Sirt3 is a tumor suppressor in lung adenocarcinoma cells

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Abstract. Sirt3, a member of the mammalian sirtuin family protein that is localized to mitochondria, is a NAD$^+$-dependent deacetylase and plays an important role in the control of metabolic activity. Recently, several studies have shown the potential role of Sirt3 in certain types of tumors such as breast cancer and hepatocellular carcinoma. However, the role of Sirt3 in lung adenocarcinoma has never been studied. In the present study, we found that Sirt3 protein expression was downregulated in human lung adenocarcinoma tissue when compared with that in adjacent normal tissue. Overexpression of Sirt3 using adenovirus significantly inhibited the growth of the A549 lung adenocarcinoma cell line. In this cell line, over-expression of Sirt3 induced apoptosis, which was evidenced by Annexin V + PI assay and cleaved caspase-3 immunoblot-ting. Furthermore, overexpression of Sirt3 increased the bax/bcl-2 and bad/bcl-x/L ratios, and promoted AIF translocation to the nucleus. Finally, Sirt3 overexpression upregulated p53 and p21 protein levels, and decreased intracellular ROS levels. Collectively, our data suggest that Sirt3 is a tumor suppressor in lung adenocarcinoma development and progression and may be a promising therapeutic target for lung adenocarcinoma.

Introduction

Worldwide, lung cancer is the most common cancer with high incidence and mortality rates (1,2). Non-small cell lung cancer accounts for nearly 80% of the disease, while lung adenocarcinoma is the most common type of non-small cell lung cancer (1). Lung adenocarcinoma is characterized by peripheral location in the lung and often has activating muta-tions in the *K-ras* oncogene (3,4). Recently, several studies have highlighted the importance of mutations of the epidermal growth factor receptor (EGFR) and other oncogenes (5,6); however, the causes and biology of lung adenocarcinoma are not yet fully understood (7,8).

In cancer cells, uncontrolled cell proliferation involves not only dysfunctions in the control of cell growth and division but also adjustments in energy metabolism that is utilized to support cancer cell proliferation. Several important mutant oncogenes (e.g., *TP53*) have been reported to be associated with abnormal glycolytic fueling (9,10). Recently, deregulated cellular energy metabolism has been viewed as a new hallmark of cancer (11). As an indispensable cell energy factory for the survival of cells, the mitochondrion is an important regulator of vital and lethal functions, particularly the intrinsic pathway of apoptosis (12). More and more data demonstrate that the mitochondrion is a critical target for cancer therapy (13,14).

Sirtuins are a conserved family of deacetylases and mono-ADP-ribosyltransferases that use NAD$^+$ as a co-substrate (15). There are 7 members of the sirtuin family in mammals (Sirt1-Sirt7) (15). These proteins are believed to be involved in stress response, metabolism and longevity (15). Sirtuins are widely expressed in different tissues and are localized in different subcellular compartments (15,16). Among the 7 members, Sirt3, Sirt4 and Sirt5 are localized in the mito-chondrion. Sirt3 is believed to be the major deacetylase within the mitochondrion, since the deacetylase activity of Sirt4 and Sirt5 is rather weak (17).

The role of Sirt3 in tumor biology has become a topic of increased interest in recent years. Ashraf *et al* (18) first reported that increased levels of Sirt3 and Sirt7 transcription are associated with node-positive breast cancer. Kim *et al* (19) demonstrated that gene deletion of Sirt3 facilitates the development of breast tumors in mice, suggesting the tumor-suppressive effect of Sirt3 in breast cancer, which was confirmed by a later study (20). A recent report showed that Sirt3 inhibits hepatocellular carcinoma cell growth through reducing Mdm2-mediated p53 degradation (21). However, the exact role of Sirt3 in other tumors is still being discovered. In

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the present study, we investigated the expression of Sirt3 in human lung adenocarcinoma tissues and explored the potential role of Sirt3 in lung adenocarcinoma.

