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Original Article

RNA interference targeting enhancer of polycomb1 exerts anti-tumor effects in lung cancer

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Abstract: Background and aim: Lung cancer is one of leading malignant tumor worldwide with a high mortality rate. A new therapy target, enhancer of polycomb1 (EPC1) knocked down by short hairpin RNA (shRNA) interference technology, for lung cancer was established to investigate its effects on lung cancer in present study. Methods: RNA interference technology was applied to down-regulate the expression of EPC1 by specific-shRNA with lentivirus vector in neoplastic human alveolar basal epithelial cells (A549 cells). The survival rate and apoptosis were respectively measured by MTT and Flow Cytometry to evaluate the effects of shRNA EPC1 on cells. Mice xenografts of HCT116 cells with shRNA EPC1 were also established to assess the effect on tumor growth. The levels of AKT and p65 were detected by western blotting. Results: The down-regulation of EPC1 by specific-shRNA with lentivirus vector was significantly decreased the survival rate and apoptosis of A549 cells, and the tumors in EPC1 shRNA transfection group had a significant lower size and weight compared with the ones with control shRNA. The protein expression of p-AKT and p65 was reduced by EPC1 shRNA in both in vitro and in vivo experiments. Conclusion: Silencing EPC1 by shRNA technology had the inhibition effects on cell proliferation and tumor growth in lung cancer, which provided a new potential target for treatment of cancers.

Keywords: Lung cancer, shRNA, EPC1

Introduction

Lung cancer is one of leading malignant tumors worldwide [1], and the incidence of lung cancer is higher in countries with high tobacco consumption, such as China. The 5-year survival for lung cancer still remains relatively low, which is mostly due to late diagnosis and limited treatment options [2, 3]. Although the diagnostic techniques for early detection and surgical procedures have made a great progress in recent years, the incidence and mortality of lung cancer is increasing most fast in China [4, 5]. Therefore, it is very important to explore new technologies and therapeutic targets in lung cancer treatment.

Enhancer of polycomb1 (Epc1), a chromatin protein, was first described to enhance the phenotypes of homozygotic mutations of the polycomb group gene in Drosophila in 1998 [6]. Previous reports elucidated that Epc1 may act as a transcription co-factor as a binding partner of other transcription factors such as E2F6 [7] or RET finger protein [8]. The expression of polycomb group family is correlated with various physiological processes including skeletal muscle differentiation [9], cell apoptosis [10] and adult T-cell leukemia/lymphoma [11]. However, the biological role of Epc1 in cancer is rarely known and the regulatory effect on lung cancer has not been reported to date.

RNA interference (RNAi), a technology to silence gene expression due to the destruction of specific mRNA molecules, has rapidly become a powerful tool for drug development [12, 13]. RNAi is also considered to be a potential strategy to against cancers [14, 15]. The introduction of synthetic siRNA or by intracellular generation of siRNA from vector driven expression of the precursor shRNA can induce RNAi [16]. Compared with siRNA, the effect of shRNA lasts longer due to its continually produced within the cells [17]. It was reported that knockdown of some key genes by shRNAs reduced tumor

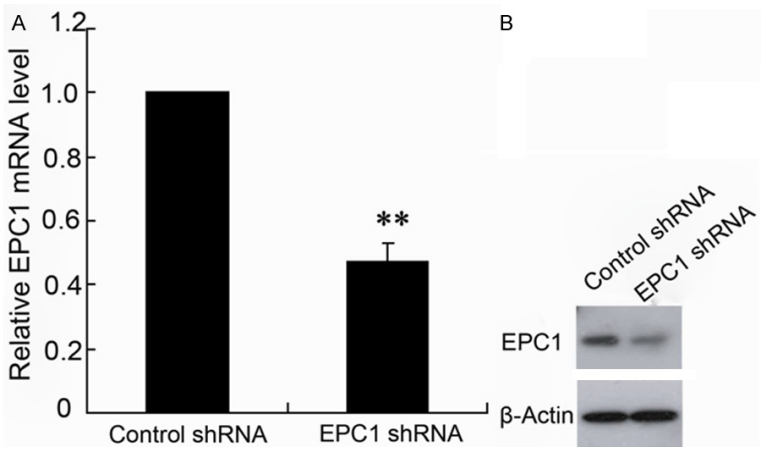

Figure 1. ShRNA suppresses the expression of EPC1 in A549 cell lines. A: The mRNA level was detected by real-time PCR; B: The protein expression was detected by western blotting.

growth in lung cancer [18]. In present study, specific-shRNA with lentivirus vector was used to knock down the expression of Epc1 of adenocarcinomic human alveolar basal epithelial cells (A549 cells) to explore the role of Epc1 in lung cancer. In addition, in vivo experiment was also performed to confirm the function of shRNA Epc1 in lung cancer.

