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CDK-associated Cullin 1 promotes cell proliferation with activation of ERK1/2 in human lung cancer A549 cells

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ABSTRACT

Lung cancer is one of the most common causes of cancer-related death in the world, but the mechanisms remain unknown. In this study, we investigated the expression of CDK-associated Cullin 1 (CAC1) in lung cancer, the effect of CAC1 on the proliferation of human lung cancer A549 cells, and the activation of signaling pathways of mitogen-activated protein kinases (MAPKs). Results showed that CAC1 expression was higher levels in human lung carcinoma than normal lung tissue, and CAC1 siRNA reduced the proliferation of lung cancer A549 cells by decreasing cell activity and cell division in vitro. The proportion of cells treated with CAC1 siRNA increased in the G1 phase and decreased in the S and G2/M phase, indicative of G1 cell cycle arrest. Furthermore, the proportions of early/late apoptosis in lung cancer A549 cells were enhanced with CAC1 siRNA treatment. It was also found that activation of extracellular signal-regulated protein kinase (ERK) and p38 signaling pathways were involved in the proliferation of A549 cells. After CAC1 siRNA treatment, p-ERK1/2 levels decreased, and meanwhile p-p38 level increased, A549 cell proliferation increased when ERK1/2 signaling is activated by PMA. Our findings demonstrated that CAC1 promoted the proliferation of human lung cancer A549 cells with activation of ERK1/2 signaling pathways, suggesting a potential cure target for treatment of human lung cancer.

1. Introduction

Lung cancer is one of the most common causes of cancer-related death and accounts for more than a million deaths yearly worldwide with non-small cell lung cancer (NSCLC) accounting for 75–85% of lung cancer. Lung cancer mortality has steadily increased because it is highly metastatic and because of the side effects which are frequently accompanied by surgery, radiotherapy and chemo-therapy. Therefore, the search for better therapeutic agents with enhanced activity against lung cancer continues. Molecular biology research indicates that lung carcinogenesis comprises numerous genetic and epigenetic events, involving a cluster of oncogenes, tumor suppressor genes, cell cycle regulators, cell adhesion molecules and DNA repair genes [1]. Over past years, induction of cell cycle arrest and/or apoptosis in lung cancer cells has been considered an influential treatment strategy [2,3]. Many researchers have focused on selectively killing cancer cells or reducing cell number through the induction of cell cycle arrest and apoptosis [4]. However, the precise mechanisms underlying lung cancer are not well defined.

CAC1 is a novel gene identified in colorectal carcinoma [5]. The GenBank accession number of this gene is AY743663, and it is located at 10q25-q26. It embraces an open reading frame sequence which encodes a 37 kDa protein of 369 amino acids. The CAC1 protein contains a cullin domain between amino acids 137 and 250, and is therefore classified as a member of the Cullin family of E3 ubiquitin ligases. CAC1 has been demonstrated to increase in colorectal cancer tissues, implicating a causal role of CAC1 in promoting tumor progression [5]. CAC1 is activated by multiple extracellular signals, including mitogens, growth factors and H_2O_2, leading to the activation of cyclin-dependent kinase 2 (CDK2), which, in turn, regulates cell proliferation and allows progression from the G1 to S phase of the cell cycle [5]. It is reported that CAC1 can promote cell proliferation in the AGS gastric cancer cell line [6]. The mitogen-activated protein kinase (MAPK) signaling cascade involves membrane-to-nucleus signaling modules that are involved in multiple physiological processes [7]. In particular, extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinases (MAPKs) are main members of the MAPK family. ERK cascades transduce the activity of extracellular and intracellular signals into enduring changes and regulate cell proliferation by regulating cellular activities and gene transcription [8].

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The role of CAC1 in regulating proliferation of colorectal carcinoma and gastric cancer is soundly evidenced [5,6]. Nevertheless, little is known about its expression and biological characteristics in lung cancer. In this experiment, we examined the effects of CAC1 on proliferation of human lung cancer A549 cells and investigated its mechanisms related to cell signaling system.

