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Original Article

Downregulation of Cited1 suppresses cell proliferation by inducing G2/M arrest of the cell cycle in non-small-cell lung cancer cell lines

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Abstract: Abnormal expression of CBP/p300-interacting transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain1 (Cited1) has been reported in several human cancers. However, little information is available regarding the effects of Cited1 on non-small-cell lung cancer (NSCLC). The present study was aimed to explore the effects of downregulation of Cited1 on NSCLC, as well as the underlying mechanism. NSCLC cell lines A549, HCC827 and H1975 cells were transfected with small interfering RNA (siRNA) against Cited1 (siCited1). After transfection, the expression of Cited1 was confirmed by quantitative RT-PCR (qRT-PCR) and Western blot. Cell viability was analyzed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and cell cycle analysis was determined by flow cytometry. In addition, expression of cyclin-dependent kinase 1 (CDK1), phosphorylated CDK1 (p-CDK1), cell division cycle 25C (CDC25), and Cyclin B1 was measured by qRT-PCR and Western blot. Both the mRNA and protein levels of Cited1 were significantly down-regulated by transfection with siCited1 ($P < 0.05$). Transfection with siCited1 significantly decreased the cell viability ($P < 0.05$) and the percentages of cells in G1 phase, while markedly increased the percentages of cells in G2/M phase in the three cell lines. Both the mRNA and protein levels of p-CDK1, CDK1, CDC25C and Cyclin B1 were statistically reduced by downregulation of Cited1 compared to the control group ($P < 0.05$). However, no significant differences were observed in CDK1. Downregulation of Cited1 suppresses cell proliferation by inducing G2/M arrest of the cell cycle in NSCLC cell lines.

Keywords: Non-small-cell lung cancer, Cited1, cell proliferation, G2/M arrest

Introduction

Lung cancer is the leading cause of cancer-related death in both men and women worldwide, and most cases are due to cigarette smoking [1]. The incidence is increasing every year worldwide [2, 3]. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of lung cancers [4, 5]. The current therapy for NSCLC includes surgical therapy, palliative treatment, chemotherapy, molecular targeting, and/or a combination. Although tremendous advance has been developed in improvement in molecular diagnosis and targeted therapies in the last decade, the outcome of advanced NSCLC remains dismal with the 5-years overall survival rate of approximately 10-15% [6]. Besides, most patients with NSCLC are diagnosed with advanced cancer, increasing the poor prognosis. Therefore, a better understanding of the molecular mechanisms involved in the pathogenesis of NSCLC might provide new insights into targeted strategy to treat NSCLC.

In recent years, the role of CBP/p300-interacting transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain (Cited1) in cancers has been paid great attention. The Cited1 gene encodes a member of the Cited family of protein, and functions as a transcriptional coactivator [7]. Its encoded protein is also known as melanocyte-specific gene 1 (MSG1). Expression of Cited1 has been found in many cells, such as melanocytes, breast epithelial cells, and testicular germ cells [8]. Abnormal expression of Cited1 has been reported in several types of cancers, such as Wilms' tumor [9], papillary thyroid carcinoma (PTC) [10,

11], hepatoblastoma [12], breast cancer [13, 14], and colorectal cancer (CRC) [15]. However, rare studies have been devoted to the functional role of Cited1 in NSCLC.

Therefore, in the present study, we aimed to uncover the functional role of Cited1 in NSCLC. Three NSCLC cell lines, A549, HCC827 and H1975 cells were transfected with small interfering RNA (siRNA) against Cited1. Then we analyzed the cell proliferation and cell cycle distribution after silencing the expression of Cited1, and investigated the underlying mechanism.

Materials and methods

Cell culture

Three NSCLC cell lines, A549, HCC827 and H1975 cells were used in the study. All the cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen Life Technologies, Carlsbad) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies), 100 U/mL penicillin (Gbico-BRL, Grand Island, NY, USA), and 100 mg/L streptomycin (Gbico-BRL) at 37°C in a humidified 95% air-5% CO_2 atmosphere.

