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Original Paper

Rsf-1 Influences the Sensitivity of Non-Small Cell Lung Cancer to Paclitaxel by Regulating NF-κB Pathway and Its Downstream Proteins

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Key Words
Rsf-1 • Non-small cell lung cancer • Paclitaxel • Resistance • NF-κB pathway

Abstract
Background/Aims: The therapeutic efficacy of paclitaxel is hampered by chemotherapeutic resistance in non-small cell lung cancer (NSCLC). Rsf-1 enhanced paclitaxel resistance via nuclear factor-κB (NF-κB) in ovarian cancer cells and nasopharyngeal carcinoma. This study assessed the function of Rsf-1 in the modulation of the sensitivity of NSCLC to paclitaxel via the NF-κB pathway. ***Methods:*** The mRNA and protein levels of the related genes were quantified by RT-PCR and Western blotting. Rsf-1 silencing was achieved with CRISPR/Cas9 gene editing. Cell cycle, migration and proliferation were tested with flow cytometry, transwell test and CCK8 test. Cell apoptosis was analyzed with flow cytometry and quantification of C-capase3. The parameters of the tumors were measured in H460 cell xenograft mice. ***Results:*** Rsf-1 was highly expressed in H460 and H1299 cells. Rsf-1 knockout caused cell arrest at the G1 phase, increased cell apoptosis, and decreased migration and cell proliferation. Rsf-1 knockout increased the inhibition of cell proliferation, the reduction in cell migration and the augment in cell apoptosis in paclitaxel treated H460 and H1299 cells. Rsf-1 knockout further enhanced the paclitaxel-mediated decrease in the volume and weight of the tumors in H460 cell xenograft mice. Helenalin and Rsf-1 knockout decreased the protein levels of p-P65, BcL2, CFLAR, and XIAP; hSNF2H knockout decreased the protein level of NF-κB p-P65 without altering Rsf-1 and p65 protein levels, while Rsf-1 and hSNF2H double knockout decreased the

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level of NF-κB p-P65, in H1299 and H460 cells. ***Conclusion:*** These results demonstrate that Rsf-1 influences the sensitivity of NSCLC to paclitaxel via regulation of the NF-κB pathway and its downstream genes.

Introduction

Lung cancer is the leading cause of cancer-related mortality around the world [1, 2]; and non–small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases. The overall 5-year survival rate for stage IIIB/IV NSCLC is 1–5%, and approximately 70% of NSCLC patients are diagnosed at an advanced stage with local metastasis [3]. Chemotherapy can prolong the life of patients with advanced non-small cell lung cancer and effectively improve their quality of life [1]. Paclitaxel (PTX), a class of diterpenoid alkaloids, is a plant anticancer drug that shows effective and wider-spectrum anti-tumor activity *in vivo* and *in vitro* [4], and is widely used as a first-line chemotherapy regimen in the treatment of malignant tumors, such as breast cancer, non-small cell lung cancer, and ovarian cancer [5]. However, its therapeutic efficacy is restricted by the increasing frequency of chemotherapeutic resistance in NSCLC [6].

A variety of genes have been implicated in the development of primary or secondary resistance to paclitaxel in NSCLC [6]. Rsf-1 (also known as HBXAP) is a gene in the chromosome 11q13.5 locus [7]. Rsf-1 encodes a histone-binding protein named remodeling and spacing factor 1 (Rsf-1), which was found to interact with human sucrose nonfermenting protein 2 homologue (hSNF2H) to form an ISWI chromatin remodeling complex [8], which is involved in chromatin remodeling and transcriptional regulation [9]. Rsf-1 is amplified and/or overexpressed in various cancers, including ovarian [7, 8], breast [10], bladder [11], esophageal [12], lung [13], colon [14], and head and neck cancers [15]. Elevated levels of Rsf-1 are correlated with poor prognosis [16]. Inhibition of Rsf-1 reduces proliferation of cancer cells [8], suggesting an important role for Rsf-1 amplification and/or overexpression in the maintenance of cell survival and growth. It has also been demonstrated that Rsf-1 contributes to paclitaxel resistance by formation of the Rsf-1/hSNF2H complex [17]. Rsf-1 enhanced paclitaxel resistance via nuclear factor-κB (NF-κB) [17, 18, 19], Akt [17] and CREB-binding protein [17] by augmenting the expression of genes necessary for the development of chemoresistance. These genes include those involved in apoptosis (CFLAR, XIAP, BCL2, and BCL2L1) [18], the cell cycle (Survivin) [19] and inflammation (PTGS2) [18] in ovarian cancer cells [17] and nasopharyngeal carcinoma [19].

