Original Article

Sirtuin SIRT6 suppresses cell proliferation through inhibition of Twist1 expression in non-small cell lung cancer

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Abstract: SIRT6 is a member of the NAD$^+$-dependent class III deacetylase sirtuin family. Current studies have revealed that SIRT6 plays important roles in the epigenetic regulation of genes expression and contribute to the proliferation, differentiation and apoptosis of cancer cells. However, the biological function of SIRT6 in lung cancer has not been elucidated. The present study showed that the mRNA and protein levels of SIRT6 were decreased in human non-small cell lung cancer (NSCLC) tissues and cell lines. MTT assay showed that overexpression of SIRT6 could inhibit the proliferation in NSCLC cells. In contrast, SIRT6 knockdown using small interfering RNA promoted NSCLC cells proliferation. On the molecular level, we found that SIRT6 inhibited the expression of Twist1 both at the mRNA and protein levels in NSCLC cells. Taken together, these results demonstrated for the first time that SIRT6 suppressed NSCLC cells proliferation via down-regulation of Twist1 expression and might provide novel therapeutic targets in the treatment of lung cancer.

Keywords: Lung cancer, cell proliferation, SIRT6, Twist1

Introduction

Lung cancer is one of the most common causes of cancer-related deaths worldwide, and the majority of lung cancers are the non-small cell lung cancer (NSCLC), which accounts for approximately 80% of all lung cancers [1]. Statistical analysis shows that approximately 40% of patients with NSCLC present with advanced-stage disease, for which 5-year survival rates are in the region of 2% [2]. During the past two decades, the platinum-based regimens have been the mainstay of lung cancer therapy. However, recent advances have begun to explore the molecular biology of the disease and developed various novel targeted agents, such as epidermal growth factor receptor tyrosine kinase inhibitors that exhibit greater efficacy than chemotherapy in patients with epidermal growth factor receptor-mutated tumors [3, 4]. Although great efforts and progressions have been made in the study of the lung cancer in recent decades, the molecular mechanism of lung cancer pathogenesis still remains elusive. Therefore, it is urgent to develop new treatment strategies for NSCLC in the clinic.

The sirtuin family comprises seven members, which are NAD$^+$-dependent protein deacetylases and/or mono-[ADP-ribosyl] transferases [5-7]. Studies demonstrated that these proteins diverge in localization and functions, with SIRT1, 2, 6, and 7 acting as critical modulators of epigenetic modifications, while SIRT3, 4 and 5 functioning mostly in the mitochondria [8, 9]. Among them, investigation of sirtuin 6 (SIRT6) shows that it is involved in the regulation of glucose metabolism, genome stability, longevity and inflammation [10-12]. In addition, recent study found that SIRT6 regulates the development of various types of cancers, such as pancreatic cancer, liver cancer, colon adenocarcinoma and breast cancer [13-15]. However, the biological role of SIRT6 in NSCLC has never been investigated. Therefore, in the present study, we examined the expression of SIRT6 in human NSCLC samples and further investigated its biological function in NSCLC cells.
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Figure 1. SIRT6 expression was down-regulated in human lung cancer tissues and cell lines. (A, B) SIRT6 expression at the mRNA and protein levels was measured by real-time PCR and western blot in human NSCLC tissues and normal tissues. *P<0.05; **P<0.01. (C, D) Real-time PCR and western blot were performed to determine the SIRT6 expression in NSCLC cell line (A549 and NCI-H23) and an immortalized human bronchial epithelial cell line (16HBE-T). *P<0.05; **P<0.01.

Materials and methods

Patients

All human tissue samples of NSCLC specimens were obtained from Peking Union Medical College Hospital. Informed consent for the use of samples was obtained from all patients before surgery, and approval was obtained from the ethic committee of Peking Union Medical College Hospital.

Cell culture

The human NSCLC cell line A549, NCI-H23 and an immortalized human bronchial epithelial cell line 16HBE-T were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO₂ at 37°C.

