Suberoylanilide hydroxamic acid overcomes erlotinib-acquired resistance via phosphatase and tensin homolog deleted on chromosome 10-mediated apoptosis in non-small cell lung cancer

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Abstract

Background: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib, are widely used to treat non-small cell lung cancer (NSCLC). However, acquired resistance is unavoidable, impairing the anti-tumor effects of EGFR-TKIs. It is reported that histone deacetylase (HDAC) inhibitors could enhance the anti-tumor effects of other antineoplastic agents and radiotherapy. However, whether the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) can overcome erlotinib-acquired resistance is not fully clear.

Methods: An erlotinib-resistant PC-9/ER cell line was established through cell maintenance in a series of erlotinib-containing cultures. NSCLC cells were co-cultured with SAHA, erlotinib, or their combination, and then the viability of cells was measured by the 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and apoptosis was determined by flow cytometry and western blotting. Finally, the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was assessed by western blotting.

Results: The half-maximal inhibitory concentration of parental PC-9 cells was significantly lower than the established erlotinib-acquired resistant PC-9/ER cell line. PC-9/ER cells demonstrated reduced expression of PTEN compared with PC-9 and H1975 cells, and the combination of SAHA and erlotinib significantly inhibited cell growth and increased apoptosis in both PC-9/ER and H1975 cells. Furthermore, treating PC-9/ER cells with SAHA or SAHA combined with erlotinib significantly upregulated the expression of *PTEN* mRNA and protein compared with erlotinib treatment alone.

Conclusions: PTEN deletion is closely related to acquired resistance to EGFR-TKIs, and treatment with the combination of SAHA and erlotinib showed a greater inhibitory effect on NSCLC cells than single-drug therapy. SAHA enhances the suppressive effects of erlotinib in lung cancer cells, increasing cellular apoptosis and PTEN expression. SAHA can be a potential adjuvant to erlotinib treatment, and thus, can improve the efficacy of NSCLC therapy.

Keywords: Acquired resistance; Erlotinib; Histone deacetylase; Lung cancer; SAHA

Introduction

Lung cancer is the most common malignant tumor and the leading cause of cancer-related death worldwide. Approximately 80% of lung cancers are classified as non-small cell lung cancer (NSCLC) according to their histopathological features. Platinum-contained chemotherapy is the standard strategy for NSCLC patients when they are diagnosed at an advanced stage. However, the anti-tumor effects of chemotherapy drugs have plateaued. Therefore, new therapeutic agents are urgently needed.

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib, are widely used for the treatment of NSCLC. Although EGFR-TKIs have been successfully applied in clinical settings, especially for NSCLC patients with EGFR mutations, most patients eventually experience relapse after an initial positive response.[1,2] Therefore, it is imperative to explore the potential mechanisms of EGFR-TKI resistance to improve the therapeutic effect in NSCLC patients. It has been reported that histone deacetylases (HDACs) play an important role in the development and progression of various cancers. HDACs have also been reported to modulate the function of various genes, particularly those involved in the cell cycle, differentiation, and apoptosis. Furthermore, HDACs can upregulate the expression of several genes that are involved in the process of neovascularization, tumor invasion, and metastasis.[3,4]

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HDAC inhibitors can modulate a variety of genes and pathways in tumor cells, and thus, exhibit strong anti-tumor effects both *in vitro* and *in vivo* by regulating epigenetic enzymes. Suberoylanilide hydroxamic acid (SAHA), a broad-spectrum HDAC inhibitor, has been approved for the treatment of cutaneous T-cell lymphoma by the Food and Drug Administration. Studies have also shown that SAHA exerts a synergistic effect when used in combination with other drugs or radiotherapy.[5-7] However, whether SAHA can be used to reverse acquired resistance to EGFR-TKIs is not fully clear, and it is a critical barrier in the development of an effective therapeutic model for NSCLC.

In the present study, we investigated the potential role of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in acquired resistance to EGFR-TKIs by establishing an erlotinib-resistant PC-9/ER cell line, and evaluated the potential role of SAHA in overcoming erlotinib-acquired resistance in PC-9/ER cells.