Materials and methods

Reagents. Antibodies against Sirt3 and apoptosis-inducing factor (AIF) were purchased from Millipore Chemicon International (Temecula, CA, USA). Antibodies against cleaved caspase-3, bax, bcl-2, bad, bcl-x/L, p53 and p21 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody against actin was obtained from Sigma-Aldrich, (St. Louis, MO, USA). The Annexin V + PI kit was purchased from Promega Corporation (Madison, WI, USA). DAPI and dichlorofluorescein diacetate (DCFH-DA) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Enhanced chemiluminescence and protease/phosphatase inhibitors were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA).

Human lung adenocarcinoma tissue. Four pairs of lung adenocarcinoma and matched normal adjacent tissue extracts were obtained from Chinese patients who underwent surgical resection for diagnosis and therapy in our hospital. Samples were obtained following informed consent according to an established protocol approved by the Ethics Committee of Central South University.

Cell culture. The A549 lung adenocarcinoma cell line was obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in 95% O_2 and 5% CO_2.

Construction of the adenovirus expressing Sirt3. The adenovirus expressing Sirt3 (Ad-Sirt3) or the control adenovirus expressing GFP (Ad-GFP) were generated using the RAPAd® CMV adenoviral expression system (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's protocol as previously described (21). Briefly, mouse Sirt3 cDNA was cloned into pacAd5 CMV-IRES vector. Then, pacAd5 CMV-IRES-Sirt3 and pacAd5 9.2-100 backbone vectors were linearized by *Pac*I. The purified linearized DNAs were cotransfected into 293 cells using Lipofectamine™ Plus (Invitrogen Life Technologies). On day 8, adenovirus-containing cells and media were harvested. Viruses were released by 3 freeze/thaw cycles and stored at -80˚C. For virus transfection, 20 μl of viral stock solution was added into the culture medium (2 ml) for 6 h.

Cell viability assay. Cell viability was evaluated by a non-radioactive Cell Counting Kit-8 (CCK-8) assay as described previously (21,22). Ad-GFP- and Ad-Sirt3-transfected cells ($5x10^3$) were seeded into 48-well plates and cultured overnight to allow attachment. After being serum-starved for 8 h, FBS was added into the medium. At 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, Switzerland). Experiments were performed in duplicate.

Quantitative real-time PCR analysis. Real-time PCR analysis was performed on an ABI Prism 7500 sequence detection system using PrimerScript® RT reagent kit (Takara Bio, Inc., Shiga, Japan). The total RNA was extracted form human tissue using TRIzol (Invitrogen Life Technologies) and 5 μg of RNA as first-strand cDNA was used. cDNA (100 ng) was amplified using primers as follows: Sirt3 sense, ACAGCAACCTCCAG CAGTACGA and antisense, CGTGTAGAGCCGCAGAAG CA; β-actin sense, GCACTCTTCCAGCCTTCCTTCC and antisense, CCGCCAGACAGCACTGTGTT. The mRNA levels of housekeeping gene β-actin were used as control.

Immunochemistry. Immunochemistry was performed as described previously (23,24). Briefly, the A549 cells were seeded onto coverslips and 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 an antibody against AIF at 37˚C for 2 h and incubation with Alexa Fluor 555-conjugated secondary antibody. DAPI was used to stain the nucleus.

Immunoblotting. Immunoblotting analyses of cell extracts were performed as described previously (22,25). Human tissues or 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 for 4 h, the membrane was incubated with a specific primary antibody and then followed by the HRP-labeled secondary antibody, and investigation using the enhanced chemiluminescence system (Pierce Biotechnology, Inc., Rockford, IL, USA).

Annexin V + PI staining assay. The Annexin V + PI assay for apoptosis was performed using flow cytometry as described previously (23). Briefly, Ad-GFP- and Ad-Sirt3-transfected cells were trypsinized, washed in ice-cold PBS and re-suspended in 1 ml of the supplied buffer ($1x10^6$ cells/ml). A 100 μl sample ($1x10^5$ cells) was incubated with 5 μl FITC-conjugated Annexin V and 5 μl PI for 30 min at 25˚C in the dark. Then, cells were analyzed immediately using a BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). The apoptotic cells were estimated as the percentage of cells that stained positive for FITC-Annexin V while remaining impermeable to PI (Annexin V+/PI-).

