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Isorhapontigenin improves the sensitivity of non-small cell lung cancer cells to gefitinib by inactivation of the SP1/EGFR pathway

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ABSTRACT: Non-small cell lung cancer (NSCLC) remains one of the most common cancers in the world. The aim of this study was to explore the therapeutic potential of isorhapontigenin (ISO) to overcome gefitinib resistance in NSCLC cells. Cell viability was higher in gefitinib-resistance A549 (A549/R) cells than that in A549 cells in response to gefitinib. Combination of gefitinib and ISO enhanced the reduction in cell viability and cell colony number induced by gefitinib or ISO alone. The number of apoptotic cells was increased in gefitinib and ISO combination group compared with A549/R cells treated with gefitinib or ISO alone. Combination of gefitinib and ISO inhibited the repairment of DNA damage in A549/R cells and repressed the activation of SP1/EGFR signaling pathway. In summary, data of this study proved that ISO enhanced the sensitivity to gefitinib through inactivation of SP1/EGFR signaling pathway in gefitinib-resistant NSCLC cells.

KEYWORDS: isorhapontigenin, sensitivity to gefitinib, non-small cell lung cancer cells, the SP1/EGFR pathway

INTRODUCTION

Lung cancer is the third most common cancer in the world with the highest mortality [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases [2]. With the development of personalized medicine, genetic alternations have been investigated and provided the treatment guidance for the selection of target drugs for NSCLC [2]. The clinical outcomes of NSCLC patients have greatly improved by immunotherapy and tyrosine kinase inhibitors (TKIs) on the basis of cancer gene testing [3]. However, the genetic mutation is very heterogenous in NSCLC [4], and not all the patient are eligible for using target therapy, making the prognosis very poor in NSCLC patients [5]. Therefore, new understanding and new drugs are required to improve the prognosis of NSCLC.

Epidermal growth factor receptor (EGFR) is the most common gene altered in NSCLC [5]. The incidence of EGFR mutation is approximately 15% in United States and approximately 50% in Asia in NSCLC patients [6]. The approval of EGFR TKIs revolutionized the standard of care in NSCLC patients with EGFR mutation [7]. Gefitinib is one of the first-generation EGFR TKIs approved in 2003 and showed a superior efficacy in EGFR-mutated NSCLC [8]. However, most patients will suffer the disease progression and acquired drug resistance with one year after treatment with EGFR TKIs [7]. Overcoming drug resistance has become a hot topic in target therapy of NSCLC.

Isorhapontigenin (ISO) is an analog of resveratrol extracted from Chinese herbs and grapes [9]. ISO showed multiple biological benefits in anti-platelet, anti-inflammation, and anticancer [10]. In human breast cancer cell lines, ISO inhibited sphingosine kinases/tubulin stabilization through inactivation of MAPK/PI3K, resulting in cell cycle arrests and cell death [9]. ISO was also reported to induce the cell apoptosis and prevent cell proliferation through inhibition of EGFR-related pathways in prostate cancer [11]. In addition, the transcription factor Specificity Protein 1 (SP1) could be inhibited through inhibition of EGFR/p38-MAPK signaling in pancreatic cancer [12]. However, no studies have explored the role of ISO in NSCLC, especially in gefitinib-resistant NSCLC. Thus, the aim of this study was to explore the therapeutic potential of ISO to overcome gefitinib resistance in non-small cell lung cancer cells (NSCLC cells).

MATERIALS AND METHODS

Cell culture and treatment

Human NSCLC cell line A549 cells were purchased from Thermo Fisher Scientific Inc. (USA) and cultured Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher) containing 10% fetal bovine serum (FBS, Thermo Fisher) and 1% Antibiotic-Antimycotic (100 X, Thermo Fisher) at 37 °C with 5% CO_2.

A549 cells were treated with an increased concentration of gefitinib (Sigma-Aldrich, USA) for 8 months to develop the acquired resistance to gefitinib (A549/R cells) [13, 14]. The concentration and treatment duration of gefitinib were: 50 nM for 2 months, 100 nM for 2 months, 150 nM for 2 months, and 200 nM for 2 months.