Methods

Materials

Human lung cancer cells PC-9 and H1975 were gifted by the Anhui Medical University Binhu Center Laboratory. Dulbecco modified Eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from Hyclone (GE Healthcare Life Sciences, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution were bought from Sigma-Aldrich (Merck KGaA, Germany). SAHA and erlotinib were purchased from Selleck Chemicals (Houston, TX, USA). The Annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI) double-staining apoptosis detection kit was obtained from 7Sea Biotech (Shanghai, China). The primary antibody against PTEN protein was acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and establishment of the erlotinib-resistant cell line

PC-9 and H1975 cells were grown in DMEM and RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified 5% CO_2 atmosphere. To establish an erlotinib-resistant PC-9/ER sub-cell line, parental PC-9 cells were cultivated in erlotinib-containing medium, in which the concentration of erlotinib was gradually increased until a final concentration of 10 nmol/L medium was reached. The cells were then washed with sterile phosphate buffer solution (PBS) and maintained in fresh drug-free DMEM medium for 24 h. When the confluence of the surviving cells reached 80%, they were exposed again to erlotinib, whose concentration was gradually increased until 1 μmol/L medium.[8]

Cell viability

Approximately 8000 cells were seeded per well in 96-well plates overnight, and then cultured in drug solutions of serial concentrations (0–5 μmol/L erlotinib for PC-9 cells; 0–8 μmol/L erlotinib and 0–4 μmol/L SAHA for PC-9/ER and H1975 cells) for 48 h. Subsequently, 200 μL of MTT (0.5 mg/mL) solution was added to each well and cells were incubated for 4 h at 37°C in dark. Thereafter, the medium was discarded and 150 μL of DMSO was added per well before gently shaking on a shaker for 10 min. Finally, the optical density was detected at 490 nm using a microplate reader (Beckman Coulter, USA). Cell viability was calculated as follows: cell viability = [experimental group optical density [OD]) − blank group OD)/(control group OD − blank group OD). The half-maximal inhibitory concentration (IC_{50}) values were calculated using SPSS 19.0 software (SPSS,Inc., Chicago, IL, USA) with the dose-response curves.[9] The combination index (CI) method reported by Chou *et al* was used to assess the interactions between erlotinib and SAHA treatment using CompuSyn program (ComboSyn Inc., Paramus, NJ, USA). The CI values less than, equal to, and greater than 1 represented synergistic, additive, and antagonistic effects between erlotinib and SAHA, respectively.[10]

Flow cytometry

Apoptotic cells were detected using the AnnexinV-FITC/PI double-staining kit. Briefly, approximately 5×10^5 cells were seeded per well and incubated overnight at 37°C, and then treated with various concentrations of drugs for 48 h. The cells were collected and resuspended in 400 μL of 1× binding buffer, and 5 μL of Annexin V-FITC was added per tube; the tubes were incubated at 4°C in dark for 15 min. Thereafter, 10 μL of PI was added to each, and the tubes were incubated for 5 min in dark; apoptotic cells were detected within 30 min.

Western blotting

After drug treatment for 48 h, we collected the cells and lysed them using cold cell lysate for 30 min. The same amounts of proteins were loaded per lane in 10% odium dodecyl sulfate-polyacrylamide gels for electrophoresis; subsequently, the proteins were electrotransferred onto polyvinylidene difluoride membranes. The proteins were blocked with Tween-Tris buffer saline buffer containing 10% fat-free milk powder. After washing with PBS, the membranes were incubated with mouse anti-human PTEN antibodies overnight at 4°C. Horseradish peroxidase-labeled anti-mouse or anti-rabbit immunoglobulin G (Cell Signaling Technology) was used as the secondary antibody. We used the enhanced chemiluminescence kit for chemiluminescence detection.

Polymerase chain reaction assay

A stem-loop reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect the expression of *PTEN*. The expression data were normalized as geometric averages of the housekeeping gene *GAPDH* to control for differences in expression levels. *PTEN* was amplified using the following genomic DNA-specific primers: forward primer of *PTEN*, 5′-TAGATTTTTCTCTTTAGATA-3′ and reverse primer, 5′-TTGCTGAACTCCTCTGG-3′; forward primer of *GAPDH*: 5′-GCTGTGAAGACCCAG-GAGAG-3′ and reverse primer, 5′-AAGCACCAG-GAAACCACTTG-3′.