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

Results

Sirt3 is downregulated in human lung adenocarcinoma tissue. As shown in Fig. 1A, Sirt3 mRNA levels were significantly downregulated (~50-60%) in human lung adenocarcinoma tissue when compared with normal adjacent tissue. Similarly, a marked downregulation of Sirt3 protein levels in human lung adenocarcinoma tissue was observed (Fig. 1B).
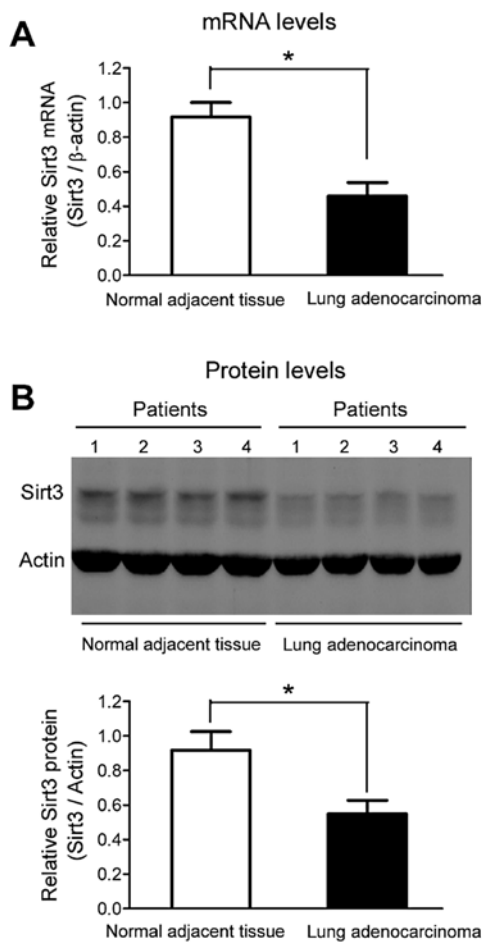
Figure 1. Downregulation of Sirt3 in human lung adenocarcinoma tissue. (A) mRNA level of Sirt3 in human lung adenocarcinoma tissue was measured by real-time PCR analysis. *P<0.05 vs. normal tissue. n=4. (B) Immunoblotting and quantitative analysis of the expression of Sirt3 in human lung adenocarcinoma and normal adjacent tissues. *P<0.05 vs. normal tissue. n=4.
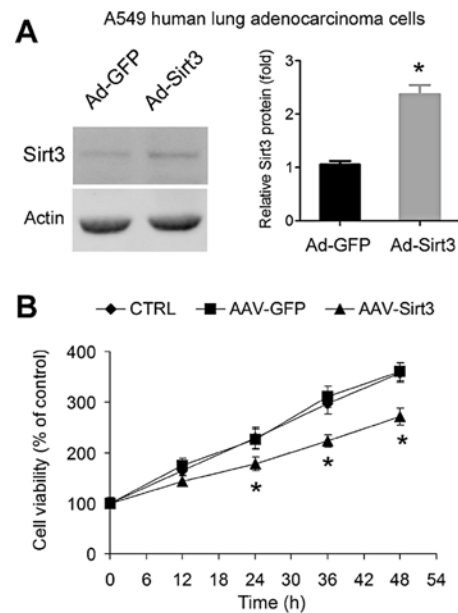