Overexpressed Rsf-1 contributes to malignant cell growth, invasion and poor overall survival [20] by regulating the NF-κB pathway activity [13] and the expression of MMP2 [13], bcl-2 [13], p-IκB [13], cyclin D1 [20] and ERK [20] in NSCLC. However, the role of Rsf-1 in conferring paclitaxel resistance to NSCLC has not been investigated. In this study, the function of Rsf-1 in the modulation of the sensitivity of NSCLC to paclitaxel was assessed by silencing Rsf-1 in NSCLC cells and xenograft mice. The involvement of the NF-κB pathway was also tested. Our results demonstrated that Rsf-1 influenced the sensitivity of NSCLC to paclitaxel via regulation of the NF-κB pathway.

Materials and Methods

Material

Helenalin (NF-κB inhibitor) was purchased from Santa Cruz Inc. (USA). The female nude mice were provided by Department of Experimental Animals, China Medical University (Shenyang, China).

Cell Culture

Non- small cell lung cancer A549, H1299, H460, and SPC cells, and human bronchial epithelial HBE cells (Institute of Cell Biology, Chinese Academy of Science, Shanghai, China) were cultured in DMEM media

containing 10% serum and 1% PenStrep (100 U/mL Penicilium and 100 μg/mL Streptomycin), at 37℃ in an incubator containing 5% CO_2.

Transfection

Transfection of H1299 cells and H460 cells with Rsf-1 CRISPR/Cas9 KO Plasmid (m), hSNF2H CRISPR/Cas9 KO Plasmid (h), and Control Plasmid (Santa Cruz Inc. USA) were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 1 mL of cell suspension was added to 10 ml of complete media in a culture flask and briefly incubated at 37℃ in an incubator containing 5% CO_2 for 24 h. Afterwards, the media was replaced with 10 ml Opti-MEM, a mixture containing 25 μl of Lenti-Easy Packaging Mix, 60 μl Lipofectamine 2000, 2895 μl of Opti-MEM, and 20 μl of the following: Rsf-1 CRISPR/Cas9 KO Plasmid, hSNF2H CRISPR/Cas9 KO Plasmid, or Control Plasmid. After the cells were incubated at 37℃ in an incubator containing 5% CO_2 for 8 h, the media was replaced with 12 ml DMEM containing 10% serum and cultured at 37℃ in an incubator containing 5% CO_2 for another 48 h. Cells were harvested and stored at -80°C for further analysis.

Western blotting

Western blotting was performed according to literature [21] with some modifications. The total protein of the cells was extracted using 500μl Lysis Buffer containing 2μl protease inhibitor Mix (Protein extract Kit, Vazyme Inc., USA). The protein concentrations were measured with BCA Protein Assay Kit (Solarbio Inc., Beijing, China) according to the manufacturer's instructions. Proteins were differentiated on polyacrylamide gel and transferred to PVDF membrane (Millipore Co., USA). Membrane was blocked in 5% skim milk in TBST, incubated with primary antibodies and secondary antibodies in TBST containing skim milk, incubated in ECL (Invitrogen), exposed to X-ray film, and developed. The signals were quantified with a densitometer. The target gene signal was normalized with β- actin or GAPDH signal and represented by the ratio of target gene signal to the internal control gene signal.

Real-time RT-PCR

Total RNA was prepared from the cells using Trizol reagent (DP431, TIANDEN, Beijing, China) according to the manufacturer's instructions. The amount and purity of the RNA were measured with a spectrophotometer. It was taken as pure if the OD260/OD280 was between 1.8 and 2.0. The primers of Rsf-1 and β-actin were designed using DNASTAR software based on the mRNA sequence of these genes in GenBank (Table1). The mRNA expression of Rsf-1 was quantified using the total RNA as a template with One Step SYBR® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (TAKARA, Dalian, China) according to the manufacturer's instructions. β-actin was used as an internal standard. The relative level of the mRNA expression of Rsf-1 was calculated with $2^{-\Delta\Delta ct}$.