2. Materials and methods

2.1. Preparation of human normal lung tissue and lung carcinoma tissue

Human normal lung tissue and lung carcinoma tissue were prepared from the First Affiliated Hospital, College of Medicine, Xi'an Jiaotong University. Informed consent was obtained before specimen collection. The experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University College of Medicine and followed the guidelines of the declaration of Helsinki. The specimen collection was conducted in accordance with the guidelines of National Institutes of Health.

2.2. Cell culture

Human lung carcinoma cell lines (A549) were provided by Environment and Genes Related to Diseases Key Laboratory of Education Ministry, Xi'an Jiaotong University College of Medicine, China. A549 cells (1×10^5 cells/mL) were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, Grand Island, NY, USA) containing 2.0 mmol/L glutamine and 20 μg penicillin–streptomycin/mL at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. siRNA synthesis and transfection

siRNA was pre-designed for CAC1 gene silencing. Human CAC1 siRNA (sense-5′ GGA UGG UGC CAU AGA UCA ATT 3′, antisense-5′ UUG AUC UAU GGC ACC AUC CGG 3′) and negative siRNA (NC-siRNA, sense-5′ UUC UCC GAA CGU GUC ACG UTT 3′, antisense-5′ ACG UGA CAC GUU CGG AGA ATT 3′) were chemically synthesized by Shanghai GenePharma Corporation (SGC, Shanghai, China). Lipofectamine™-2000 (Invitrogen, Carlsbad, CA, USA) was used to optimize siRNA transfection which was performed in serum-free RPMI-1640. Lipofectamine and siRNA were diluted separately in serum-free RPMI-1640 and incubated for 5 min at room temperature. Then the two solutions were softly mixed and incubated for 15 min. The obtained Lipofectamine-siRNA complexes contained siRNA at a density of 300 nM, and the complexes would be diluted to desired transfection concentrations and added to the plated cells in future experimental procedures.

2.4. MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]-based assay was applied to estimate the effect of CAC1 on A549 cell proliferation. Cells were seeded into 96-well plates (5000 cells/well in 200 μl medium) and incubated for 1 days at 37 °C in 5% CO_2. A549 cells were treated with different concentrations of Lipofectamine-siRNA complexes (30, 60 and 90 nM) for 1, 2 and 3 days, respectively. Cells cultured with complete medium were used as blank control, those treated with negative siRNA (NC-siRNA, 60 nM) and used as negative control. Then, the siRNA (60 nM) group cells were treated with PMA (phorbol 12-myristate 13-acetate, 100 nM) (Sigma, St Louis, MO, USA) activating ERK1/2 and incubated for 1, 2 and 3 days. At the end of culture, 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) solution was added per well and the cells were incubated for another 4 h at 37 °C. Supernatants were removed and formazan crystals were dissolved in 150 μl of dimethylsulfoxide (Sigma, St Louis, MO, USA). Finally, optical density was determined at 490 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). In each assay, five parallel wells were made, and the results were collected as the mean of more than three independent experiments.

2.5. Flow cytometry analysis for cell cycle

To analyze cell cycle, DNA content per duplicate was analyzed using FACSort Cellquect software (BD Biosciences, San Jose, CA, USA). Cells were cultured in 6-well plates for 1 day and treated by 60 nM siRNA for 2 days. At the end of culture, cells were fixed in 75% ice-cold ethanol overnight at 4 °C. The fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 30 min at room temperature in the dark and analyzed by fluorescence-activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA, USA). The cells were excited at 488 nm, and the emission was collected through a 630-nm filter. Totally 20,000 cells were collected from each sample. The cell cycle distribution was evaluated by calculating the proportion of cells in G0/G1, S, and G2/M stages. In each independent experiment, three parallel wells were made, and the procedures were carried out in triplicate. Data obtained were presented as mean ± SEM.

2.6. Apoptosis analysis

Cells were incubated in 6-well plates for 1 day and treated by 60 nM siRNA for 2 days, then harvested and washed twice with PBS. The cells were stained by incubation with 5 μL FITC-Annexin V and 10 μL PI at 250 μg/ml for 10 min in the dark at room temperature. Cells then were washed with PBS and examined using flow cytometry. Quantification of apoptosis was determined by counting the number of cells stained by FITC-labeled Annexin V. The apoptosis of cells was detected using the Annexin V/PI Apoptosis Detection Kit by FACS. Early apoptotic cells were identified with PI negative and FITC Annexin V positive; cells that were in late apoptosis or already dead were both FITC Annexin V and PI positive.

