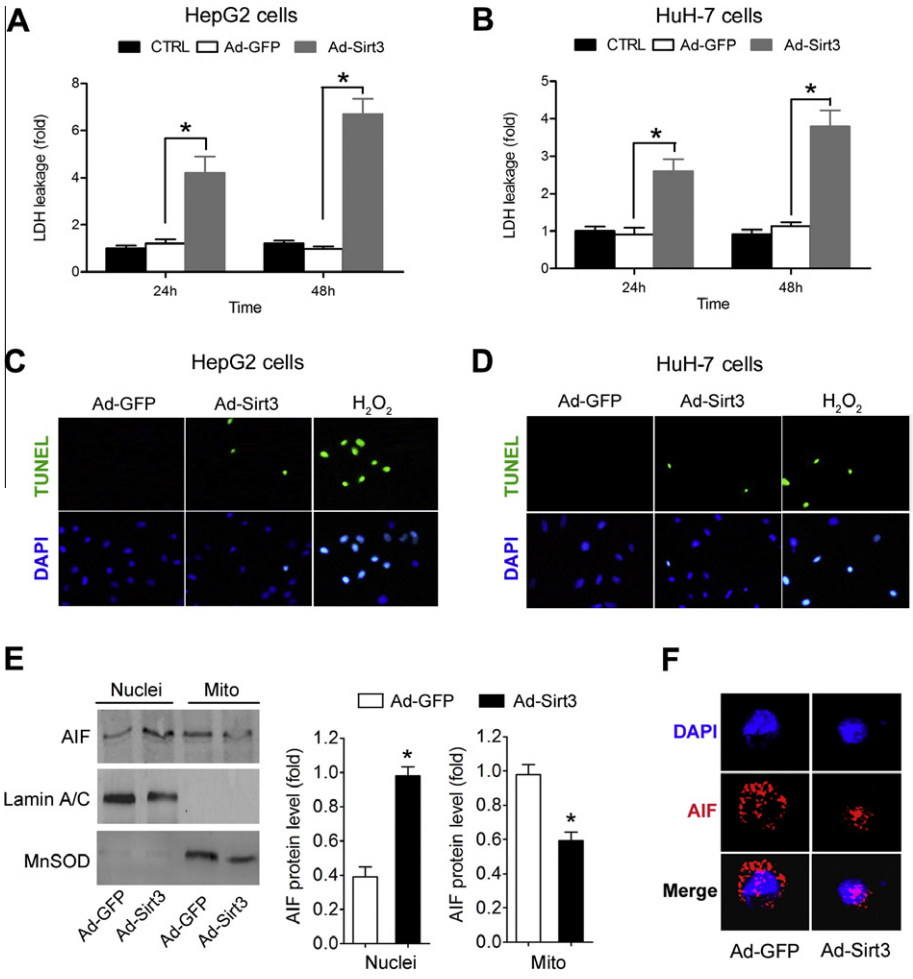

Fig. 2. Apoptosis of HCC cells induced by Sirt3 overexpression. (A and B) LDH concentration in culture medium of HepG2 and HuH-7 cells. $^{*}P < 0.05$ versus Ad-GFP (vector control). $N = 8$. (C and D) TUNEL assay showing the apoptosis in HepG2 and HuH-7 cells induced by Sirt3 overexpression and H_2O_2 (positive control). DAPI was used to stain nuclei. (E) Immunoblotting showing the nuclear translocation of AIF induced by Sirt3 overexpression. (F) Immunofluorescence showing the nuclear translocation of AIF induced by Sirt3 overexpression.