Figure 2. Sirt3 overexpression inhibits lung adenocarcinoma cell growth. (A) Immunoblotting demonstrating the overexpression of Sirt3 using adenovirus in A549 lung adenocarcinoma cells. *P<0.05 vs. Ad-GFP (vector control). n=4. (B) Cell growth curve of A549 lung adenocarcinoma cells transfected with Ad-GFP or Ad-Sirt3. *P<0.05 vs. Ad-GFP (control). n=8.
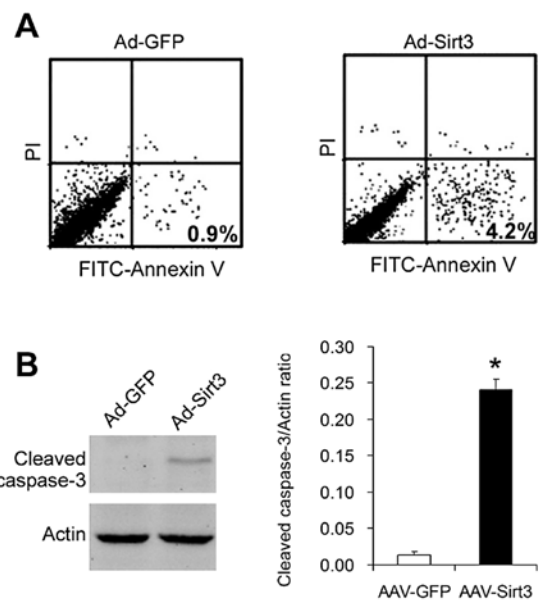

Figure 3. Apoptosis of lung adenocarcinoma cells induced by Sirt3 overexpression. (A) Annexin V + PI assay demonstrating the proportion of apoptotic cells in Sirt3-overexpressing A549 lung adenocarcinoma cells (Ad-Sirt3) and control A549 lung adenocarcinoma cells. (B) Immunoblotting demonstrating cleaved caspase-3 expression in Sirt3-overexpressing and control cells. *P<0.05 vs. Ad-GFP (control). n=5.

Overexpression of Sirt3 inhibits lung adenocarcinoma cell growth. We next tested whether overexpression of Sirt3 influences A549 lung adenocarcinoma cell growth *in vitro*. Overexpression of Sirt3 using adenovirus (Fig. 2A) significantly inhibited the growth of A549 cells (Fig. 2B).

Overexpression of Sirt3 induces apoptosis in lung adenocarcinoma cells. We studied the effects of overexpression of Sirt3 on the apoptosis of lung adenocarcinoma cells. First, we analyzed Ad-GFP- and Ad-Sirt3-transfected cells with the FITC-Annexin V + PI staining assay. As shown in Fig. 3A, the ratio of apoptotic cells (Annexin V+/PI-) was ~0.9% in the control Ad-GFP-transfected cells, whereas the apoptotic ratio increased to 4.2% in Ad-Sirt3-transfected cells.

Then, we analyzed the levels of cleaved caspase-3, a key mediator and marker of apoptosis. As shown in Fig. 3B, cleaved caspase-3 expression was detected in the cells overexpressing Sirt3 (Ad-Sirt3-transfected cells) but not in the control cells (Ad-GFP-transfected cells).

Overexpression of Sirt3 modulates apoptotic-related proteins in lung adenocarcinoma cells. The Bcl-2 family proteins and AIF are important determinants regulating cellular apoptosis.

Overexpression of Sirt3 increased the bax-bcl-2 ratio and bad-bcl-x/L ratio (Fig. 4A). Moreover, immunofluorescence analysis (Fig. 4B) showed that Sirt3 overexpression induced AIF nuclear translocation.