2.7. Real-time quantitative PCR (Q-PCR) analysis

Normal lung tissue, lung carcinoma tissue and A549 cells were treated by 60 nM siRNA for 2 day using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA was extracted from the tissues and cells, quantified by spectrophotometry (Nano-Drop™ Wilmington, USA). The RNA was reverse transcribed to cDNA using a reverse transcriptase kit (PrimeScript™ RT reagent Kit, TaKaRa Bio. Co. Ltd., USA). Relative abundance of each mRNA sample was quantitated by Q-PCR using specific primers and the SYBR® Premix Ex Taq™II (TaKaRa. Bio. Co. Ltd., USA). Primers for human CAC1 (forward 5′-GCAGCATATT CAGAAAGTTCAGA-3′; reverse 5′-CATTTACAGCCTAATGCCTTTACT-3′) and human β-actin (forward 5′-TGGCACCCAGCACAATGAA-3′; reverse 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′) were designed and synthesized by TaKaRa Biotechnology. Real-time PCR reactions were carried out using an iQ multicolor real-time PCR detection system (Bio-Rad, USA). Cycle threshold values were obtained from the Bio-Rad iQ5 2.0 Standard Edition optical System software (Bio-Rad, USA). Data were analyzed using the ΔΔCt method and β-actin served as an internal control. The results were presented as mean ± SEM of three separate experiments.

2.8. Western blot analysis

For Western blotting analysis, A549 cells were treated by 60 nM siRNA for 1 day. Normal lung tissue, lung carcinoma tissue and the A549 cells were lysed in RIPA lysis buffer. Insoluble material was removed by centrifugation at 12,000 rpm for 15 min at 4 °C. Protein were subjected to electrophoresis using 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked for 2 h in 5% non-fat dry milk in TBST (10 mM Tris–HCl and 0.05% Tween 20). The membrane was incubated with primary antibodies overnight at 4 °C and then incubated with secondary antibody for 2 h at room temperature. The primary monoclonal antibodies included rabbit monoclonal anti-ERK1/2 (1:2000, Cell Signaling, Danvers, MA, USA), mouse monoclonal anti-P-ERK1/2 (1:2000, Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-p38 (1:2000, Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-p-p38 (1:1000, Cell Signaling, Danvers, MA, USA), mouse monoclonal anti-CAC1 (1:1000, GeneTex, USA), and mouse monoclonal anti-β-actin (1:5000, Santa Cruz, CA, USA). The membranes were incubated in the dark with ECL (Amersham) for chemiluminescence detection. The luminescent signal was detected by CCD camera, recorded and quantified with Syngene GBox (Syngene, UK). The experiment was carried out in triplicate for each group, and results were presented as mean ± SEM.

2.9. Statistical analysis

Statistical analysis was performed in SPSS 13.0 software (version 13.0). The results were analyzed by one-way ANOVA. Student's *t*-tests were used to assess the difference between groups; $P < 0.05$ was considered statistically significant. The quantitative data are presented as mean ± SEM.

3. Results

3.1. Expression of CAC1 in human lung carcinoma

Using real-time Q-PCR and Western blotting, we analyzed the change of mRNA and protein of CAC1 in human lung carcinoma. The results from real-time Q-PCR showed that CAC1 mRNA was higher levels in human lung carcinoma than normal lung tissue (Fig. 1A) ($P < 0.05$). CAC1 protein expression in western blot examinations showed the same changing trend as CAC1 mRNA (Fig. 1B) ($P < 0.05$).