Cell transfection

SiRNA transfection was used to suppress the expression of Cited1. Briefly, these three lines (2×10^5 cells/per well) were seeded on 96 well-plates and incubated for 24 h. Thereafter, Cited1 siRNA or its negative control (Genepharma, Shanghai, China) were transfected into the three lines cells respectively. The transfection was carried out by using Lipofectamine 2000 (Invitrogen, USA), following the manufacturer's instruction. After 48 h of transfection, the cells were harvested for further analyses.

Cell viability

Cell viability was analyzed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, the three lines were cultured on 12-well plates (5×10^4 cells/well). After 48 h of transfection with siCited1, 20 μL of 10 mg/mL MTT (Sigma-Aldrich, St Louis, MO) were added to 0.2 mL of culture medium and were incubated for 4 h at 37°C. Dimethylsulfoxide (DMSO; Sigma-Aldrich) was then added to dissolve the MTT. Absorbance at 590 nm was read on a microplate reader (Bio-Rad, Richmond, CA).

Cell cycle analysis by flow cytometry

The three cell lines were plated in 96-well plates and transfected with siCited1 for 48 h at 37°C. The cells were collected and fixed with cold 70% ethanol at 4°C overnight. After washing with phosphate buffer saline (PBS), the cells were then incubated with propidium iodide (PI) (50 μg/mL, Sigma-Aldrich) with RNase A (100 μg/mL) for 1 h at room temperature in the dark. Thereafter, flow cytometry was performed to analyze the percentage of cells in sub-G1, G1, S, and G2/M phases using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Expression of CDK1, CDC25C, Cyclin B1 mRNA in the three cell lines was analyzed using qRT-PCR after transfection with siCited1 for 48 h. Total RNA was extracted from these three lines with Trizol reagent (Life Technologies) according to the manufacturer's recommendation. The reverse transcription reaction was performed using the reverse transcription kit (Applied Biosystems, Fostercity, CA). The expression mRNAs levels were determined by SYBR green-based quantitative RT-PCR (SYBR Green PCR Master Mix, Applied Biosystems, Carlsbad, CA). GAPDH was used as a loading control. Data of genes was analyzed using $2^{-\Delta\Delta Ct}$ statistical method. Reactions were carried out in triplicate.

Western blot

For Western blot analysis, the three cell lines were transfected with siCited1 for 48 h, and were collected for protein extraction and concentration determination. The protein density was measured using BCA assay kit (Pierce, Rockford, IL) according to the manufacturer's instruction. Proteins samples were then subjected to a 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose blotting membranes (Bedford, MA, USA). Thereafter, the membranes were blocked in 5% defatted milk powder in Tris Buffered Saline With Tween (TBST) buffer for 2 h at room tempera-