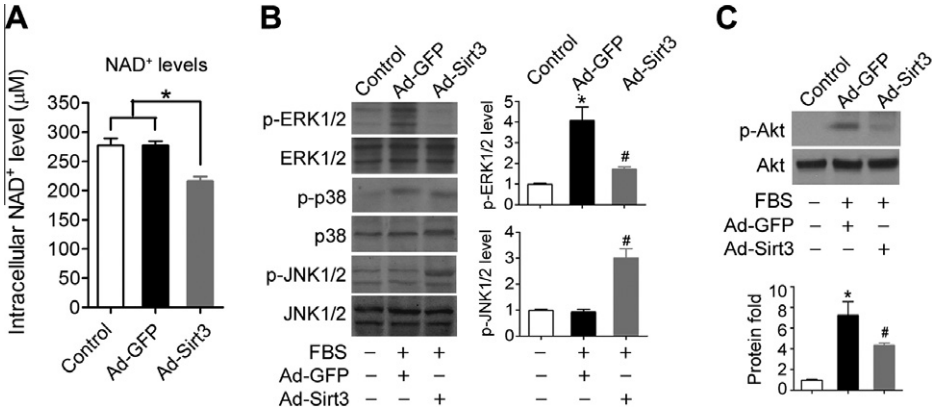

Fig. 3. Influence of Sirt3 overexpression on intracellular signaling pathways. (A) NAD^+ levels in Ad-GFP or Ad-Sirt3 transfected HepG2 cells. $^{*}P < 0.05$ versus Ad-GFP. $N = 8$. (B) Representative immunoblotting images and quantitative analysis of phosphorylation of ERK1/2, p38 and JNK signalings. $^{*}P < 0.05$ versus without FBS. $^{\#}P < 0.05$ versus Ad-GFP. $N = 4$. (C) Representative immunoblotting images and quantitative analysis of phosphorylation of Akt. $^{*}P < 0.05$ versus without FBS. $^{\#}P < 0.05$ versus Ad-GFP. $N = 4$.

protein level through attenuating Mdm2-mediated p53 degradation. These data clearly suggests that Sirt3 plays an important role in HCC development and progression.

The first important finding of our study is that there is down-regulation of Sirt3 in human HCC tissue. A large-scale proteomics analyses have revealed that acetylation was found in more than 20% of mitochondrial proteins, including many metabolism enzymes [30]. The status of mitochondrial protein acetylation is dictated by the energy status of the cell, suggesting that acetylation could be a critical regulatory mechanism that underlying the