Overexpression of Sirt3 upregulates the p53 signaling pathway in lung adenocarcinoma cells. Next, we studied
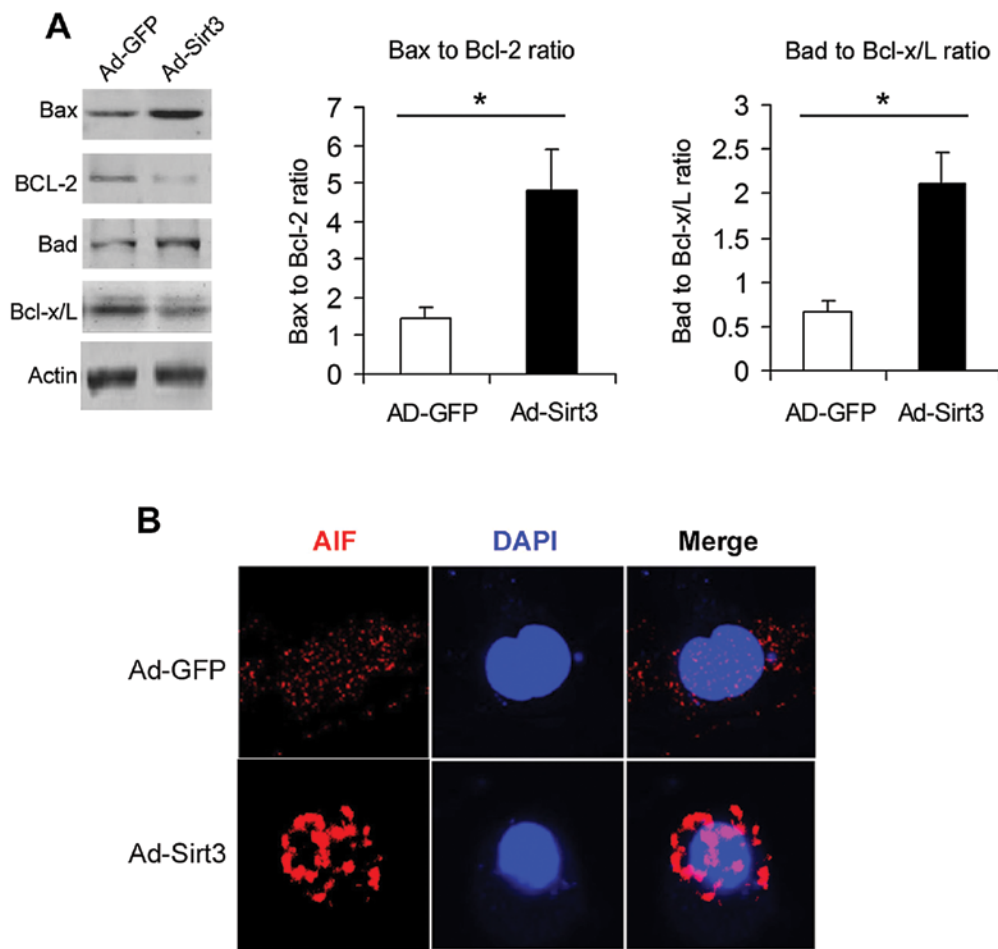
Figure 4. Influence of Sirt3 overexpression on apoptosis-related proteins in lung adenocarcinoma cells. (A) Representative images of immunoblotting and quantitative analysis of bax, bcl-2, bad and bcl-x/L. Actin was used as a loading control. *P<0.05 vs. Ad-GFP. n=6. (B) Representative images of immunofluorescence showing the nuclear translocation of AIF induced by Sirt3 overexpression. AIF was stained by the AIF-specific primary antibody and then the Alexa 555-conjugated secondary antibody. DAPI was used to stain the nucleus.
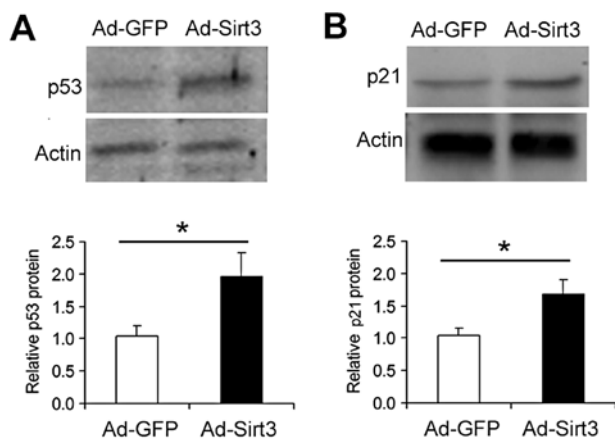

Figure 5. Sirt3 overexpression upregulates p53 and p21 in lung adenocarcinoma cells. (A) Representative images of immunoblotting and quantitative analysis of p53 protein levels. *P<0.05 vs. Ad-GFP. n=4. (B) Representative images of immunoblotting and quantitative analysis of p21 protein levels. *P<0.05 vs. Ad-GFP. n=4.

the influence of Sirt3 overexpression on p53 signaling in A549 cells. Compared with the Ad-GFP-transfected cells,

Ad-Sirt3-transfected cells exhibited increased protein levels of p53 (Fig. 5A) and p21 (Fig. 5B), a p53-downstream factor.