ISO (Aladdin, China) was dissolved into dimethyl sulfoxide and stored at −20 °C. On the day of experiment, ISO solution was diluted with culture medium to the treatment concentration. The cells were treated with gefitinib or ISO for 48 h and collected for further experiments.

MTT assay and colony formation assay

3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay and colony formation assay were used to evaluate cell proliferation. The 10^4 cells/well of A549 or A549/R cells were seeded in 96-well plates and treated with ISO or gefitinib according to the experiment design. Cell viability was measured using the commercial MTT Assay Kit (Abcam, UK) according to manufacturer's protocol. Colorimetric detection was conducted at 570 nm using a Varioskan LUX multimode microplate reader (Thermo Fisher).

For cell colony formation assay, A549 or A549/R cells were cultured in sterile 6-well plates at the density of 200 cells/well. After treated as above, cells were fixed with FIX & PERM Cell Fixation and Permeabilization Kits (Thermo Fisher) and then stained with 0.1% (w/v) crystal violet for 30 min. The dyed cell colonies were observed and quantified under an inverted microscope (Keyence, China).

Flow cytometry

Cell apoptosis was examined using flow cytometry. After treated as above, cells were suspended and fixed using 10% methanol. After fixation, the cells were stained using Annexin V-FITC/PI apoptosis assay kit (NeoBioscience, China). Twenty thousand stained cells were collected for each experiment using CytoFLEX flow cytometer (Beckman, USA). The proportion of cells in early and late apoptosis was calculated.

Western blotting

After treated as above, cells were collected and lysed to extract protein using RIPA buffer (Beyotime, China). Protein concentration was quantified using Bicinchoninic Acid Kit (Merck KGaA, Germany). Fifteen μg of total protein was separated using 10% polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Merck KGaA). The transferred membranes were blocked using 5% of fat-free milk for 1 h at room temperature followed by probing with proper primary antibodies overnight at 4 °C. Finally, the membranes were probed with secondary antibody for 2 h at room temperature. The protein expression was visualized using Ultra High Sensitivity ECL Kit (GLPBio, China) and detected using Invitrogen iBright Imaging Systems (Invitrogen, USA). The primary antibodies used in this study (Cell Signaling, USA) were: γH2AX (#7631, 1:1000 dilution), p-DNA-PK (#68716, 1:2000 dilution), Rad51 (#8875, 1:1500 dilution), SP1 (#5931, 1:1500 dilution), p-EGFR (#2244, 1:500 dilution), EGFR (#4267, 1:2000 dilution), and β-actin (#4967, 1:10000 dilution).

Statistical analysis

Statistical analysis was performed using SPSS statistics (IBM, USA). All data were presented as mean ± SEM. Statistical difference was analyzed using student *t*-test between 2 groups and one-way ANOVA among multiple groups. The combination index (CI) value of the GEF and ISO drug-drug interactions was calculated using Chou-Talalay's CI method by CompuSyn program (Compusyn Inc., USA) [15]. CI < 1, $= 1$, and > 1 represented synergism, additive effect, and antagonism, respectively. Statistical significance was defined as p-value less than 0.05.

RESULTS

ISO improved the sensitivity to gefitinib in gefitinib-resistant NSCLC cells.

Results of MTT assay showed that the cell viability was higher in A549/R cells (IC_{50}, 62.19 μm) than that in A549 cells (IC_{50}, 12.75 μm) after treated with gefitinib (Fig. 1A). The CI was less than 1 (Fig. 1B), meaning there was a synergistic effect between gefitinib and ISO. Cell viability was reduced by ISO in a concentration-dependent manner in A549/R cells (Fig. 1C). Cell viability and the number of cell colonies were reduced in A549/R cells by gefitinib or ISO alone (Fig. 1D,E,F). This reduction was further enhanced by combined treatment with gefitinib and ISO in A549/R cells (Fig. 1D,E,F). These data demonstrated that ISO enhanced gefitinib-induced cell death in gefitinib-resistant NSCLC cells.

ISO promoted cell apoptosis and prevented the repairment of DNA damage in gefitinib-resistant NSCLC cells.