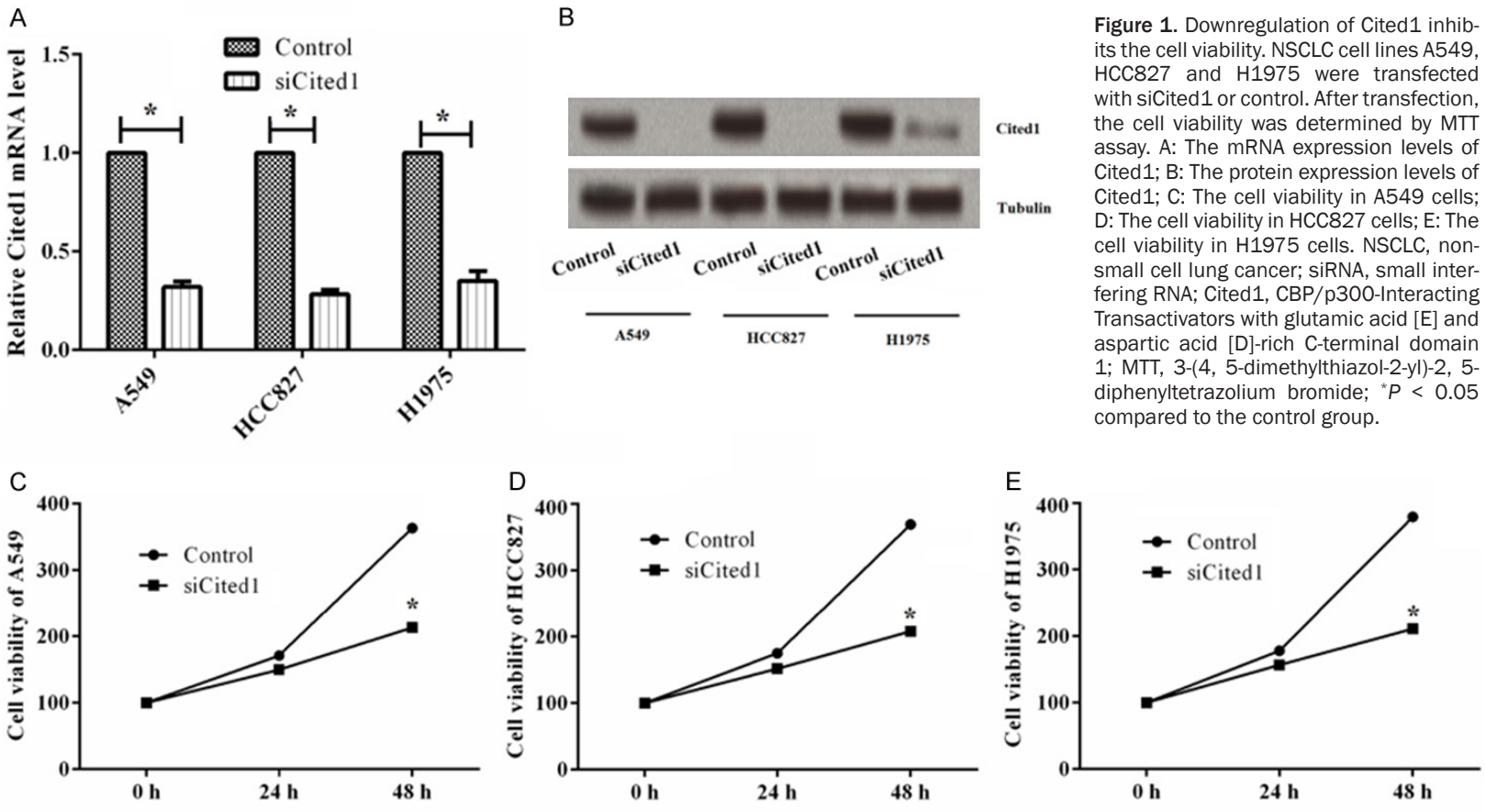

Figure 1. Downregulation of Cited1 inhibits the cell viability. NSCLC cell lines A549, HCC827 and H1975 were transfected with siCited1 or control. After transfection, the cell viability was determined by MTT assay. A: The mRNA expression levels of Cited1; B: The protein expression levels of Cited1; C: The cell viability in A549 cells; D: The cell viability in HCC827 cells; E: The cell viability in H1975 cells. NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; Cited1, CBP/p300-Interacting Transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain 1; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; $^{*}P < 0.05$ compared to the control group.