Overexpression of Sirt3 decreases oxidative stress in lung adenocarcinoma cells. Finally, we studied the influence of Sirt3 overexpression on oxidative stress in A549 cells. The reactive oxygen species (ROS) generation measured by the DCFH-DA assay (Fig. 6A) revealed that overexpression of Sirt3 significantly decreased the total ROS level in A549 cells (Fig. 6B). Moreover, overexpression of Sirt3 attenuated the superoxide anion level in A549 cells (Fig. 6B).

Discussion

In the present study, we initially demonstrated that Sirt3 was markedly downregulated in lung adenocarcinoma tissue when compared with that in the normal adjacent tissue. Using adenovirus-mediated overexpression, we found that Sirt3 overexpression inhibited the growth of A549 lung adenocarcinoma cells. Further analyses, including Annexin V + PI assay, cleaved caspase-3 immunoblotting, bax-bcl-2 ratio, bad-bcl-x/L ratio and AIF translocation, showed that Sirt3 overexpression promoted apoptosis in A549 lung adenocarcinoma cells.
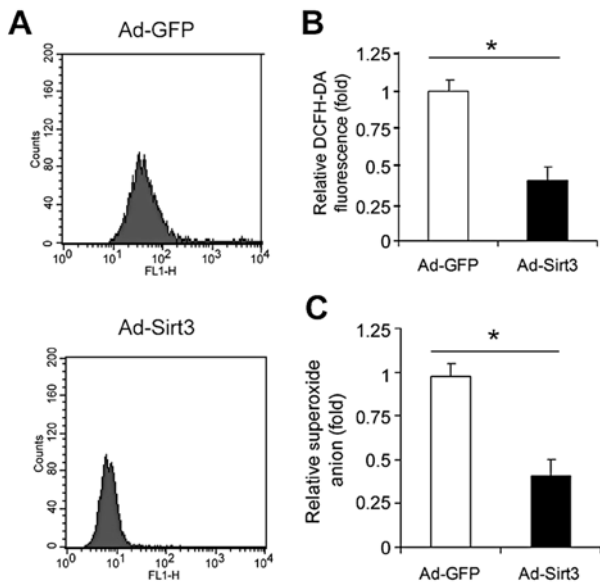
Figure 6. Sirt3 overexpression decreases the ROS level in lung adenocarcinoma cells. (A and B) Dichlorofluorescein diacetate (DCFH-DA) fluorescent intensity was assessed by flow cytometric analysis and shown in the histogram. *P<0.05 vs. Ad-GFP. n=8. (C) Assessment of the superoxide anion level in lung adenocarcinoma cells. *P<0.05 vs. Ad-GFP. n=8.

Furthermore, Sirt3 overexpression downregulated ROS and superoxide anion levels in A549 lung adenocarcinoma cells. These data clearly suggest that Sirt3 is a tumor suppressor through the induction of apoptosis in lung adenocarcinoma cells.

The first important finding of our study is that Sirt3 was downregulated in human lung adenocarcinoma tissue. Acetylation was found in >20% of mitochondrial proteins, including many longevity regulators and metabolism enzymes in a large-scale proteomics analysis (26), implicating the wide influence of acetylation modification among mitochondrial proteins. As acetylation modification is mainly mediated by sirtuins and Sirt3 is the major deacetylase of sirtuins in mitochondria (17), Sirt3 is believed to be a potent regulator in mitochondria. Furthermore, the energy status dictates the status of mitochondrial protein acetylation, suggesting that Sirt3-mediated mitochondrial acetylation may be a critical regulatory mechanism underlying the adaptive response to energy stress. In line with the potential role of Sirt3 in mitochondrial biology, metabolically active tissues with high oxidative capacity, such as skeletal muscle, liver, brain, kidney and adipose tissue, express Sirt3 abundantly (27). The downregulation of Sirt3 in lung adenocarcinoma may lead to enhanced acetylation status and ROS generation in mitochondria, which are tightly associated with higher cancer risk (28,29).