3.2. Effect of CAC1 on the proliferation of A549 cells

To determine whether CAC1 plays an important role in the proliferation of A549 cells, cells were treated with CAC1 siRNA (30, 60 and 90 nM) for 1, 2 and 3 days. MTT assay was employed to analyze the cell activity. The results showed that CAC1 siRNA at the concentration of 60 and 90 nM significantly decreased the activity of the cells (Fig. 2A) ($P < 0.05$). Due to the significant differences on cell activity in the treatment groups, CAC1 siRNA (60 nM) group was chosen to be used in the following experiments. Because the cell cycles are involved in the regulation of cell growth, we examined the processes using a flow cytometer 2 days after treatment. The cell cycles were arrested significantly at G1/G0 (Fig. 2B) ($P < 0.05$). After CAC1 siRNA had been transfected into A549 cells, we analyzed knockdown efficiency of CAC1 siRNA in mRNA and protein levels. The mRNA and protein expression of CAC1 of A549 cells decreased significantly to 43.08 ± 7.22% and 21.59 ± 7.98% in siRNA groups compared with control (Fig. 2C and D) ($P < 0.05$).

3.3. Effect of CAC1 on apoptosis of A549 cells

To examine the possible effects of CAC1 on cell death, we observed the measurement of apoptosis by Annexin-V/PI staining. We found that the proportion of early apoptotic (24.88 ± 3.54%) and late apoptotic (19.34 ± 3.12%) increased significantly after siRNA treatment, the proportion of non affected cells decreased in siRNA group (Fig. 3) ($P < 0.05$). These findings showed that CAC1 may interrupt apoptosis of A549 cells.

3.4. CAC1 promoted the proliferation of A549 cells through activating ERK1/2 signaling pathways

To explore the possible molecular mechanisms of CAC1 in the proliferation of A549 cells, the cells were treated by 60 nM siRNA for 1 day, and then the expressions of phosphorylated ERK and p38 were measured. No significant change was observed in the total protein expression of each signaling pathway, but the expression of phosphorylation of ERK and p38 evidently changed in treatment groups. The ratio of p-MAPK/total MAPK was used to indicate the phosphorylation and activation of the proteins. It was found that the ratio of p-ERK1/2 to ERK1/2 was remarkably decreased 3.72-fold after treatment with siRNA, when compared to that in the control group (Fig. 4A) ($P < 0.05$). By contrast, the ratio of p-p38 to total p38 was significantly increased by nearly 2.31-fold in the group treated with siRNA compared to that in the control group (Fig. 4B) ($P < 0.05$). MTT assay showed that the cell activity of siRNA (60 nM)+PMA (100 nM) group significantly increased comparing with siRNA (60 nM) group at 1, 2 and 3 days ($P < 0.05$), and there were no significant changes in the cell activity comparing with control and NC-siRNA groups at 1, 2 and 3 days (Fig. 4C) ($P > 0.05$).

4. Discussion

The ubiquitin–proteasome system plays an important role in maintaining the balance between normal growth and uncontrolled proliferation by regulating a large variety of cellular proteins [9]. The cullin family of ubiquitin ligases, including CUL1, 2, 3, 4A, 4B, 5 and 7, represents the largest class of E3 ligases [10]. Cullin-mediated substrate degradation involves a wide range of cellular processes such as proliferation, apoptosis, and differentiation. Once the cell regulatory mechanisms of cullin encounter malfunctions or perturbations, accumulation of oncoproteins or excessive degradation of tumor suppressors will inevitably occur, which may provoke cells into malignant transformation and tumorigenesis [9]. Being a novel member of the cullin family, CAC1 expression patterns have been extensively investigated in a previous study [5]. Examination of the distribution of CAC1 showed that it is expressed in normal stomach, colon, small intestine, lung, liver, kidney, heart, muscle, uterus, mammary gland, brain, spleen, lymph node [5]. CAC1 is highly conserved both in mammals (NCBI data base) and in other species also suggests that this gene may play an important role in cell biology. Kong et al. found that CAC1 is highly expressed in colorectal cancer tissues and cancer cell lines [5]. We found that CAC1 expression was also higher levels in human lung carcinoma than normal lung tissue. In addition, it is reported that CAC1 knockdown by RNAi inhibits cell proliferation in HeLa cells and AGS cells [5,6]. Gene silencing by RNA interference is a powerful method for analyzing gene function which inhibits gene expression through sequence-specific degradation of a target mRNA [11]. In our experiments, siRNA was adopted to specifically silence CAC1, we successfully transfected NCsiRNA and CAC1-siRNA into human lung cancer A549 cells. The results