Plasmid construction, siRNA and transfection

The cDNA fragment encoding SIRT6 and Twist1 was isolated with Takara RNA PCR kit (Takara, Japan) using total RNAs from lung cancer cell line. The primers sequences were as following: SIRT6 forward, 5’-AAGCTGGAGCCCAAGGAGGAA-3’ and reverse, 5’-AAGAATGTGCCAAGTGAAGA-3’; Twist1 forward 5’-ATGGCTTACCA-
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Figure 2. SIRT6 inhibited the proliferation of NSCLC cells. A. SIRT6 expression was determined by western blot in A549 cells transfected with plasmids encoding SIRT6-flag. B. MTT assay was used to measure the proliferation of NSCLC cells A549 after transfection with plasmids encoding SIRT6-flag. C, D. Real-time PCR and western blot were used to measure SIRT6 expression in A549 cells after transfection with siRNA oligos. *P<0.05; **P<0.01. E. The cells proliferation was measured using the MTT assay in A549 and A549 cells transfected with SIRT6 siRNA oligos.

TACGATGTCCAGAT-3' and reverse, 5'-ATGCAGGCAGTGTCCAGCTGC-3'.

PCR products were cloned into pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). siRNA SIRT6 were purchased from Invitrogen (Carlsbad, CA, USA). Cells were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instruction.

**Real-time PCR**

Total RNAs were isolated from tissues or cells by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Tokyo, Japan) according to the manufacturer’s instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Tokyo, Japan) on ABI 7500 system (Applied Biosystems, Foster, CA, USA).

**Western blot analysis**

Cells were harvested by trypsination, lysed in buffer and prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After immunoblotting, the membranes were blocked in PBS/0.1% Tween-20 with 5% nonfat dry milk, and incubated with primary antibodies against SIRT6, Twist1 and GAPDH (Santa Cruz, CA, USA). GAPDH was used as a
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Figure 3. Overexpression of SIRT6 decreased the expression of Twist1. A. Real-time PCR was applied to measure the mRNA expression of Twist1 in A549 cells over-expressing SIRT6. *P<0.05; **P<0.01. B, C. Western blot was performed to determine the Twist1 protein expression in A549 cells over-expressing SIRT6. Relative band intensities of each protein were quantified by densitometry. *P<0.05; **P<0.01.

loading control. The proteins were visualized by the enhanced chemiluminescence method and intensity of protein bands was quantified by densitometry.

Statistical analysis

All data were presented as mean ± SD and treated for statistics analysis by SPSS program. Comparison between groups were determined by ANOVA and statistical significance was indicated as *P<0.05.

Results

SIRT6 is down-regulated in NSCLC tissues and cell lines

Firstly, we examined the mRNA expression of SIRT6 in 36 paired NSCLC and adjacent non-tumor normal tissues by real-time PCR. Data showed that mRNA expression of SIRT6 in NSCLC tissues was significantly down-regulated compared with the normal tissues (Figure 1A). Western blot analysis indicated that the SIRT6 protein expression was also down-regulated in NSCLC samples (Figure 1B). Furthermore, SIRT6 expression in NSCLC cell lines were analyzed by real time PCR and western blot. We found that SIRT6 was obviously decreased both at the mRNA (Figure 1C) and protein (Figure 1D) levels in two NSCLC cell lines compared with normal human bronchial epithelial cell line 16HBE-T, suggesting that SIRT6 expression was down-regulated in NSCLC tissues and cell lines.

Overexpression of SIRT6 inhibited the proliferation of NSCLC cells

To further investigate the biological role of SIRT6 in NSCLC, A549 cells were transfected
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Figure 4. Down-regulation of SIRT6 increased the expression of Twist1. A. Real-time PCR was used to measure the mRNA levels of Twist1 after knockdown of SIRT6 in A549 cells. *P<0.05; **P<0.01. B, C. Western blot was used to measure the Twist1 protein expression after down-regulation of SIRT6 in A549 cells. Relative band intensities of each protein were quantified by densitometry. *P<0.05; **P<0.01.

with plasmids containing SIRT6 (Figure 2A). MTT assay showed that SIRT6 overexpression significantly inhibited the proliferation of NSCLC cells (Figure 2B). In contrast, down-regulation of SIRT6 with small interfering RNA (siRNA) in A549 cells was confirmed by real time PCR and western blot (Figure 2C and 2D). Consequently, cell growth was obviously enhanced after SIRT6 knockdown in NSCLC cells (Figure 2E). Similar results were also observed in NCI-H23 cells (data not shown). Taken together, our results demonstrated that SIRT6 could inhibit the proliferation of NSCLC cells.