Previously, significant reduction in the Sirt3 copy number was found in human breast cancer (30) and hepatocellular carcinoma (21,31). Moreover, loss of Sirt3 in vitro led to tumorigenesis (19), and Sirt3 levels were decreased in human breast cancer (19,20). In breast cancer cells, Finley et al (20) demonstrated that SIRT3 mediates metabolic reprogramming by destabilizing hypoxia inducible factor 1α (HIF-1α), a transcription factor that controls glycolytic gene expression.

Murine tumors lacking Sirt3 exhibit abnormally high levels of ROS that directly induce genomic instability and cellular metabolic reprogramming (20). Bell et al (32) also showed that Sirt3 suppressed tumor growth via inhibition of HIF-1α. All these data suggest the inhibitory effects of Sirt3 in tumor. However, we also noted a contradictory finding. Sirt3 was upregulated in oral cancer and was required to protect oral cancer from stress-mediated cell death by various stimuli (33). In our studies, we found that Sirt3 was downregulated in human lung adenocarcinoma tissue, supporting the tumor-suppressive role of Sirt3 in lung adenocarcinoma. Subsequent functional analysis also showed that Sirt3 overexpression inhibited A549 lung adenocarcinoma cell proliferation.

The inhibitory effect of Sirt3 overexpression on lung adenocarcinoma cell growth was very obvious in vitro, which prompted us to study apoptosis. Using Annexin V + PI assay and cleaved caspase-3 immunoblotting, we confirmed the apoptosis-inducing effects of Sirt3 in A549 lung adenocarcinoma cells. After revealing the pro-apoptotic feature of Sirt3, we observed that Sirt3 overexpression was modulated by apoptotic signaling pathways (bax, bcl-2, bad, bcl-x/L and AIF). Bcl-2 family proteins are known to play a pivotal role in the induction of mitochondrial caspase activation and in the regulation of apoptosis (34). AIF is a flavoprotein that is normally confined to the mitochondrial intermembrane space, yet translocates to the nucleus to induce peripheral chromatin condensation and triggers large-scale DNA degradation to fragments of ~50 kbp (35). In our study, overexpression of Sirt3 increased bax-bcl-2 and bad/bcl-x/L ratios and induced AIF nuclear translocation. We postulated that these changes may contribute to the apoptotic-inducing effects of Sirt3 in A549 lung adenocarcinoma cells.

Finally, we provide evidence showing that Sirt3 overexpression was associated with an upregulated p53 protein level and a decreased ROS level in lung adenocarcinoma cells. Sirt1, another member of the sirtuin family, has been found to be able to directly deacetylate p53 and increase p53 protein level (36,37). Recently, a study demonstrated that Sirt3 also enhanced the p53 protein level in hepatocellular carcinoma cells (21). In agreement with these studies, we observed that Sirt3 overexpression upregulated p53 and its downstream factor p21 in lung adenocarcinoma cells. Giving that p53 is a well-known tumor suppressor (38), we hypothesized that p53 upregulation is quite likely to contribute to the tumor-suppressive effect of Sirt3 in lung adenocarcinoma cells.

Collectively, we demonstrated that the expression of Sirt3 was decreased in human lung adenocarcinoma tissue. Overexpression of Sirt3 exhibited an obvious antitumor effect in the A549 lung adenocarcinoma cell line through induction of apoptosis. Our finding concerning the regulation of lung adenocarcinoma cell growth by Sirt3 may provide an important focus for the further understanding of lung adenocarcinoma and novel therapeutic interventions.

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