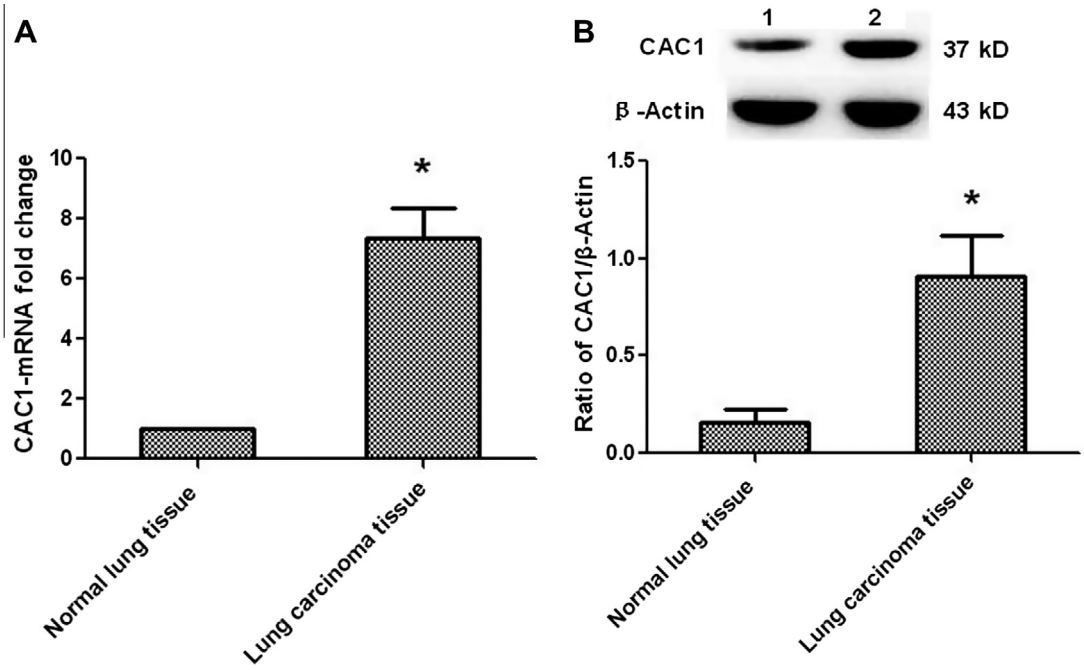

Fig. 1. Expression of CAC1 in human lung carcinoma. (A) Real-time Q-PCR products. (B) Western blotting analysis. β-Actin was used as a loading control (*$P < 0.05$, compared with normal lung tissue, $n = 30$).