Methods and materials

Cell culture

The human A549 lung cancer cell line was purchased from the China Center for Type Culture Collection (Wuhan, China), it was cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, UK) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin and 1% nonessential amino acids.

Construction of plasmids and stable cell lines

The expression of Epc1 in A549 cells was silenced by shRNA interference. The sequence of Epc1 gene was obtained from NCBI (GenBank Access No. FJ905304.1) and the EPC1 shRNA primers after annealing were linked to the pUCTP vector at 24°C for 2 h. The ligation products were subjected to transform into the GeneHogs cells and the positive colonies were screened by antibiotic resistance and identified by PCR. The final DNA preparations were confirmed by sequencing (Sangon, China). A non-silencing shRNA sequence was adopted to act as a control shRNA. Plasmids containing EPC1 shRNA or control shRNA were prepared in 293T cells for 24 h followed by transfecting into A549 cells.

Establishment of xenograft

All experiments with animals were approved by an animal committee for ethics of the First Affiliated Hospital, College of Medicine, Zhejiang University. 5×10^6 A549 cells transfected with EPC1 shRNA or control shRNA were suspended in 100 μL PBS and respectively injected into the flanks of twelve 4-week-old BALB/c athymic nude mice (Gene-Cell, China) to establish lung cancer xenograft model. The tumor size was measured every five days and the formula of tumor size was calculated as following: π × length × width^2/6 [19]. Thirty days after injections of A549 cells, the mice were sacrificed and tumors were removed and weighted.

MTT assay

The effects of shRNA on viability of A549 cells were determined by an MTT assay (Beyotime, China) following the operation instructions. In brief, 1×10^4 Cells/well was incubated in 96-well plates. After 48 h of shRNA interference, the solution of MTT was added to each well and incubated for 4 h at 37°C, and the absorbance value of supernatant with DMSO (100 μL/well) was measured at 490 nm using a spectrophotometer.

Cell apoptosis analysis by flow cytometry and TUNEL assay

A549 cells were transfected with the EPC1 shRNA or Control shRNA. The apoptosis of A549 cells treated with shRNA was detected and quantified by flow cytometry. Briefly, cells were resuspended in 100 μL binding buffer to obtain a suspension with 1×10^6 cells/mL, and 5 μL Annexin V-EGFP and 5 μL PI were supplemented into cell supplement. After incubation for 15 min in the dark, the stained cells were detected by FACScan (Becton Dickinson, USA). The percentage of cell apoptosis was calculated using Cell-Quest software (Becton Dickinson, USA).