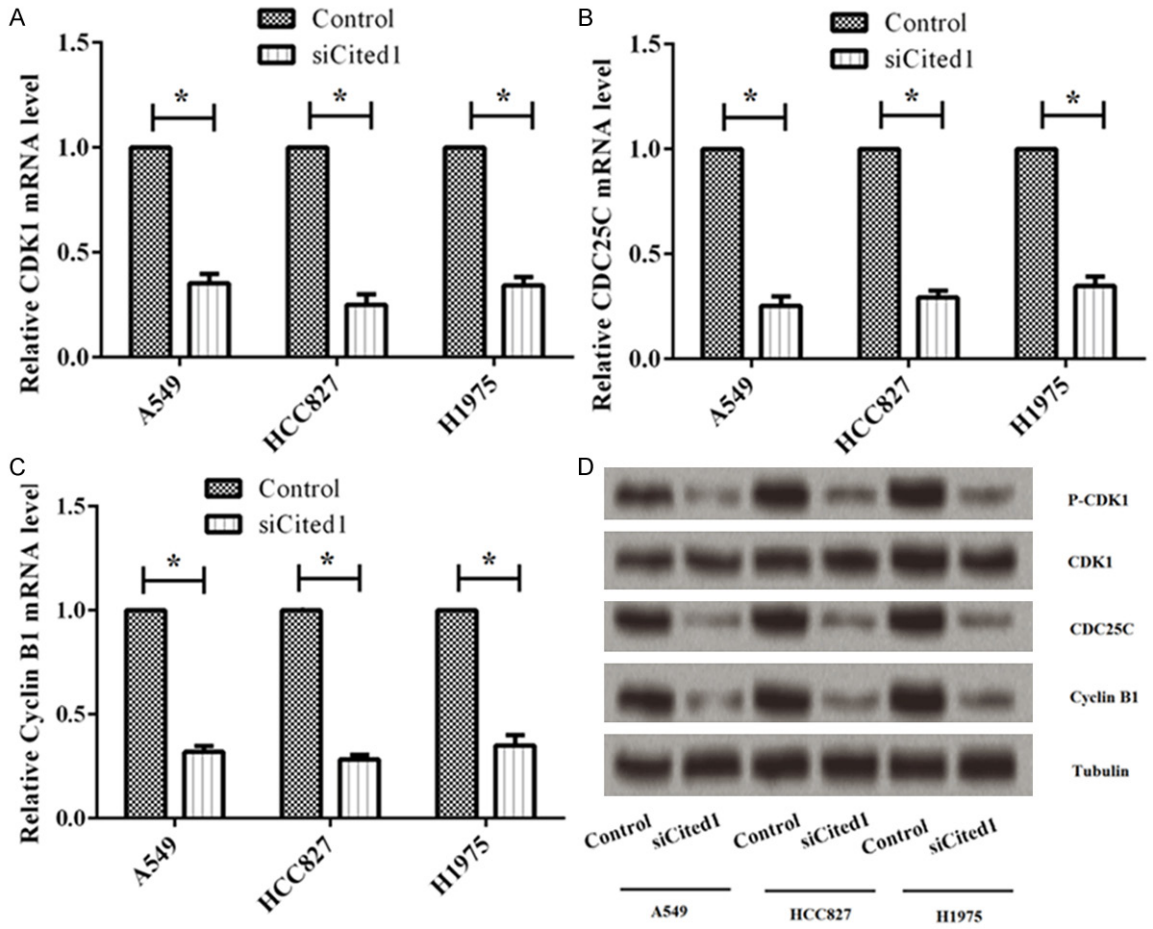

Figure 2. Downregulation of Cited1 reduced the expression of CDK1, CDC25C and Cyclin B1. The expression levels of p-CDK1, CDK1, CDC25C and Cyclin B1 were determined by Qrt-PCR and/or Western blot. A: Relative mRNA expression level of CDK1; B: Relative mRNA expression level of CDC25C; C: Relative mRNA expression level of Cyclin B1; D: The protein expression levels of p-CDK1, CDK1, CDC25C and Cyclin B1. CDK1, cyclin-dependent kinase 1; p-CDK1, phosphorylated cyclin-dependent kinase 1; CDC25C, cell division cycle 25C; siRNA, small interfering RNA; Cited1, CBP/p300-Interacting Transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain 1; *P< 0.05 compared to the control group.