Immunofluorescence (IF) staining

IF staining was performed according to literature [22]. Cells were fixed in 4% paraformaldehyde on slides, incubated in 0.5% Triton X-100/PBS, blocked with goat serum, detected with primary antibody and florescent-labelled secondary antibody, stained with DAPI (Calbiochem, San Diego, USA), mounted, and observed with laser scanning confocal microscopy.

Table 1. Primers used for real-time RT-PCR

Genes	GenBank NO.	Primers
RSF-1	NM_016578.3	Forward: 5'-AGGAGGCGCTCTCAAGTGGGCTCCT-3' Reverse: 5'-GATCCGCAGAGGAGCCCACTTGAGA-3'
GAPDH	NM_001256799.2	Forward: 5'-GGCACCGCAGGCCCCGGGATGCTAG-3' Reverse: 5'-CTGCGCACTAGCATCCCGGGGCCTG-3'

Flow cytometry test of cell cycle

10 ml of cell suspension (1-5×10^6 cells/mL) was spun at 500-1000 rpm for 5 min. The cell pellet was washed with PBS once and fixed in 3.70% ice ethanol at 4℃ for 2 hours. After

washing with PBS twice, the cells were suspended in PBS containing 50 mg/L RNase A and 25μg/ml PI and incubated in dark at 25℃ for 15 min. The cells at different phases were measured using flow cytometry with an excitation wave length of 488nm and an emission wave length greater than 560 nm to detect PI signal.

Flow cytometry test of apoptosis

10 ml of cell suspension (1-5×10^6 cells/mL) was spun at 500-1000 rpm for 5 min. The cell pellet was washed with PBS once and fixed in 70% ice ethanol at 4℃ for 2 hours. After the fixation solution was removed, the cells were suspended in 3 ml PBS for 5 min. This was filtered once with a 400 gauge filter and centrifuged at 1000 rpm for 5 min, and then the cell pellet was stained in 1 ml PI staining solution containing 50 mg/L RNase A and 25μg/ml PI at 4℃ in the dark for 30 min. Both the living and dead cells were measured using flow cytometry with an exciting wave length of 488 nm and an emission wave length greater than 630 nm.

Transwell test of the invasion capacity of the cells

H460 cells, H1299 cells, transfected H460 cells and transfected H1299 cells were starved in the media without serum for 12 hours, and then they were harvested and washed with PBS twice. The cells were suspended in DMEM containing BSA at a density of 5×10^5cells/ml. Cells for all of the four lines were divided into two portions: one portion was not treated and the other portion was added with 4μg/ml of paclitaxel. 500μl of DMEM containing 20% FBS was added to the lower chamber of each transwell on a 24 well plate. 200μl of cell suspension was seeded into the upper chambers of the transwells and cultured at 37°C in an incubator containing 5% CO_2 for 24h. The transwell chambers were taken out. After the media in the upper chambers were absorbed, the transwell chambers were moved into wells containing 800μl of methanol, and fixed at room temperature for 30 min. The transwell chambers were taken out again. The solution in the upper chambers was absorbed and the cells on the upper surface of the membrane on the bottom of the upper chamber were removed, and the transwell chambers were moved into wells containing 800μl of crystal violet staining solution and stained at room temperature for 15-30 min. After being rinsed in PBS several times, the transwell chambers were taken out, the solution in the upper chamber was absorbed, and the membrane was lifted and turned over to air dry. After the membrane was transferred to a slide and sealed, it was observed under a microscope and photographed.

CCK8 test of cell proliferation

Cells were harvested at exponential phase and suspended in DMEM media at 5×10^5 cells/ml. After 100μl of cell suspension was added to each well and incubated at 37℃ for 24 hours, paclitaxel was added, and the cells were incubated. 10μl CCK8 (CCK8 kit, Dojindo, Japan) was added to each well and incubated for 1-4 hours. The signal was measured at absorption wave lengths of 450 -490 nm, and reference wave lengths of 600-650nm. Each sample was tested as triplicate. Zero control was set with wells containing DMEM media and CCK8, but no treatment reagents.