Statistical analysis

All data are presented as mean ± standard deviation of three or more independent experiments. The Student's *t* test was used for comparison between two groups and the one-way analysis of variance followed by the Bonferroni *post-hoc* test was used for comparing three or more groups. A *P* value of <0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using the SPSS software version 19.0

Results

Co-administration of SAHA and erlotinib synergistically inhibits NSCLC cell proliferation

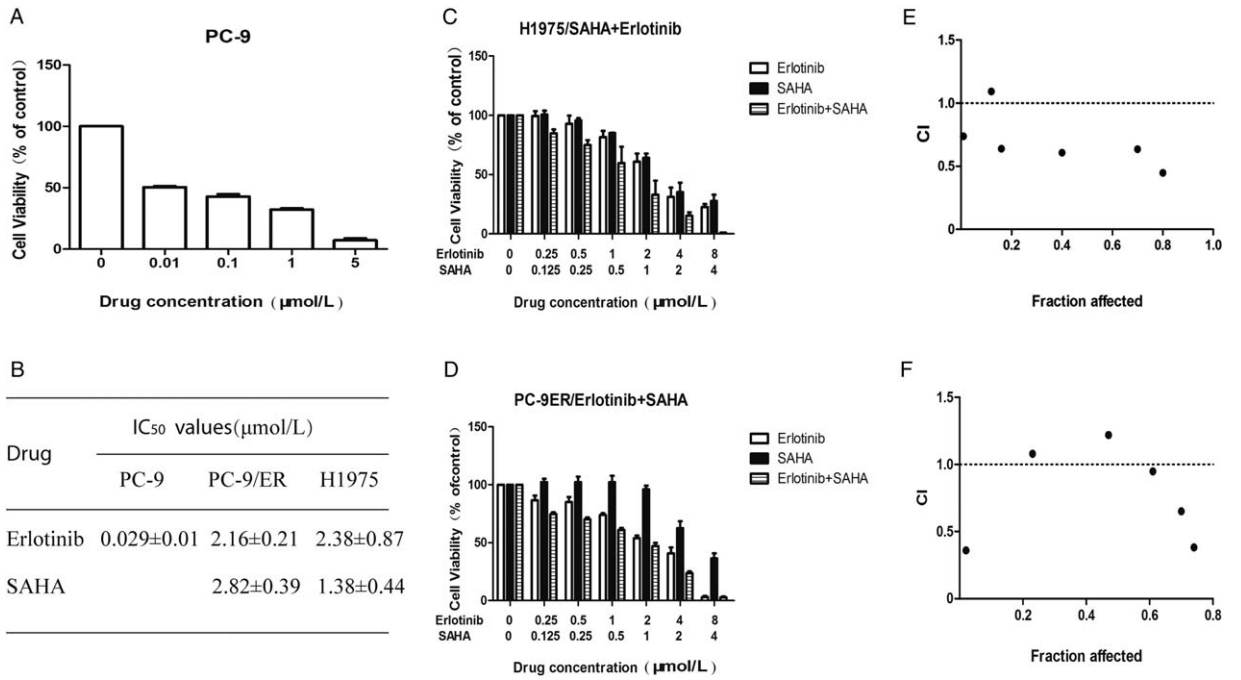
To investigate whether SAHA could overcome acquired resistance to EGFR-TKIs, we first established an erlotinib-acquired resistant PC cell line (hereinafter named PC-9/ER) by exposing PC-9 cells to increasing concentrations of erlotinib. As shown in Figure 1A and 1B, the IC_{50} value was 0.029 μmol/L for PC9 cells; however, it was 2.16 ± 0.21 and 2.38 ± 0.87 μmol/L for PC-9/ER cell lines and H1975 cell lines (harboring a T790M mutation in EGFR) [Figure 1B], respectively. PC-9/ER cells had a resistance index (RI) of 74.48 ($RI = IC_{50}^{PC\text{-}9/ER}/IC_{50}^{PC\text{-}9}$), which indicated that we successfully constructed an erlotinib-acquired resistant PC cell line. To elucidate the mechanism of EGFR-TKI acquired resistance, PC-9, PC-9/ER, and H1975 cells were cultured with a serial gradient concentration of erlotinib, and the MTT assay was performed to assess erlotinib, SAHA, or their combination on cell viability. Treatment with erlotinib or SAHA alone only slightly lowered the proliferation of H1975 [Figure 1C] and PC-9/ER cells [Figure 1D], whereas co-administration of erlotinib and SAHA significantly decreased the viability of H1975 [Figure 1C] and PC-9/ER [Figure 1D] cell lines. The CIs were calculated to assess the interaction between erlotinib and SAHA, and we found that the combined SAHA and erlotinib treatment had a synergistic action on the proliferation of both H1975 [Figure 1E] and PC-9/ER [Figure 1F] cells at most concentrations.