SIRT6 inhibited Twist1 expression in NSCLC cells

We furthermore investigated the molecular mechanism underlying the inhibitory effects of SIRT6 on NSCLC cells, data showed that mRNA expression of Twist1 was reduced after overexpression of SIRT6 in A549 cells (Figure 3A). Western blot analysis showed that Twist1 protein expression was significantly decreased (Figure 3B and 3C). In contrast, down-regulation of SIRT6 with siRNA obviously elevated the Twist1 expression both at mRNA (Figure 4A) and protein levels (Figure 4B and 4C). These results showed that SIRT6 negatively regulated Twist1 expression.

SIRT6 inhibited NSCLC cells proliferation by down-regulation of Twist1

In order to further investigate how SIRT6 functions an inhibitory effect on NSCLC cells proliferation; the expression of Twist1 was up-regulated after transfection with plasmids encoding Twist1 as shown by real time PCR and western blot (Figure 5A and 5B). As a result, the inhibitory effects of SIRT6 on cell proliferation were partially reversed after overexpression of Twist1 in A549 cells (Figure 5C). Taken together, these data showed that SIRT6 could suppress NSCLC cell proliferation by down-regulation of Twist1.
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Discussion

It has been reported that sirtuin family members play crucial roles in carcinogenesis [8, 9]. But the biological function of SIRT6 in NSCLC cells remains poorly understood. In the current study, we for the first time investigated the biological function of SIRT6 in NSCLC tissues and cells lines.

Sirtuin are protein deacetylases/ADP ribosyl transferases that target a wide range of cellular proteins in the nucleus, cytoplasm, and mitochondria for post-translational modification by acetylation or ADP ribosylation, therefore modulating the expression levels of many genes [16]. Their aberrant expression is closely related with tumor initiation and development [17]. During the past several years, the sirtuin family member SIRT6 has been deeply investigated and current evidence show that SIRT6 participates in metabolism, inflammation, and carcinogenesis [14]. In our research, we found that both the mRNA and protein expression of SIRT6 were significantly decreased in NSCLC tissues and cells lines.

Increasing studies indicate that SIRT6 is involved in regulation of both establishment and maintenance of cancers. Loss of SIRT6 leads to tumor formation, whereas SIRT6-deficient cells display elevated tumor growth [18]. A recent study demonstrated that SIRT6 overexpression induces massive apoptosis in a variety of cancer cell lines but not in normal, non-transformed cells, which is mediated by the activation of both the p53 and p73 apoptotic signaling cascades [19]. In pancreatic cancer cells, SIRT6 enhances the expression of pro-inflammatory cytokines and chemokines, such as IL8 and TNF, and promotes cell migration [20]. In liver cancer, SIRT6 represses survivin expression and NF-kB activation, which markedly impairs the initiation and development of cancer cells [21]. In our study, overexpression of SIRT6 in NSCLC cells significantly inhibited cell proliferation, whereas SIRT6 knockdown promoted the NSCLC cell prolifera-

Figure 5. SIRT6 inhibited NSCLC cells proliferation by down-regulation of Twist1. A549 cells over-expressing SIRT6 were transfected with plasmids encoding Twist1. Expression of Twist1 at the mRNA (A) and protein (B) levels was detected by real time PCR and western blot. *P<0.05; **P<0.01. (C) MTT assay was used to measure the cell proliferation in A549 cells over-expressing SIRT6 and Twist1.
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...tion, implicating that SIRT6 acts as a negative regulator of growth in NSCLC cells.

Twist1 belongs to a family of basic helix-loop-helix (bHLH) transcription factors that regulates mesodermal cell fate in Drosophila [22]. Recent investigation has shown that Twist1 is a potential oncogene and over-expressed in numerous tumor cells such as breast cancer, hepatocellular carcinoma and lung cancer [23-25]. Twist1 plays an important role in apoptosis and drug resistance of the tumor, as well as in epithelial-mesenchymal transition, tumor proliferation and metastasis [26-28]. Our study found that SIRT6 could significantly down-regulate the expression of Twist1. In addition, the inhibitory effect on cell proliferation was diminished after over-expression of Twist1 in NSCLC cells, indicating that anti-proliferative activity of SIRT6 was mediated by Twist1.

In conclusion, our results demonstrated that SIRT6 suppresses the proliferation of NSCLC cells via inhibition of Twist1 expression and might provide novel therapeutic targets in the NSCLC treatment.

Disclosure of conflict of interest

The authors have declared no potential conflicts of interest.

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