ture. Subsequently, the membranes were probed with the following primary antibodies overnight at 4°C: anti-Cited1 antibody (ab87978, Abcam, Cambridge, UK), anti-CDK1 antibody (ab18, Abcam, Cambridge, UK), anti-p-CDK1 antibody (ab47329, Abcam, Cambridge, UK), anti-CDC25C antibody (#4688, Cell Signaling Technology Inc., Beverly, MA), and anti-Cyclin B1 antibody (#12231, Cell Signaling Technology Inc., Beverly, MA). Tubulin (#5335, Cell Signaling Technology Inc., Beverly, MA) was used as a reference control. After 2 h of incubation with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature, the membranes were subjected to enhanced chemiluminescence and densitometric analysis.

Statistical analysis

All the experiments were performed in triplicate. The data are shown as the mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism 6 software (GraphPad, San Diego, California, USA). Statistical significance was analyzed by t tests or one-way analysis of variance (ANOVA). Statistical significance was defined as a $P < 0.05$.

Results

Downregulation of Cited1 inhibited the cell viability

To investigate the effects of Cited1 on NSCLC, A549, HCC827 and H1975 cells were first

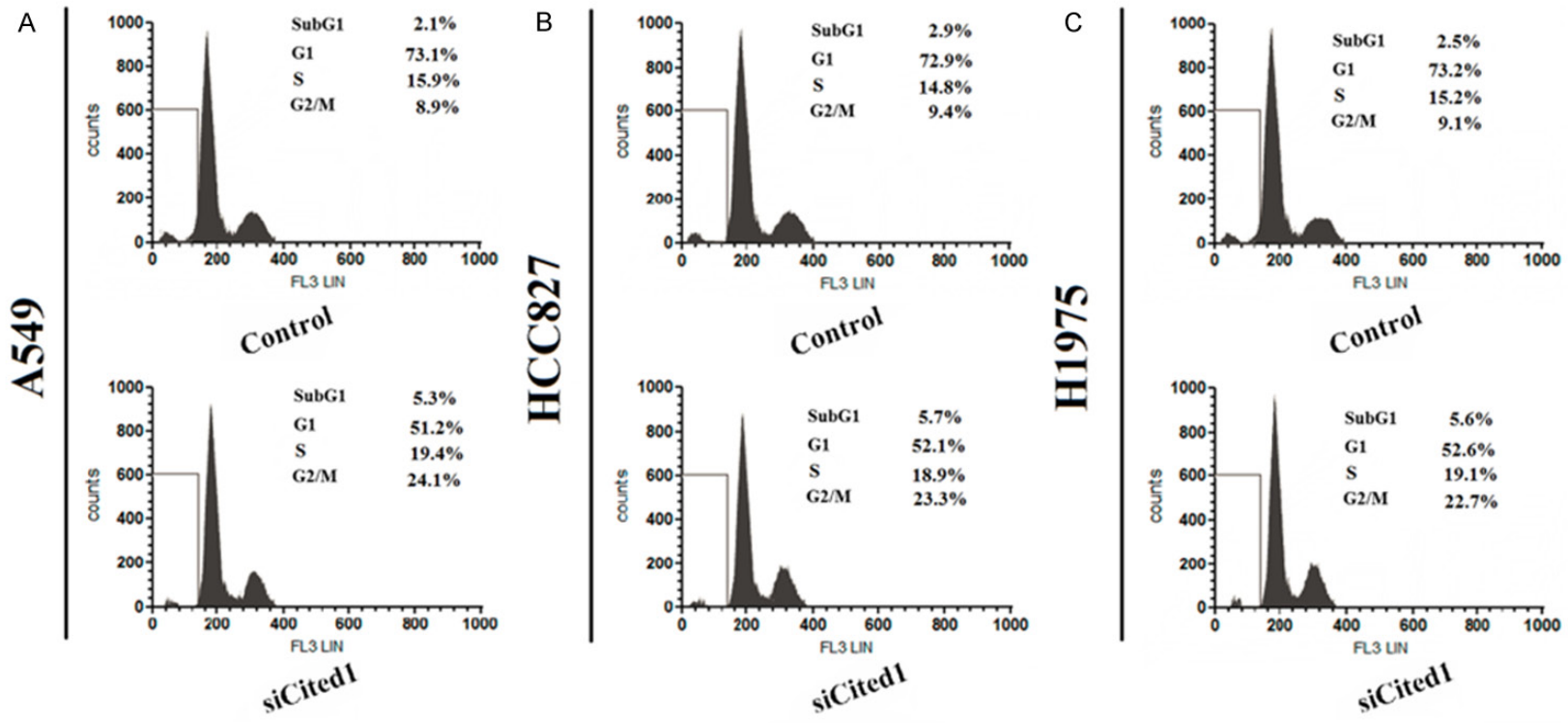

Figure 3. Downregulation of Cited1 induces G2/M phase arrest of cell cycle progression. Flow cytometric analysis was performed to assess the cell cycle distribution after transfection with siCited1. A: The cell cycle distribution in A549 cells; B: The cell cycle distribution in HCC827 cells; C: The cell cycle distribution in H1975 cells. Cited1, CBP/p300-Interacting Transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain 1; siRNA, small interfering RNA.

transfected with siCited1 or control. The expression levels of Cited1 were confirmed by qRT-PCR and Western blot. As expected, both the mRNA and protein levels of Cited1 were significantly down-regulated by transfection with siCited1 in A549, HCC827 and H1975 cells compared to the control group ($P < 0.05$) (**Figure 1A** and **1B**). After transfection, the cell viability was determined by MTT assay. As shown in **Figure 1C-E**, the results showed that the cell viability was significantly decreased in A549, HCC827 and H1975 cells by transfection with siCited1 at 48 h compared to their control group ($P < 0.05$). No significant difference was found at 0 and 24 h. The results indicated that downregulation of Cited1 exerted anti-proliferation effects on NSCLC cells.

Downregulation of Cited1 reduced the expression of p-CDK1, CDC25C and cyclin B1

Furthermore, we investigated the G2/M regulated genes in A549, HCC827 and H1975 cells. The expression levels of p-CDK1, CDK1, CDC25C and Cyclin B1 were analyzed by qRT-PCR and/or Western blot. We found that expression levels of p-CDK1, CDC25C and Cyclin B1 were statistically reduced by downregulation of Cited1 compared to the control group in A549, HCC827 and H1975 cells ($P < 0.05$). However, no significant differences were observed in protein expression levels of CDK1 in the three cell lines (**Figure 2A-D**). The results suggested that downregulation of Cited1 induced G2/M phase arrest might by decreasing the expression of p-CDK1, CDC25C and Cyclin B1 in NSCLC cells.

Downregulation of Cited1 induced G2/M phase arrest of cell cycle progression

To better understand whether the cell cycle distribution of A549, HCC827 and H1975 cells were affected by down-regulation of Cited1, flow cytometric analysis was performed to assess the cell cycle distribution. As indicated in **Figure 3A-C**, the cell cycle data showed that the transfection with siCited1 significantly decreased the percentages of cells in G1 (73.1% vs 51.2% for A549 cells; 72.9% vs 52.1% for HCC827 cells; and 73.2% vs 52.6% for H1975 cells), while increased the percentages of cells in G2/M (8.9% vs 24.1% for A549 cells; 9.4% vs 23.3% for HCC827 cells; and 9.1% vs 22.7% for H1975 cells). The results demonstrated that downregulation of Cited1 induced G2/M phase arrest of cell cycle progression in NSCLC cells.