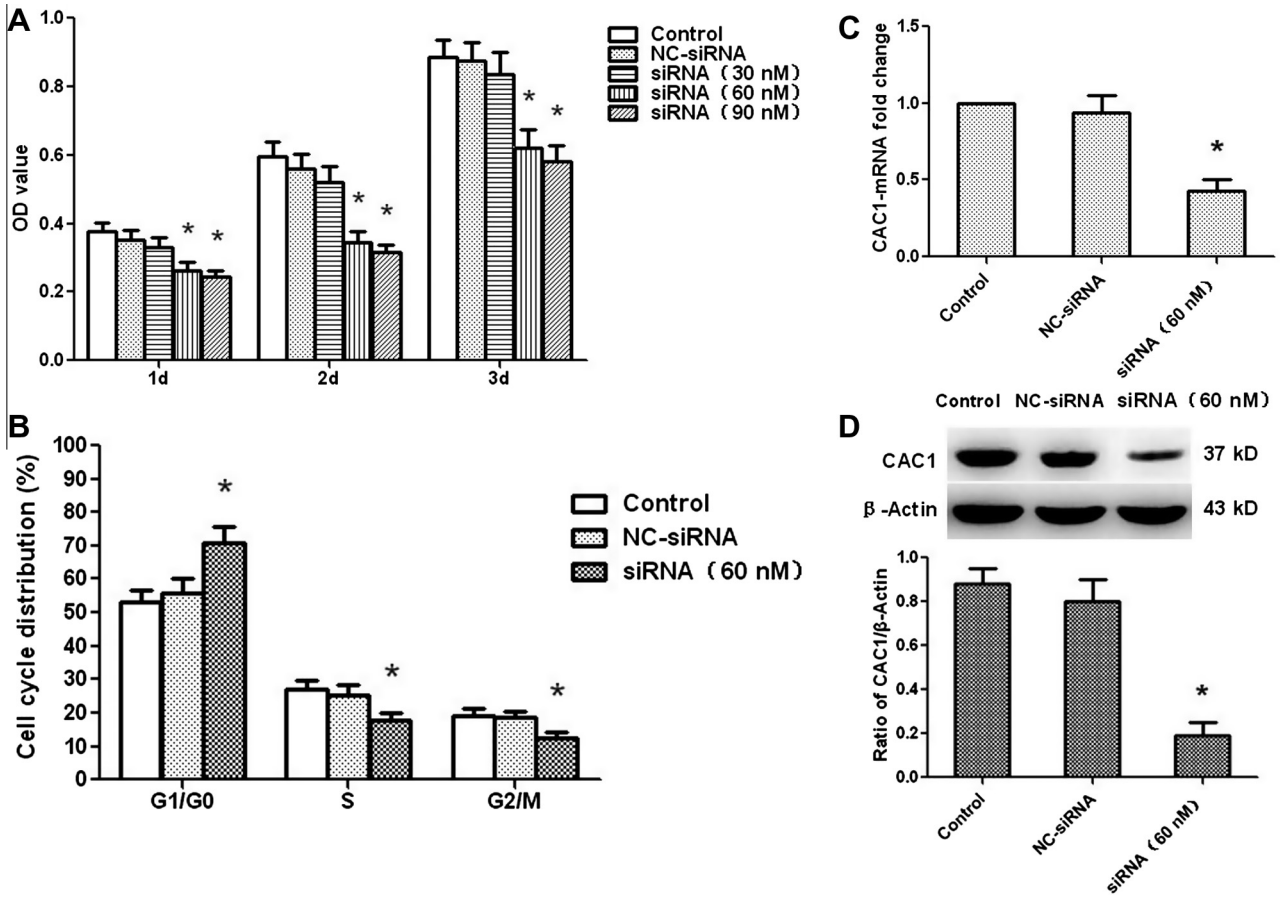

Fig. 2. Effects of CAC1 on the growth of A549 cells. (A) MTT assay showed that CAC1 siRNA decreased the activity of A549 cells at 1, 2, and 3 days. (B) The results of flow cytometry analysis of the cell cycle in A549 cells were visualized via PI staining. The data show the percentage of cells in the G1/G0, S, and G2 phases. G1/G0 phase cells significantly increased after siRNA (60 nM) treatment. (C) Real-time Q-PCR results showed that knockdown efficiency of siRNA in A549 cells. (D) Western blotting analysis results showed that knockdown efficiency of siRNA. β-Actin was used as a loading control (*$P < 0.05$, compared with control group, $n = 3$).

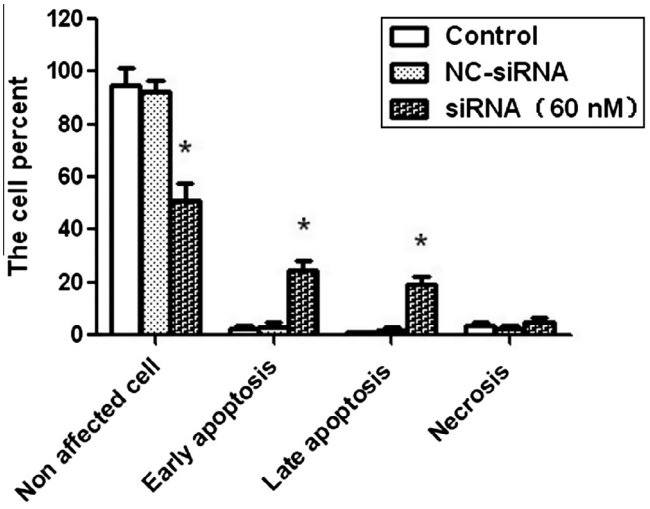

Fig. 3. CAC1 silencing enhanced apoptosis in A549 cells. The results of flow cytometry analysis of apoptosis were visualized using Annexin-V/PI staining. The data show the percentage of non-affected, early apoptotic, late apoptotic, and necrotic ($^{*}P < 0.05$, compared with control group, $n = 3$).

showed that 60 nM CAC1-siRNA inhibited CAC1 expression and decreased cell proliferation in A549 cells.