The number of apoptotic cells were increased in A549/R cells by gefitinib or ISO (Fig. 2). Combination of gefitinib and ISO further increased the number of apoptotic cells in A549/R cells (Fig. 2). Phosphorylation of histone 2AX (H2AX) at serine 139 (γH2AX) was upregulated by gefitinib or ISO, which was further upregulated by combined treatment with gefitinib and ISO in A549/R cells (Fig. 3A,B). Combination of gefitinib and ISO inhibited phosphorylation of DNA dependent protein kinase (DNA-PK), and gefitinib or ISO alone induced overexpression of ATPase, Rad51 in A549/R cells (Fig. 3A,C,D). These results confirmed that ISO accelerated cell apoptosis and prevented the repairment of DNA damage in gefitinib-resistant NSCLC cells.

ISO inhibited SP1/EGFR signaling pathway.

The expression of EGFR did not show any changes after treated with gefitinib, ISO, or combination of gefitinib

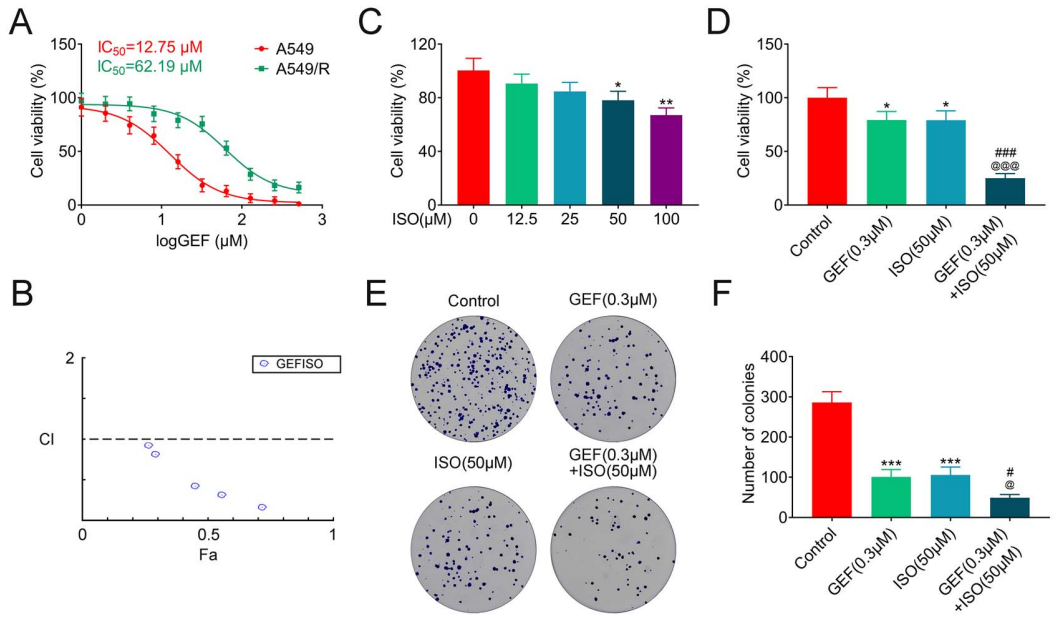

Fig. 1 ISO improved the sensitivity to gefitinib in gefitinib-resistant NSCLC cells. To check the cell response to GEF and ISO, A549 and A549/R cells were treated with different concentrations of GEF, ISO, or both for 48 h. Cell viability was measured using MTT assay after treated with different concentrations of (A) GEF, (C) ISO, and (D) the combination of GEF and ISO; (B) the combination index was calculated; and cell colony formation was also measured; (E) the images and (F) statistical results of cell colony formation. * $p < 0.05$ vs. Control; ** $p < 0.01$ vs. Control; *** $p < 0.005$ vs. Control. ISO: Isorhapontigenin; NSCLC: non-small cell lung cancer; GEF: Gefitinib; A549/R cells: GEF-resistant A549 cells.