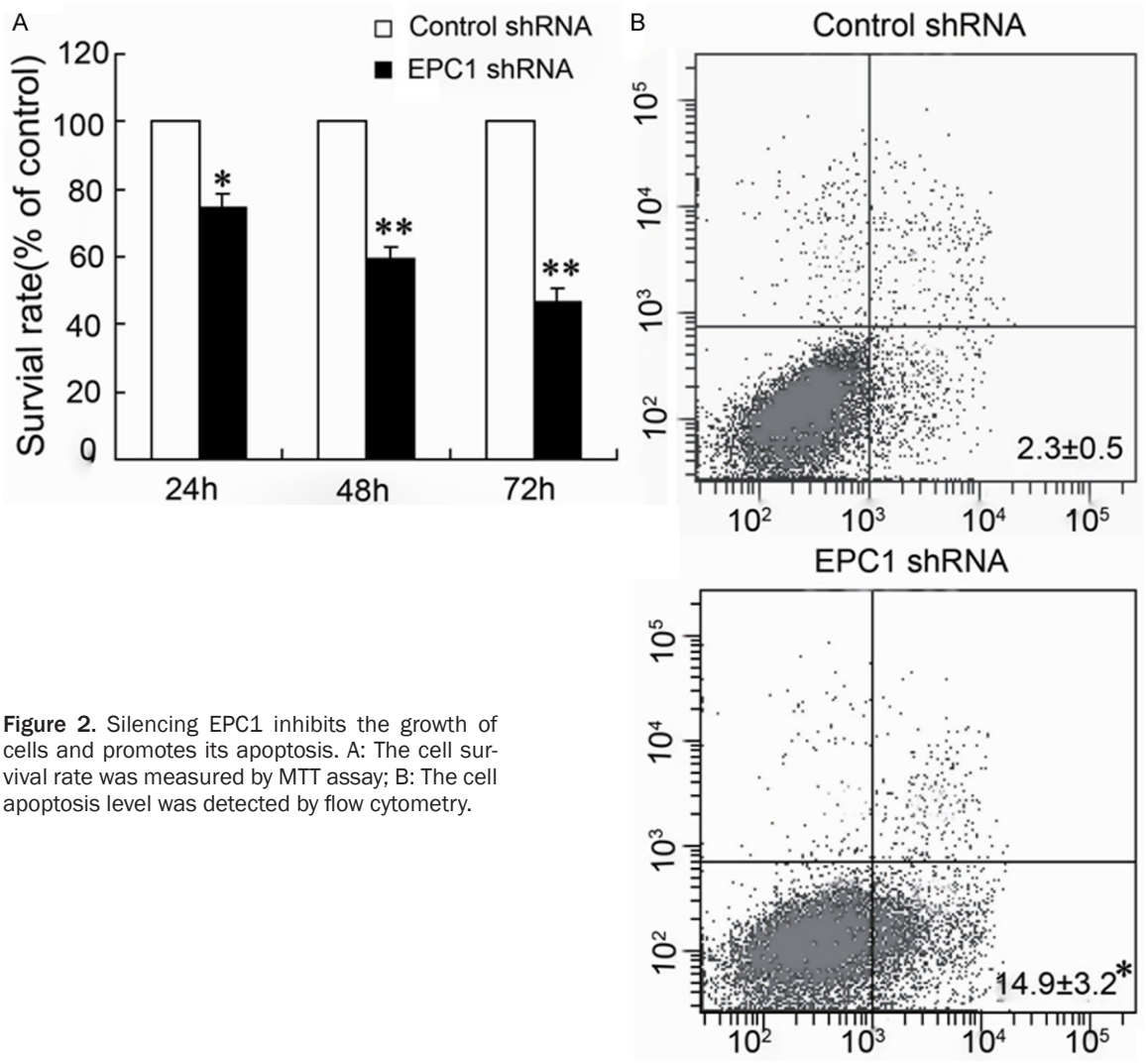

Figure 2. Silencing EPC1 inhibits the growth of cells and promotes its apoptosis. A: The cell survival rate was measured by MTT assay; B: The cell apoptosis level was detected by flow cytometry.

The cell apoptosis in tumor tissue was analyzed by TUNEL assay with APO-BRDU kit (Sigma, Germany) following the instructions strictly. The percentage of TUNEL positive cells was calculated as followed: TUNEL-positive cells/total number of cells) × 100%.

Real-time PCR

The A549 cells transfected with EPC1 shRNA or control shRNA were digested by trypsin. Total RNA of cells was extracted by TRIzol reagent (Takara, China) according to the manufacturer's instructions. 500 ng RNA was used to do reverse transcription with ImProm-II™ (Promega, USA). The level of mRNAs was quantified by real-time PCR using TransStartTM SYBR Green qPCR Supermix (TransGen Biotech, China), and with β-actin acted as an internal normalized reference.

Western blotting

In the logarithmic phase of cell growth, cells were harvested and centrifugated at 10,000× g for 15 min (4°C). The supernatants were collected to extract protein, and the protein concentration was quantified by BCA Protein Assay Kit (GenStar Biosolutions, China). Equal amounts of proteins were loaded onto the SDS-PAGE for electrophoresis and transferred to PVDF membranes. The membranes were incubated with primary antibodies after blocking with 5% Bull Serum Albumin for 2 h. Three times washing with 0.1% Tween, the membranes were then incubated with secondary antibody for 1 h. The protein expression was observed by autoradiography (Protein Simple, USA). β-actin was a control protein to evaluate the expression of target proteins.