Correlation between PTEN loss and erlotinib resistance

Western blotting was performed to investigate whether PTEN contributed to erlotinib-acquired resistance. We found that PC-9/ER cells expressed lower levels of PTEN than PC-9 cells. However, no difference in PTEN expression was observed between H1975 and PC-9 cells [Figure 2A]. The above results suggest that the loss of PTEN may be associated with erlotinib resistance. Finally, to explore whether SAHA overcomes erlotinib resistance by modulating PTEN expression, we used both RT-PCR and western blotting to measure the expression of PTEN in PC-9/ER cells after treatment with different concentrations of SAHA (0.25–4 μmol/L). As shown in Figure 2B and 2C, treating PC-9/ER cells with 2 μmol/L SAHA significantly increased the PTEN protein [Figure 2B] and mRNA [Figure 2C] levels.

Erlotinib and SAHA synergistically induced apoptosis in PC-9/ER cells

To explore the effects of SAHA on apoptosis, we treated both PC-9/ER and H1975 cells with drugs at their IC_{50} values (1.5 μmol/L SAHA and 2 μmol/L erlotinib for H1975 cells; 2 μmol/L erlotinib and 2.5 μmol/L SAHA



B

Drug	IC50 values(μmol/L)		
	PC-9	PC-9/ER	H1975
Erlotinib	0.029±0.01	2.16±0.21	2.38±0.87
SAHA		2.82±0.39	1.38±0.44

Figure 1: SAHA + erlotinib inhibits cell proliferation. (A) Viability of PC-9 cells; (B) The IC_{50} of SAHA and erlotinib; (C) H1975; and (D) PC-9/ER cells treated with SAHA or erlotinib, or their combination. Drug combination indices (CIs) in H1975 (E) and PC-9/ER (F) cells. IC_{50}: Half-maximal inhibitory concentration; SAHA: Suberanilohydroxamic acid.