Test of formation of tumors in nude mice

The female nude mice (6-week old, body weight 18-20g) were raised under pathogen-free conditions at 23 ± 2°C with 60 ± 5% humidity. Animals were divided into four groups: (1) mice transplanted with H460 cells; (2) mice transplanted with H460 cells and treated with paclitaxel; (3) mice transplanted with H460 Rsf-1KO cells; and (4) mice transplanted with H460 Rsf-1KO cells and treated with paclitaxel. Cells in the exponential phase were suspended in complete DMEM media at 2×10^7 cells/ml. 0.1ml of the cell suspension was injected subcutaneously in the back of the mice. When the tumors became visible, the tumors were measured by the length (L) and width (d) every day. The volumes of the tumors were calculated using the formula V= 0.5×L×d^2, and growth curves of the tumors were plotted as the volume of the tumor versus the time. Ten days after the tumors were formed, the mice were injected with paclitaxel at 10 mg/kg every other day through the tail vein. Thirty days after tumor formation, the mice were sacrificed and the tumors were dissected out and weighed.

Statistical analysis

All data were collected from the experiments performed in triplicate. Statistical analysis was carried out using the Graphpad Prism 5. Comparison between groups was performed using paired t test. Multiple

comparisons were performed using one-way repeated measures ANOVA, followed by Bonferroni post hoc test to determine the significance of the differences. Final results were presented as the mean ± SD. $P < 0.05$ was taken as statistically significant.

Results

Knockout of Rsf-1 in H460 and H1299 cells

Rsf-1 was highly expressed in H460 and H1299 cells at the protein level (Fig. 1A, B) and the mRNA level (Fig. 1C), and less expressed in A549 and SPC cells. After transfection by the vector Rsf-1 CRISPR/Cas9 KO Plasmid, no Rsf-1 protein was detectable with IF in both H460 and H1299 cells (Fig. 2B). Western blotting analysis showed that Rsf-1 protein levels dramatically decreased in the transfected H460 and H1299 cells compared with the corresponding control cells (Fig. 2A). These results indicated that Rsf-1 was successfully knocked out in H460 and H1299 cells.

Effect of Rsf-1 knockout in H460 and H1299 cells

Flow cytometry analysis (Fig. 3A to D) showed that Rsf-1 knockout significantly increased the cells in G1 phase, decreased the cells in S phase, and inhibited the transit from G1 phase to S phase in H460 and H1299 cells ($p < 0.05$). Rsf-1 knockout significantly increased cell apoptosis ($p < 0.05$) in both H1299 and H460 cells (Fig. 3E to H). Migration analysis showed that the migration ability was significantly decreased in H1299 (Fig. 4A, C, E) and H460 cells (Fig. 4F, H, J) after Rsf-1 was knocked out ($p < 0.05$).

Influence of Rsf-1 knockout on the effect of paclitaxel in H460 and H1299 cells

In both H1299 (Fig. 4A to E) and H460 (Fig. 4F to J), paclitaxel alone and Rsf-1 knockout alone significantly ($p < 0.001$) decreased the cell migration ability; Rsf-1 knockout further significantly