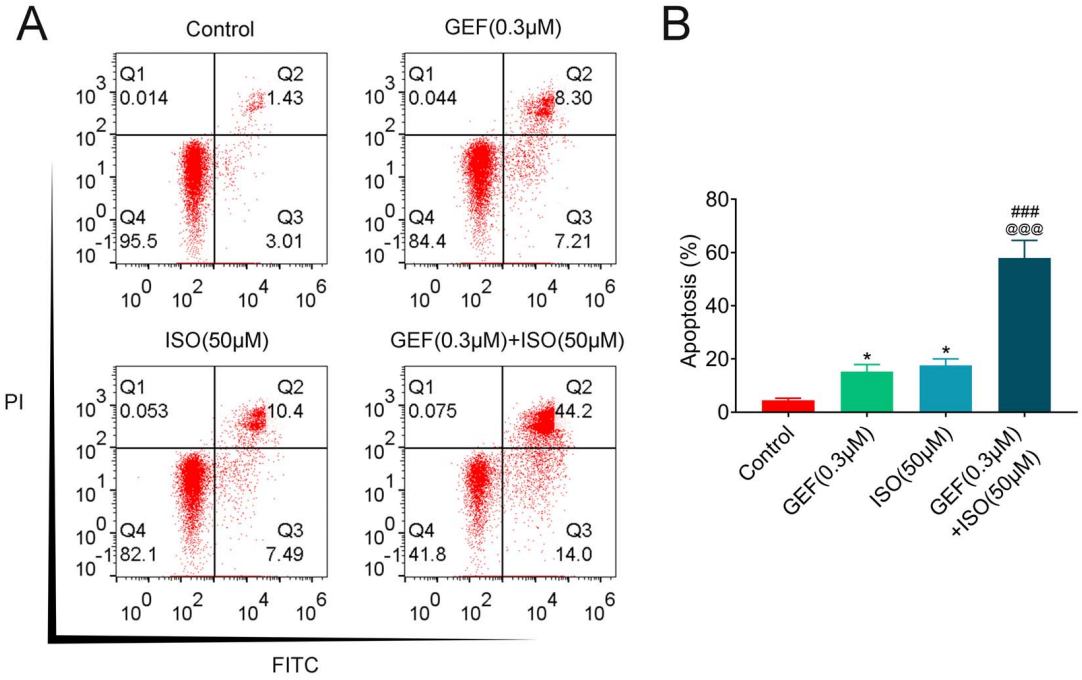

Fig. 2 ISO promoted cell apoptosis in gefitinib-resistant NSCLC cells. To investigate the synergistic effects of ISO and GEF on cell apoptosis, A549/R cells were treated with GEF and ISO alone or combined for 48 h, and flow cytometry was used to examine the cell apoptosis; (A) The dot plot and (B) statistical results of cell apoptosis. * $p < 0.05$ vs. Control; $^{\#\#\#}$ $p < 0.005$ vs. GEF; $^{@@@}$ $p < 0.005$ vs. ISO.