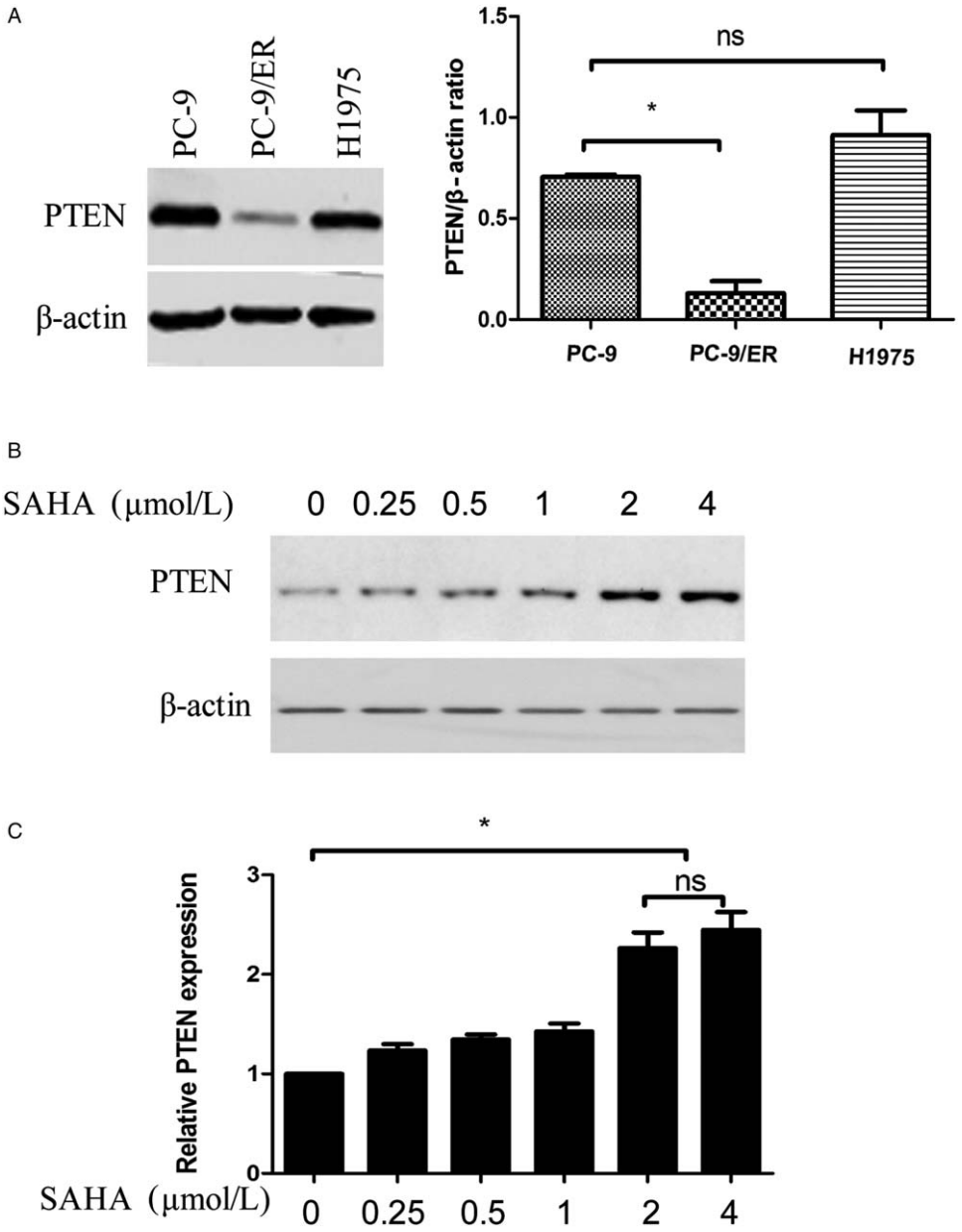


Figure 2: SAHA treatment increases PTEN expression. (A) PTEN expression in PC-9, H1975, and PC-9/ER cells. PC-9/ER cells were treated with SAHA, and then PTEN (B) protein and (C) mRNA levels were measured. $^{*}P < 0.05$, ns represents $P > 0.05$. PTEN: Phosphatase and tensin homolog deleted on chromosome 10; SAHA: Suberanilohydroxamic acid.

for PC-9/ER cells) for 48 h, and apoptotic cells were detected by flow cytometry assay. Our data confirmed that SAHA combined with erlotinib significantly increased apoptosis in H1975 cells (52.73% ± 2.63%) compared with SAHA (29.8% ± 1.55%) or erlotinib (10.12% ± 0.99%) treatment alone [Figure 3A]. Similarly, treatment of PC-9/ER cells with a combination of erlotinib and SAHA increased the apoptosis rate to 43.6% ± 4.99%, which was significantly higher than that with erlotinib (16.09% ± 1.75%) or SAHA (14.25% ± 1.19%) alone [Figure 3B].

SAHA increases PTEN expression in PC-9/ER cells

To understand the effect of SAHA on PTEN expression, we assessed the expression of PTEN in PC-9/ER and H1975 cells following treatment with SAHA. The cells were incubated with SAHA at IC_{50} values for 48 h. The expression of PTEN in H1975 cells increased after SAHA treatment alone or combined with erlotinib, whereas erlotinib treatment alone had no significant effect [Figure 4A and 4B] on PTEN expression. In PC-9/ER cells, PTEN expression was obviously increased following treatment with SAHA alone or in combination with erlotinib, compared with control cells and cells treated with erlotinib alone [Figure 4C and 4D].

Discussion

Over 60% of NSCLC cases are accompanied by high EGFR expression, which is also an indicator of poor prognosis.[9,11] Acquired resistance is emerging as a serious problem