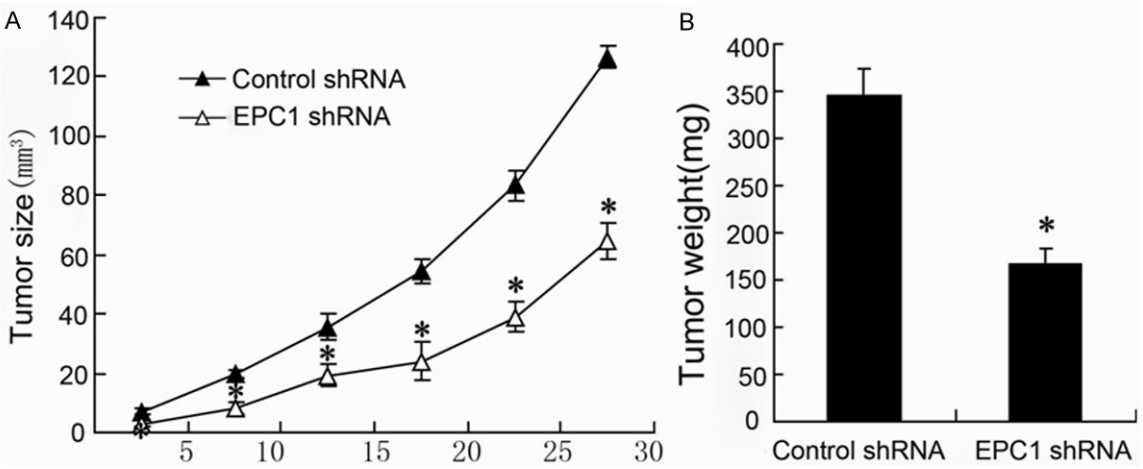

Figure 3. Silencing EPC1 inhibits the growth of tumor in vivo. The mice were sacrificed after thirty days. The tumor size (A) and tumor weight (B) were measured.

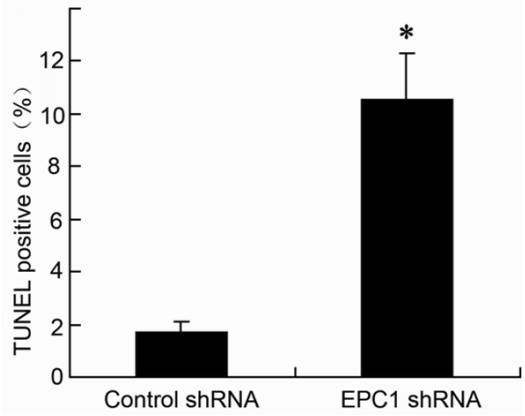

Figure 4. Silencing EPC1 promotes the cells apoptosis in lung tumor. TUNEL Assay of tumor tissue was applied to calculate the percentage of TUNEL-positive cells.

Statistics

Statistical analyses were performed using SPSS 18.0. The method of Independent Sample T test was performed to do data comparison between two groups. The data in present study were expressed as the mean ± standard deviation. $P < 0.05$ was considered as significant. $P < 0.01$ was regarded as highly significant.

Results

shRNA suppresses EPC1 in A549 cell lines

Quantitative PCR and western blotting were used to evaluate the effect of knockdown by shRNA transfection on A549 cells. As shown in **Figure 1**, both mRNA and the protein levels of EPC1 were significantly decreased by shRNA transfection in A549 cells, which implies that shRNA was effective to silence the expression of EPC1.

Silencing EPC1 inhibits cells growth and promotes its apoptosis

The A549 cells treated with EPC1 shRNA had a markedly lower survival rate compared with the control ones, and the inhibition effect of EPC1 shRNA in cells was in a time-dependent manner (**Figure 2A**). In another hand, the silencing EPC1 resulted in a significant increase in apoptosis of cells (**Figure 2B**).

Silencing EPC1 inhibits the growth of tumor in vivo

To better understand the effect of EPC1 on lung cancer, a stable transfection of A549 cell line with EPC1 shRNA was established in nude mice. As shown in **Figure 3**, the size and weight of tumor were significantly decreased in group with EPC1 shRNA transfection compared with the group treated with control shRNA.

Silencing EPC1 promotes the cells apoptosis in vivo

A TUNEL assay was employed to investigate the role of EPC1 shRNA in the cells apoptosis *in vivo*. The result (**Figure 4**) showed that the TUNEL positive cells in group transfected with EPC1 shRNA was five times more than that in

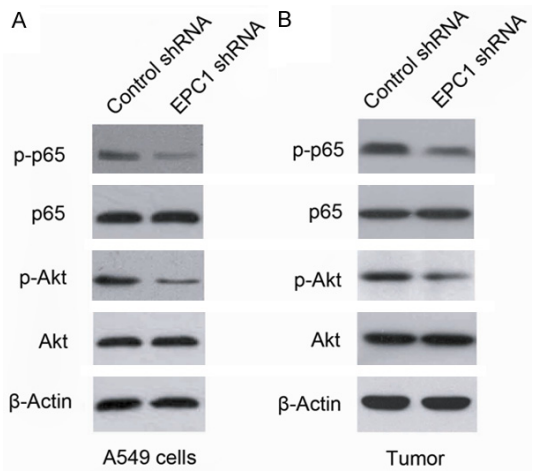

Figure 5. EPC1 shRNA suppresses the level of AKT and p65 expression. The protein levels of p65 and Akt were detected by western blotting in A549 cells (A) and in lung tumor (B).

control group, which indicated that EPC1 shRNA promoted the cell apoptosis in tumor.