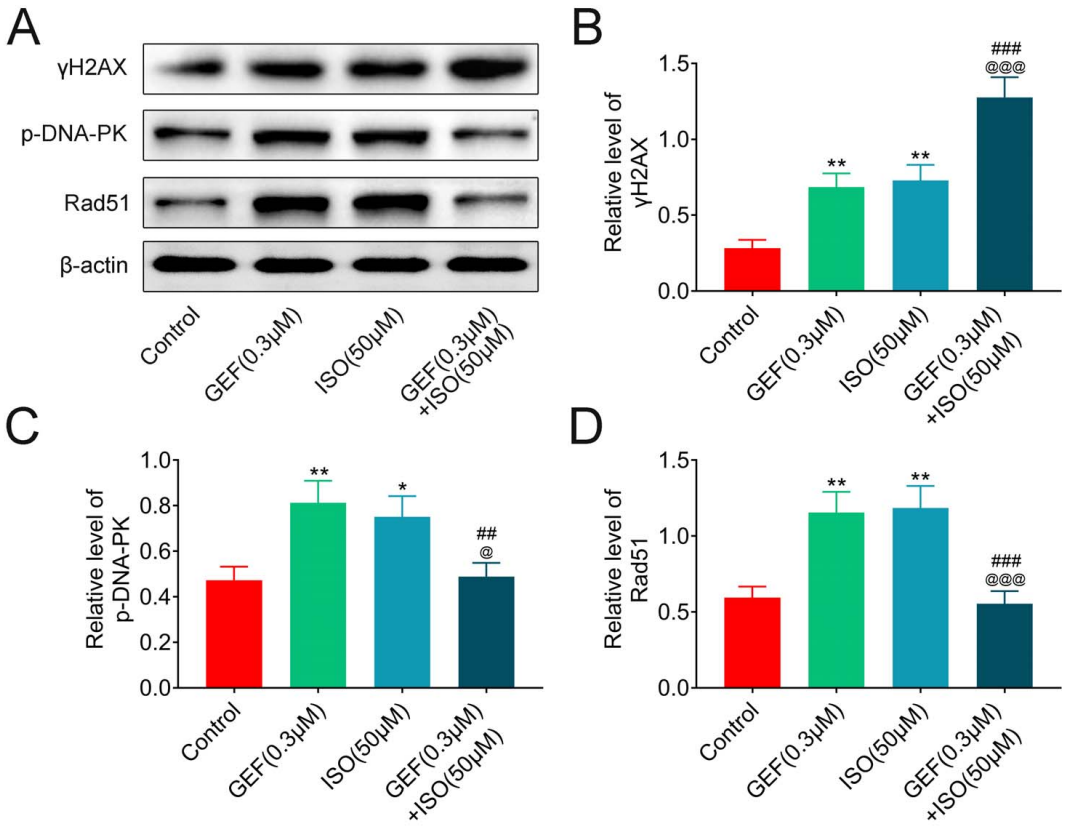

Fig. 3 ISO reduced repair of DNA damage in gefitinib-resistant NSCLC cells. To investigate the synergistic effects of ISO and GEF on DNA damage and repairment, A549/R cells were treated with GEF and ISO alone or combined for 48 h, and Western blotting was used to measure the protein expression related to DNA damage and repairment; (A) Western blotting results of protein expression; statistical results of protein expression of γH2AX (B), p-DNA-PK (C), and Rad51 (D). * $p < 0.05$ vs. Control; ** $p < 0.01$ vs. Control; $^{\#\#\#}$ $p < 0.005$ vs. GEF; $^{@@@}$ $p < 0.005$ vs. ISO.

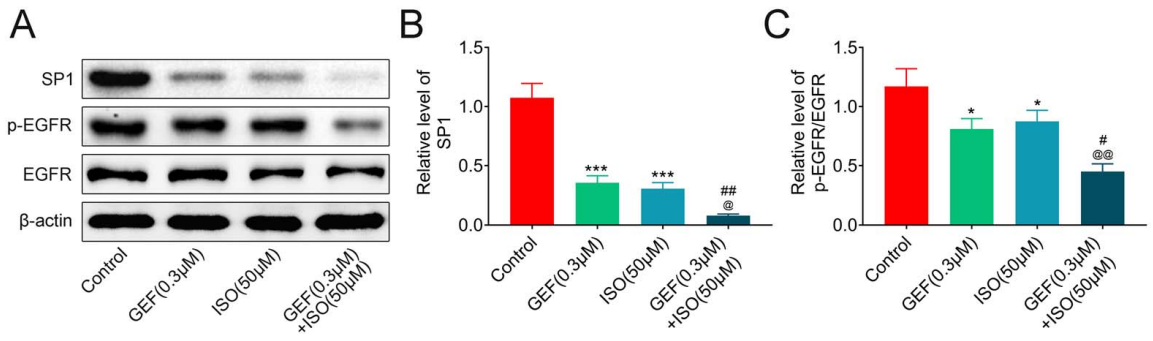

Fig. 4 ISO inhibited SP1/EGFR signaling pathway. To investigate the mechanism of ISO reversing GEF resistance, A549/R cells were treated with GEF and ISO alone or combined for 48 h, and Western blotting was used to measure the change of protein expression; (A) Western blotting results of protein expression; statistical results of protein expression of SP1 (B); and p-EGFR (C). * $p < 0.05$ vs. Control; *** $p < 0.005$ vs. Control; $^{\#}$ $p < 0.05$ vs. GEF; $^{\#\#}$ $p < 0.01$ vs GEF; $^{@}$ $p < 0.05$ vs. ISO; $^{@@}$ $p < 0.01$ vs. ISO.