Discussion

Recently, an increasing number of studies have shown that Cited1 plays critical roles in cancer pathogenesis, indicating that Cited1 could provide new insights into the etiology of human cancers [8, 9, 16-20]. However, the functional role of Cited1 in NSCLC was unclear. In the present study, we provided the first evidence of the silencing effects of Cited1 on NSCLC. The results showed that the cell viability was significantly decreased by transfection with siCited1 in A549, HCC827 and H1975 cells compared to the control group. The levels of p-CDK1, CDC25C and Cyclin B1 were statistically down-regulated by downregulation of Cited1 compared to the control group. The percentages of cells in the G1 phase were statistically decreased, while the percentages of cells in the G2/M phase in the three cell lines were markedly increased by transfection with siCited1. Our results suggest that downregulation of Cited1 suppresses cell proliferation by inducing G2/M arrest of the cell cycle in NSCLC cell lines.

Cited1 was originally found in a mouse melanoma cell line [8]. It has been reported that Cited1 is expressed in many progenitors, such as kidney, placenta, heart, and axial skeleton [21, 22]. Cited1 is considered as an important co-ordinator during morphogenesis of renal epithelial [23] and development of mammary gland [24]. It also is involved in regulation of nuclear mothers against DPP homolog (SMAD) 2/3 signaling, Wnt signaling and transforming growth factor (TGF)-beta receptor signaling pathway [23, 25]. Higher expression levels of Cited1 have been reported in PTH [10, 26], malignant melanoma [19, 27], and Wilms' tumor [9, 17]. A recent study has suggested that suppression of Cited1 inhibits intestinal tumorigenesis [18]. Therefore, we speculated that Cited1 might be involved in the development and process of NSCLC.

To ensure the speculation, siCited1 were first transfected into NSCLC cell lines A549, HCC827 and H1975. After confirming the successful transfection, the cell viability was determined by MTT. Our data showed that transfection with siCited1 significantly decreased the

cell viability compared to the control group in the three cell lines, suggesting that suppression of Cited1 exerts an anti-proliferation effect on NSCLC cells. Murphy *et al.* has found that overexpression of Cited1 in human hepatoblastoma Hep293TT cells induces cell proliferation [12], and Méniel *et al.* has suggested that absence of Cited1 represses s intestinal tumourigenesis in $Apc^{Min/+}$ mice [18]. Our results were similar with the previous two studies. We further explored the underlying mechanism regarding to the anti-proliferation of suppression of Cited1 by analyzing G2/M regulated genes in A549, HCC827 and H1975 cells. It has been well demonstrated that the cell cycle progression from the G2 to the M phase is controlled by activation of CDK1 [28-30]. The activity of CDK1 is dependent upon binding of cyclin B [28]. The activation of the CDK1/cyclin B complex is sustained by phosphorylation of CDC25C, which is considered as a rate-limiting step for G2 entry into mitosis [31]. Moreover, dephosphorylation of CDK1 is catalyzed by CDC25C [32]. Therefore, we assessed whether downregulation of Cited1 changed the mRNA and protein expression levels of CDK1, cyclin B1, and CDC25C, as well as the phosphorylation of CDK1. As indicated in our study, the results showed that both the expression levels of p-CDK1, cyclin B1, and CDC25C were statistically down-regulated by transfection with Cited1. Suppression of CDC25C would lead to inactivation of CDK1, subsequently result in dissociation of cyclin B1 and prevent cell cycle progression from the G2 phase to the M phase. We confirmed the results by analyzing cell cycle distribution. The results showed that the percentages of cells in the G1 phase were statistically decreased, while the percentages of cells in the G2/M phase were markedly increased in all the three cell lines. The data suggested that the anti-proliferation effect on NSCLC cells by suppression of Cited1 might be via induction of G2/M phase arrest.

In conclusion, the study suggests that downregulation of Cited1 suppresses cell proliferation by inducing G2/M arrest in NSCLC cell lines. Inhibition of Cited1 expression may provide new insights into potential targeted therapies that abrogate cancer cell proliferation.

Disclosure of conflict of interest

None.

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