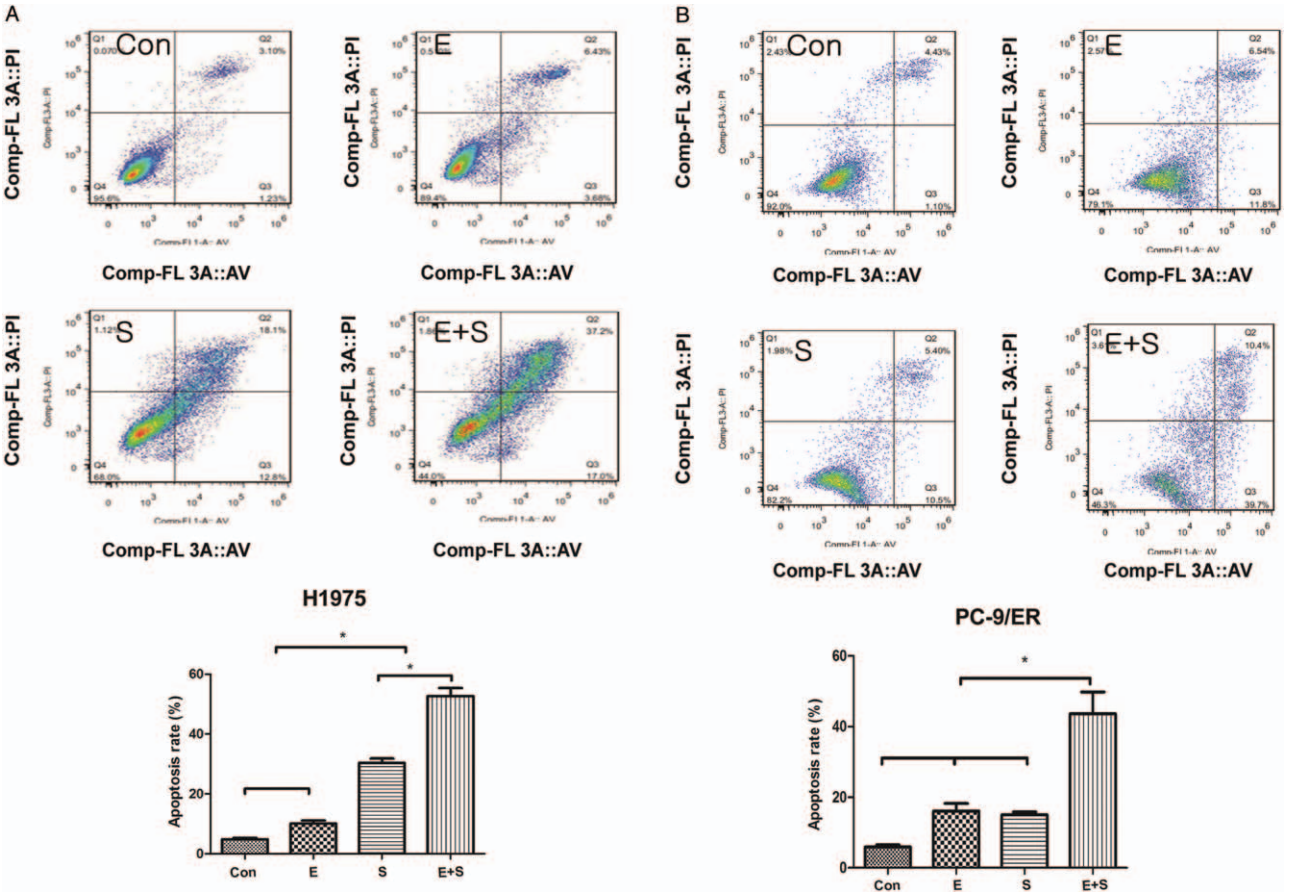


Figure 3: Combination of SAHA and erlotinib increases erlotinib-resistant cell apoptosis. Treatment of H1975 (A) or PC-9/ER cells (B) with either SAHA or erlotinib alone, or their combination. Con: Control, E: Erlotinib, S: SAHA, E + S: Erlotinib combined with SAHA. $^{*}P < 0.05$. SAHA: Suberanilohydroxamic acid.