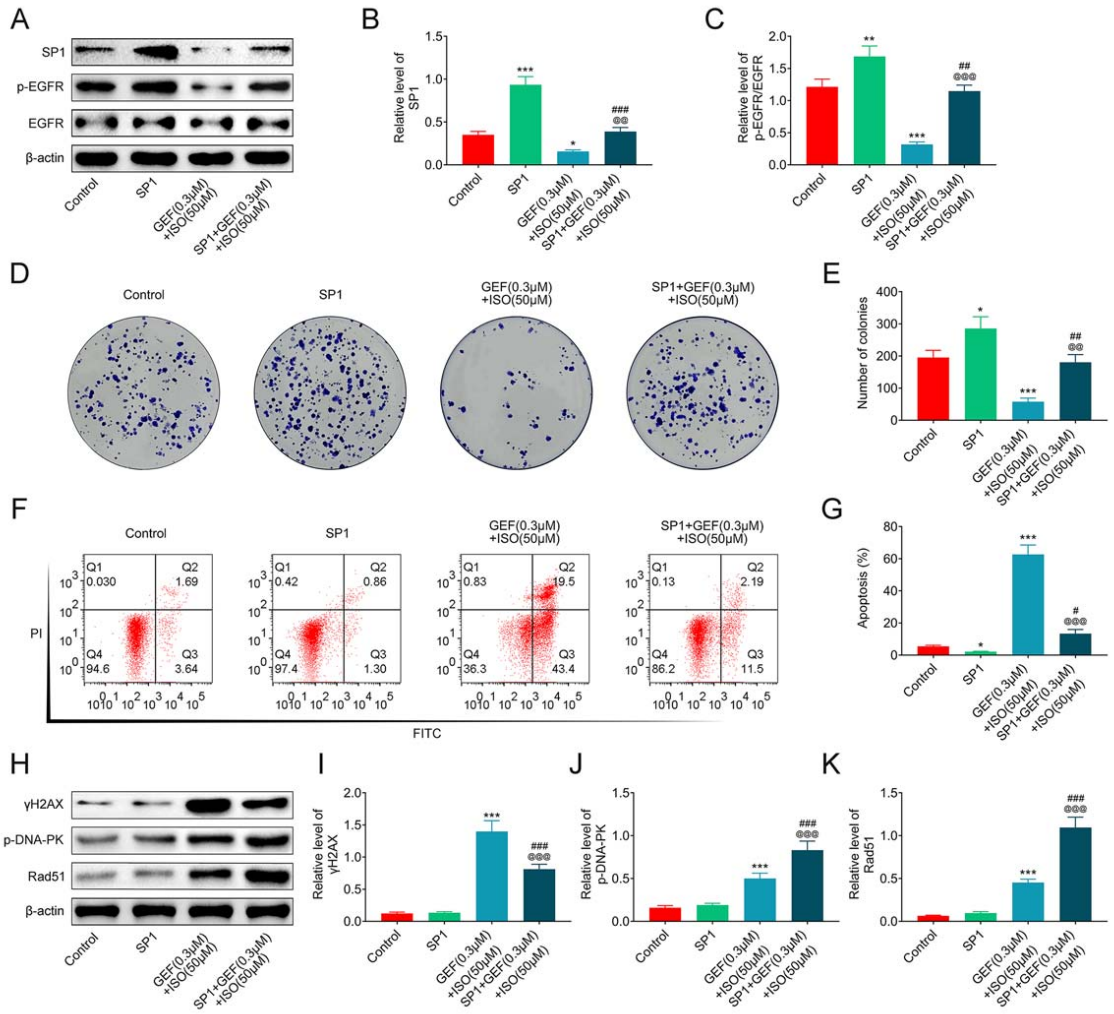

Fig. 5 Upregulation of SP1 reversed the effects of ISO on gefitinib-resistant NSCLC cells. Cells were transfected with SP1 plasmid or control vector and then cultured for 12 h. After that, cells were treated with GEF and ISO spontaneously for 48h for further analysis. Protein expression was analyzed using Western blotting (A, B, C, H, I, J, and K); cell proliferation was examined using cell colony formation assay (D and E). Cell apoptosis was determined using flow cytometry (F and G).