The first gap (G1) phase of the mammalian cell cycle is a unique period when cells respond to environmental signals to determine cell fate such as proliferation, survival, differentiation and cellular senescence [12]. There are many important cell cycle regulators, including CDK2, CDK4, CDK6, cyclinD and cyclinE, which can govern the cellular progression through the G1 phase of the cell cycle [13]. Kong et al. reported that CAC1 expression and distribution showed regular alteration with the cell cycle, wherein CAC1 expression was the highest in G1/S, was lower in the middle of S phase, and increased again in S/G2 phase, the proportion of cells in G1 increased after CAC1 knockdown in HeLa cells [5]. CAC1 was capable of binding to CDK2 and stimulating its kinase activity at the G1/S phase transition without greatly changing the expressions of cyclinA, cyclinE, cyclinD1, CDK2, RB, PTEN, and so on [5,14]. In this experiment, we demonstrated that CAC1 knockdown induced G1 cell cycle arrest in A549 cells, which was consistent with findings in a previous study [5,6]. These results suggest that CAC1 may drive more cells crossing G1/S node and entering into cell cycle, resulting in cell proliferation in human lung cancer A549 cells.

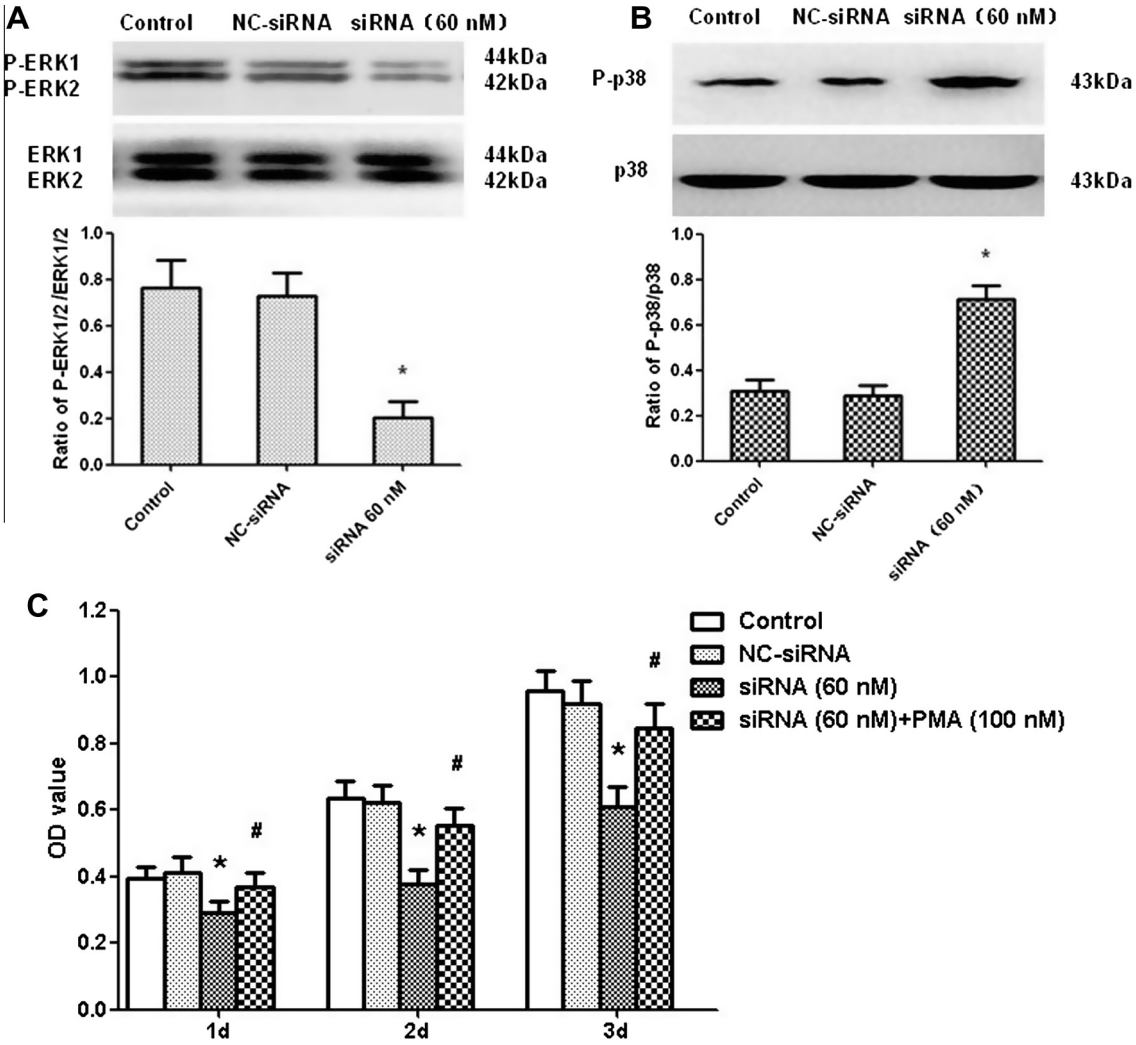

Fig. 4. Expressions of phosphorylated ERK and p38 after treatments, and effect of ERK1/2 activation on proliferation after treatments. (A) Phosphorylated and total ERK1/2. (B) Phosphorylated and total p38. Data were expressed as a ratio of the normalized percentage of p-MAPKs and MAPKs. (C) MTT assay showed that the cell activity of siRNA group increased when ERK1/2 is activated by 100 nM PMA ($^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with siRNA group, $n = 3$).

The RAS MAPK signaling pathway includes membrane-to-nucleus signaling modules that are involved in the regulation of multiple biological and physiological processes such as survival, proliferation, apoptosis, differentiation and transformation. ERK and p38 MAPKs are main members of the MAPK family, which play an important role in proliferation, differentiation, migration, stress responses, inflammation and apoptosis [15–18]. The ERK cascade can be activated by growth factors and transmit signals to promote cell proliferation and survival [19,20]. ERK interacts with more than 170 