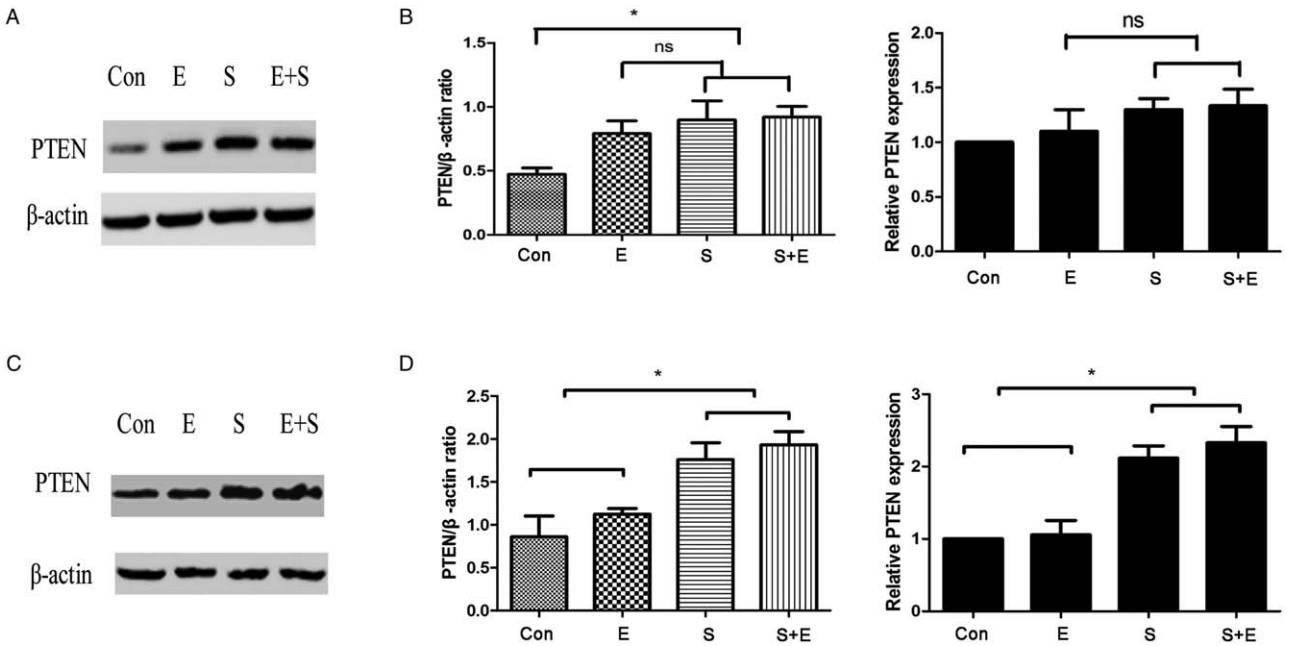


Figure 4: SAHA increases PTEN expression. PTEN protein (A) and mRNA (B) levels in H1975 and PC-9/ER cells (C and D) after treatment with SAHA (S) or erlotinib (E) or their combination (E + S). Con: Control. $^{*}P < 0.05$, ns: $P > 0.05$. PTEN: Phosphatase and tensin homolog deleted on chromosome 10; SAHA: Suberanilohydroxamic acid.

hampering the treatment effects of EGFR-TKI agents. To overcome EGFR-TKI resistance, several generations of EGFR-TKIs have been developed for treating NSCLC patients, such as afatinib and HKI-272 (second-generation EGFR-TKIs) and WZ4002 and AZD9291 (third-generation EGFR-TKIs). However, resistance is inevitable. There are several mechanisms that contribute to EGFR-TKI resistance, including EGFR T790M mutations, Mesenchymal-epithelial transition factor/hepatocyte growth factor signal axis over-activation, and Mesenchymal-epithelial transition factor amplification.[10,12] Among all the reported mechanisms, EGFR T790M secondary mutations and Mesenchymal-epithelial transition factor amplification are responsible for approximately 50% of all acquired resistance to EGFR-TKIs; however, the reasons in the remaining cases are unclear. Therefore, it is important to clarify the mechanisms of resistance to develop new therapeutic agents. *PTEN* is a tumor suppressor gene located at the 10q23 locus. PTEN deletion is related to tumorigenesis and poor prognosis in several tumors.[13] In lung cancer, the loss of PTEN is related to poor prognosis and EGFR-TKI resistance.[14] In the present study, we established an erlotinib-acquired resistance PC-9 cell line and found that the expression of *PTEN* in PC-9/ER cells was significantly lower than that in its parental PC-9 cells. This suggests that the loss of *PTEN* may be an underlying reason contributing to EGFR-TKI resistance in NSCLC cells. Our results were highly consistent with an earlier study, which showed PTEN loss was associated with tumor cells' resistance to cetuximab in head and neck squamous cell carcinoma patients.[15] In addition, it is well known that the target of HDACs is PTEN, and several reports have demonstrated that inhibition of HDACs could upregulate the expression of PTEN.[16-18] SAHA has been reported to promote apoptosis in a number of tumor cells and inhibit the growth and proliferation of these cells.[19-22] SAHA (2 μmol/L) could significantly increase the effect of EGFR-TKIs to induce the death of H358 and A549 cell lines, indicating that the sensitivity of tumor cells to EGFR-TKIs was potentially restored by HDAC inhibitors.[23] Furthermore, Neale *et al* reported that the addition of SAHA at IC_{50} doses (20 μmol/L for H520 and 7 μmol/L for A549) for 48 h considerably potentiates the anti-proliferative activity of carfizomib.[24] Additionally, a phase I/II study demonstrated that gefitinib at 250 mg/day combined with vorinota at 400 mg/day biweekly was feasible and well-tolerated.[25]