and ISO (Fig. 4). Expressions of Specificity Protein 1 (SP1) and phosphorylation of EGFR were suppressed by gefitinib or ISO alone (Fig. 4). Combination of gefitinib and ISO further inhibited the expression of SP1 and phosphorylation of EGFR in A549/R cells (Fig. 4), suggesting that ISO inhibited the activation of SP1/EGFR pathway. Overexpression of SP1 elicited the upregulation of SP1 and p-EGFR, while treatment with gefitinib and ISO inhibited this upregulation of SP1 and p-EGFR (Fig. 5A,B,C). Upregulation of SP1 increased the number of cell colonies, while treatment with gefitinib and ISO reduced the number of cell colonies (Fig. 5D,E). SP1-induced inhibition of cell apoptosis was reversed by treatment with gefitinib and ISO (Fig. 5F,G). Overexpression of SP1 inhibited the gefitinib- and ISO-induced upregulation of γH2AX, p-DNA-PK, and Rad51 induced (Fig. 5H,I,J,K).

DISCUSSION

Acquired drug resistance is the major reason for developing disease progression after targeted therapy [7]. The mechanism of acquired drug resistance remains unclear. Therefore, more and more pre-clinical and clinical studies focus on investigating new drugs as the alternative therapy to overcome drug resistance. In the present study, ISO was found to prevent cell proliferation and induce cell apoptosis in gefitinib-resistant NSCLC cells. Repairment of DNA damage was also suppressed by co-treatment with GEF and ISO. All these effects were shown to be mediated by inactivation of SP1/EGFR signaling pathway, demonstrating that ISO may overcome gefitinib resistance and inhibit

cell growth in NSCLC.

H2AX is a histone variant of the H2A family, and phosphorylation of H2AX at serine 139 is termed as "γH2AX", which is a useful indicator for DNA double strand break (DSB) [16]. Results of Western blotting showed that combination of ISO and gefitinib induced the upregulation of γH2AX in A549/R cells, implying the synergistic effects on DNA damage to kill cancer cells. γH2AX could be recognized by DNA-PK [16]. DNA-PK was rapidly phosphorylated in response to DNA damage to repair DSB [17]. In the present study, combination of ISO and gefitinib downregulated the phosphorylation of DNA-PK, thus preventing DSB repair process. Moreover, ISO plus gefitinib also inhibited the expression of Rad51, an ATPase contributing to DSB repair [18]. These findings manifested that ISO repressed the repairment of DNA damage and enhanced the sensitivity to gefitinib to promote cell death in gefitinib-resistant NSCLC cells. EFGR has been reported to induce the DNA-PK activation and upregulate Rad51 [19–21]. It was observed in the present study that both DNA-PK and Rad51 were upregulated by single treatment with GEF or ISO, implying that DSB was slightly repaired in GEF-resistant NSCLC cells. This might be one of the mechanisms of drug resistance, which was reversed by co-treatment with GEF and ISO, further confirming that ISO could reverse GEF-resistance in NSCLC cells.

SP1 is a family member of Sp transcription factors and is overexpressed in many cancers [22]. Upregulation of SP1 is highly associated with poor prognosis, so inhibition of SP1 has been proposed as the new direction for cancer treatment [22]. In this study, ISO plus gefitinib enhanced the ISO or gefitinib alone-induced suppression of SP1, suggesting that SP1 mediated the ISO-induced anti-cancer effects in A549/R cells. SP1 level was correlated with the expression of EGFR in human gastric cancer, and SP1 was able to activate the downstream cascade of EGFR pathways through interaction with half LIM domain protein 1 (FHL1) in glioblastoma [23]. ISO plus gefitinib inhibited the phosphorylation of EGFR in A549/R cells, implying that ISO prevented the activation of SP1/EGFR signaling pathway. Furthermore, previous study has proved that inactivation of SP1/EGFR signaling pathway could overcome gefitinib resistance through induction of autophagy and promote cells death in NSCLC cells [24]. Taken together, it could be concluded that ISO improved the sensitivity to gefitinib and induced cell death through preventing the activation of SP1/EGFR signaling pathway in gefitinib-resistant NSCLC cells, providing a potential supplemental treatment for gefitinib-resistant NSCLC.

In conclusion, ISO was found to prevent cell proliferation and induce cell apoptosis in gefitinib-resistant NSCLC cells. Repairment of DNA damage was also suppressed by ISO. All of these effects were proved to be mediated by inactivation of SP1/EGFR signaling pathway, suggesting that ISO may overcome gefitinib resistance and inhibit cell growth in gefitinib-resistant NSCLC cells.

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