EPC1 shRNA suppresses the expression of AKT and p65

To investigate the possible mechanism underlying the anti-tumor effect of EPC1 shRNA on lung cancer, the proteins of AKT and p65 were detected by western blotting. As shown in **Figure 5**, the total protein levels of p65 and Akt had no difference between groups with or without EPC1 shRNA treatment. However, EPC1 shRNA significantly decreased the phosphorylation level of p65 in both A549 cells and tumor (**Figure 5A**, **5B**), and the expression of phosphorylated Akt was also down-regulated in vitro and in vivo experiments (**Figure 5A**, **5B**).

Discussion

Lung cancer is the leading cause of cancer-related mortality not only in china but also around the world [20]. Non-small cell lung cancer (NSCLCs) arises from the epithelial cells of the central bronchi to terminal alveoli [21]. The new approach to lung cancer treatment is one focus of global research. In present study, the role of EPC1 was evaluated in lung cancer. shRNA technology was used to silence the expression of EPC1 *in vitro* and *in vivo*. The major finding of this study was that EPC1 shRNA markedly promoted the cell apoptosis in A549 cells and significantly inhibited the tumor growth in mice transfected with A549 cells. In addition, the effects of EPC1 on lung cancer were probably related to the regulation of p-AKT and p-p65 expression.

shRNA is a sequence of RNA that makes a tight hairpin turn that can be used to silence target gene expression via RNA interference [22]. Due to the ability of shRNA to provide specific and long-lasting effects on gene silencing, shRNA has caused wide public attention for gene therapy applications [18, 22, 23]. In our study, the results of real-time Quantity PCR and western blotting showed that EPC1 shRNA effectively down-regulated the mRNA and protein expression of EPC1 in A549 cells.

EPC1 encodes a member of the polycomb group family, which is a component of the NuA4 histone acetyltransferase complex and can act as both a transcriptional activator and repressor. The encoded protein has been linked to cell apoptosis, DNA repair, skeletal muscle differentiation, gene silencing, and adult T-cell leukemia/lymphoma [9-11]. The reports related to EPC1 are very few. However, recent study showed that EPC1 acted as a critical oncogenic cofactor in acute myeloid leukemia [10]. In present research, we found that EPC1 shRNA significantly decreased the survival rate and increased the apoptosis in A549 cells, which supplies an anti-proliferation effect on cancer cells. The further study of *in vivo* experiments confirmed that EPC1 shRNA inhibited the growth of tumor and promoted the apoptosis of tumor cells in mice. The findings above provided a new idea to treatment of lung cancer.

To further clarify the molecule mechanisms of EPC1 shRNA involved in inhibition of tumor growth, the proteins of phosphorylated AKT and p65 were detected by western blotting. AKT is a critical signaling node downstream of phosphoinositide 3-kinase (PI3K), which is a signal transduction protein that figures prominently in mechanisms of carcinogenesis and chemoresistance [24-26]. Inhibition of AKT has also been reported to be a useful strategy to inhibit the cell proliferation in NSCLCs [27, 28]. In this study, EPC1 shRNA markedly decreased the expression of p-AKT in both *in vivo* and *in vitro*. p65, a protein involved in heterodimer formation, nuclear translocation and activation of NF-κB, which is required for NSCLC metastasis [29]. Our study found that the expression of

p-65 was down-regulated in A549 cells transfected with EPC1 shRNA. The findings described above indicated a possible mechanism underlying the anti-tumor effects of silencing EPC1 in cancer cells.

In conclusion, the present study revealed that silencing EPC1 by shRNA technology had the inhibition effects on lung cancer cells proliferation and tumor growth, and the regulation of NF-κB and AKT pathways was probably involved in its molecular mechanism. Therefore, EPC1 is a potential target for treatment of lung cancer, but a further research on its mechanism will be done in the future.

Disclosure of conflict of interest

None.

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