proteins including many substrates [21,22]. The interaction of ERK with different proteins in response to distinct stimuli may influence substrate specificity and lead to precise biological outcomes. The ERK1/2 pathway has long been associated with cell proliferation and survival [23,24]. It is reported that ERK1/2 activation may promote cell proliferation and survival in colon cancer, gastric cancer, resistant breast cancer, bladder cancer, cervical cancer and lung cancer [25–29]. Recently, it has been shown that CDK can govern multiple biological and physiological processes by regulating ERK1/2 activation [30,31]. In this experiment, we find that CAC1 siRNA suppressed the phosphorylation and activation of ERK1/2 in human lung cancer A549 cells, and A549 cell proliferation increase when ERK1/2 signaling is activated by PMA in CAC1 siRNA group. However, the ERK signaling cascades seem to be far more complex. More work is needed to uncover the mechanisms of CAC1 and ERK pathways on lung cancer cell proliferation.

In addition, the phosphorylation of p38 MAP kinase could induce cell apoptosis, and inhibition of p38 with selective inhibitor SB203580 can reduce the cell death [32–34]. It was reported that activation of p38 MAPK cascade to cause HCT116 colorectal cancer cell apoptosis [6]. In the present study, phosphorylation of p38 MAPK is found to have increased in human lung cancer A549 cells after the treatment with CAC1 siRNA. These results suggest that CAC1 may reduce apoptosis of A549 cells by regulating the phosphorylation of p38 MAPK signaling. Cell proliferation is involved in cell growth and cell death, the expression of p-ERK and p-p38 resulted in a new balance between cell growth and cell death after CAC1 knockout.

In conclusion, we found that CAC1 expression was higher levels in human lung carcinoma than normal lung tissue, and CAC1 promoted the proliferation of human lung cancer A549 cells in vitro and reduce the apoptosis of the cells, and CAC1 knockout may decreased the expression of phosphorylation of ERK1/2 and increased phosphorylation of p38. The results suggest a potential cure target for treatment of human lung cancer. However, more work is needed to uncover the mechanisms of CAC1 promoting the proliferation of lung cancer cells.

Acknowledgments

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