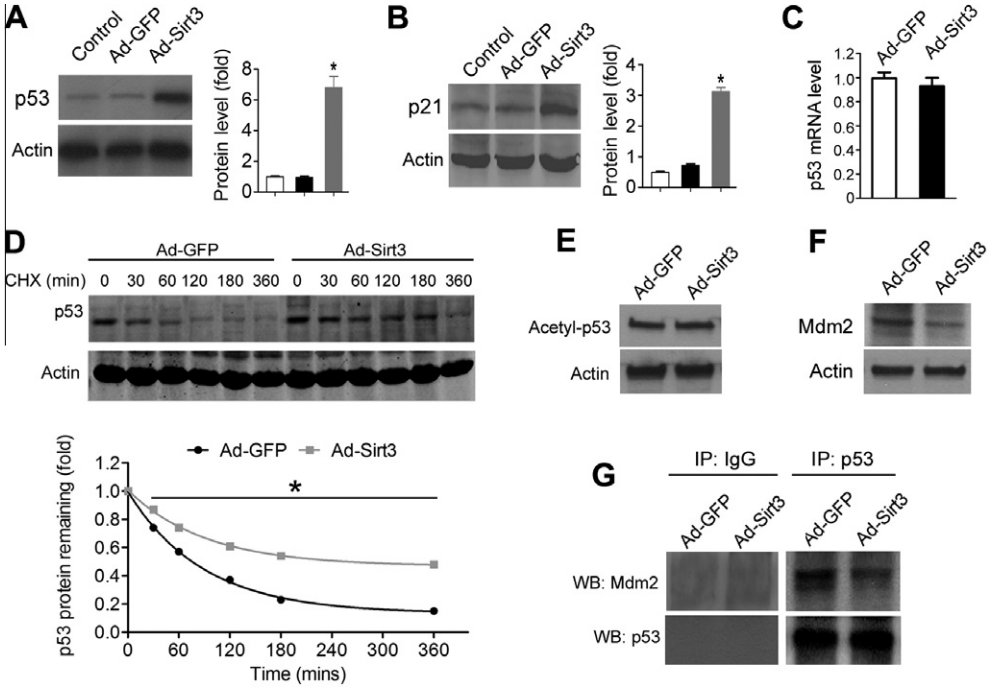

Fig. 4. Sirt3 overexpression upregulates p53 protein level through attenuating Mdm2-mediated p53 degradation. (A and B) Representative immunoblotting images and quantitative analysis of p53 and downstream p21 protein levels. $^{*}P < 0.05$ versus Ad-GFP. $N = 4$. (C) Influence of Sirt3 overexpression on mRNA level of p53. NS, no significance. (D) HepG2 cells transfected with Ad-GFP or Ad-Sirt3 were serum starved and treated with 150 μg/mL CHX to block protein synthesis. The p53 protein levels were measured to determine degradation curve of p53. $^{*}P < 0.05$ versus Ad-GFP. $N = 4$. (E) Influence of p53 on acetylation of p53. NS, no significance. (F) Sirt3 overexpression downregulated Mdm2 protein level. (G) HepG2 cells transfected with Ad-GFP or Ad-Sirt3 were serum starved and then the p53-Mdm2 interaction was determined. IgG was used as a negative control for immunoprecipitation. IP, immunoprecipitation and IB, immunoblot.

adaptive response to energy stress. Sirt3 has been demonstrated to be the major enzyme regulating acetylation of mitochondrial protein [9]. In agreement with the potential role in mitochondrial biology, Sirt3 is highly expressed in tissues with metabolically active tissues with high oxidative capacity, such as skeletal muscle, liver, brain, kidney and adipose tissue [31]. In mitochondria, Sirt3 regulates acetylation and ROS generation, which are tightly associated with cancer genesis and development [32,33]. SIRT3$^{-/-}$ MEFs displayed higher stress-induced superoxide levels, genomic instability and tumor-permissive phenotype, which could be prevented by overexpression of superoxide dismutase, indicating that the elevated levels of ROS in SIRT3$^{-/-}$ mice might contribute to a cancer-prone phenotype and Sirt3 functioned as a tumor-suppressor gene [14]. Therefore, the downregulation of Sirt3 in human HCC tissue might lead to higher ROS level and thereby increase the cancer risk. We did not have fresh HCC tissue, so we cannot determine the ROS level in HCC tissue. However, a previous report showed that the ROS level in human HCC tissue was much higher than normal tissue, thus supporting our results [34].

The inhibitory effect of Sirt3 overexpression on HCC cell growth was very obvious *in vitro*, which prompted us to study apoptosis. After finding the pro-apoptotic feature of Sirt3, we observed that Sirt3 overexpression modulated several important survival and proliferative signaling pathways (ERK1/2, Akt and JNK). ERK1/2 and Akt are two well-established survival and proliferative signaling pathways, whereas JNK is a pro-apoptotic kinase in many circumstances [35]. The changes in these critical signaling pathways in HCC cells would of course affect HCC growth. The inhibitory effects of Sirt3 on ERK1/2 and Akt signaling pathways have been previously reported by Sundaresan et al. [36] in hypertrophic cardiomyocytes, while the modulating effect of Sirt3 on JNK was reported by Jing et al. [37] in skeletal muscle. Consequently, our study is the first to show the effect of Sirt3 on ERK1/2 and Akt as well as on JNK signaling pathway in cancer cells.

We provided evidence showing Sirt3 overexpression upregulated p53 protein level without altering p53 mRNA level in HCC cells, suggesting that Sirt3 may modulate p53 via post-transcriptional regulation such as acetylation and ubiquitination. Sirt1, another member of sirtuins family, has been found to be able to directly deacetylate p53 [38,39]. However, there was no evidence showing that Sirt3 can deacetylate p53. In our study, we did not detect difference of acetyl-p53 level between control and Sirt3 overexpressed cells. Interestingly, we detected reduced Mdm2 level in Sirt3 overexpressed cells. Mdm2-p53 loop is a widely recognized model. Mdm2 interacts with p53 and acts an ubiquitin ligase E3 of p53 and mediates p53 degradation. Thus, we can propose that the Sirt3 overexpression-induced downregulation of Mdm2 may attenuate p53 degradation and thereby increase p53 protein level.

Collectively, we demonstrate that the expression of Sirt3, a tumor suppressor, was lost in HCC. Overexpression of Sirt3 in HCC cell lines exhibited anti-tumor effect through reducing Mdm2-mediated p53 degradation. Our results indicate that the regulation of HCC cell growth by Sirt3 could provide an important area for HCC understanding and therapeutic intervention.

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