The anti-tumor effects of SAHA remain unclear in EGFR-TKI-resistant lung cancers. In this study, treating PC-9/ER cells with SAHA alone or in combination with erlotinib significantly increased the PTEN levels compared with erlotinib treatment alone. However, no significant difference was observed in H1975 cells, although SAHA induced apoptosis when used in combination with erlotinib. This may be due to the effect of HDAC inhibitors on cell apoptosis. SAHA has been proved to promote leukemia cell apoptosis in the G0/G1 phase by upregulating the expression of cyclin-dependent kinase inhibitor p21 and causing cell cycle arrest.[26,27] These results suggest that SAHA could enhance the sensitivity of tumor cells to EGFR-TKIs by upregulating PTEN in certain cell types.

Recent studies have indicated that the pro-apoptotic effects of HDAC inhibition occur in almost all tumor cells. Several researchers have reported that HDAC could regulate the expression of both pro-apoptotic and anti-apoptotic proteins to regulate tumor cell apoptosis.[28] Inhibition of HDAC-induced apoptosis was mediated directly by activating the endogenous (mitochondrial) and exogenous (death receptor) apoptosis pathways, as well as indirectly by improving the sensitivity of tumor cells to apoptosis. Inhibition of HDAC activity can upregulate B-cell lymphoma 2 modifying factor (BMF) expression and promote BMF-mediated cellular apoptosis.[29] Furthermore, inhibition of HDAC reduces the expression of cellular: Fas-associating protein with a novel death domain (FADD)-like interleukin beta-converting enzyme and promotes the formation of death-inducing signal complex, which could further cause caspase-8 activation. Here, we demonstrated that SAHA in combination with erlotinib can significantly increase apoptosis in NSCLC cells, but the underlying mechanism requires further investigation.

A significant reduction in PTEN level in NSCLC cell lines has been reported.[30] Furthermore, the knockdown of PTEN significantly increased the viability of cells, whereas overexpression of PTEN significantly decreased the viability of NSCLC cells.[31] A previous study showed that NSCLC H1650 cell line, with the loss of PTEN, was resistant to erlotinib, and that susceptibility to erlotinib-induced apoptosis was increased by PTEN reconstitution. Moreover, apoptotic cell fraction was significantly reduced by PTEN silencing in PC-9 cells when treated with erlotinib.[32] Together, these data suggest that the absence of PTEN may be one of the mechanisms of EGFR-TKI resistance.[33,34] Similarly, the present study showed that the loss of PTEN was associated with resistance to erlotinib. It has also been reported that the inhibition of PTEN is a key factor in the process of cell apoptosis, which mainly depends on the phosphorylation and dephosphorylation of Akt, and Caspase 3 is activated and poly ADP-ribose polymeras (PARP) cleavage is promoted through overexpression of PTEN. Additionally, PTEN loss leads to a significant decrease in the apoptosis of EGFR mutant cells; it also significantly reduces the sensitivity of EGFR-TKIs by activating Akt and EGFR. PTEN is a well-known target of HDACs. Not surprisingly, the expression of PTEN is upregulated by HDAC inhibition.[35,36] SAHA could considerably increase BIM protein expression as well as cleaved poly ADP-ribose polymeras (and cleaved caspase-3, when combined with gefitinib.[37] In addition, TSA, an HDAC inhibitor, potentiated apoptosis in oral squamous cell carcinoma cells via the activation of PTEN and inactivation of AKT.[18] The present study similarly showed that SAHA could increase PTEN expression in PC-9/ER cell lines and increase apoptosis. Overall, SAHA may overcome erlotinib-acquired resistance via PTEN-mediated apoptosis in NSCLC; however, further experimental evidence is required to confirm this, including knockdown of PTEN and expression of downstream apoptosis-associated protein.[38]

Our results suggest that the combination of SAHA and erlotinib could produce a synergistic anti-tumor effect in NSCLC. This finding has a substantial effect in the search for more effective treatments for human lung cancer. Furthermore, we revealed that the loss of PTEN

contributed to erlotinib resistance and that SAHA could upregulate PTEN expression and increase tumor cell apoptosis. The specific underlying molecular mechanisms remain to be elucidated.

Conflicts of interest

None.

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