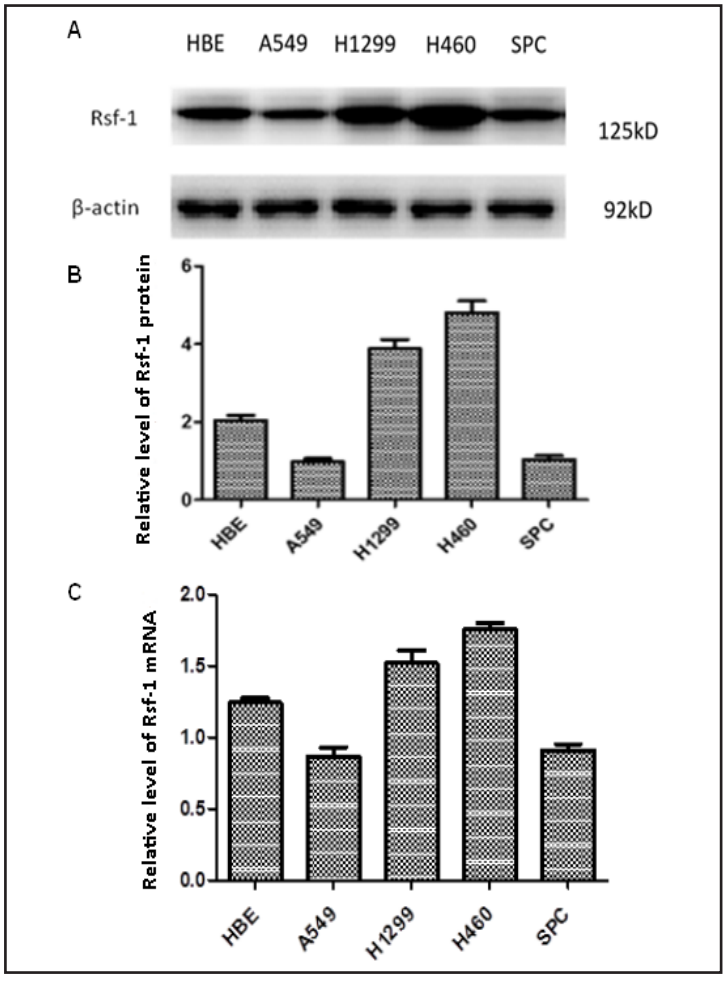

Fig. 1. Expression levels of Rsf-1 mRNA and protein in cultured cell lines. Panel A: Rsf-1 protein was detected in the cell lines with Western blotting; panel B: quantitative volume of Rsf-1 protein in the cell lines; panel C: quantitative volume of Rsf-1 mRNA was detected with Real-time RT-PCR. HBE, A549, H1299, H460 and SPC represent the corresponding cell lines; β-actin was used as an internal standard for Western blotting. GAPDH was used as an internal standard for Real-time RT-PCR. Rsf-1 mRNA and protein were highly expressed in cell lines of H1299 and H460.

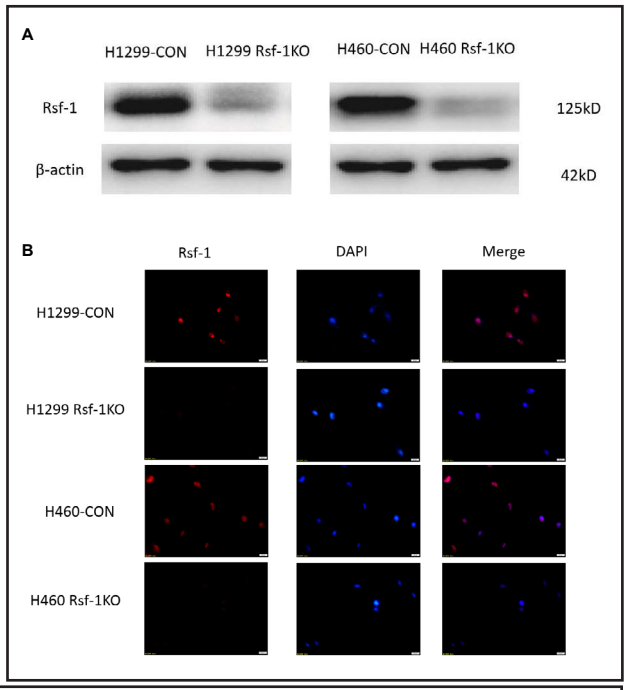

Fig. 2. Expression of Rsf-1 protein detected in the cells of H1299 and H460 with Rsf-1 knockout. Panel A: expression of Rsf-1 protein detected with Western blotting; H1299-CON, control H1299 cells; H1299 Rsf-1KO, H1299 cells with Rsf-1 knocked out; H460-CON, control H460 cells; H460 Rsf-1KO, H460 cells with Rsf-1 knocked out. Most Rsf-1 protein was depleted in H1299 cells with Rsf-1 knockout and H460 cells with Rsf-1 knockout. β-actin was used as an internal standard. Panel B: expression of Rsf-1 protein detected with IF; H1299-CON means control H1299 cells; H1299 Rsf-1KO means H1299 cells with Rsf-1 knockout; H460-CON means control H460 cells; H460 Rsf-1KO means H460 cells with Rsf-1 knockout. No Rsf-1 protein was detected in H1299 cells with Rsf-1 knockout and H460 cells with Rsf-1 knockout.

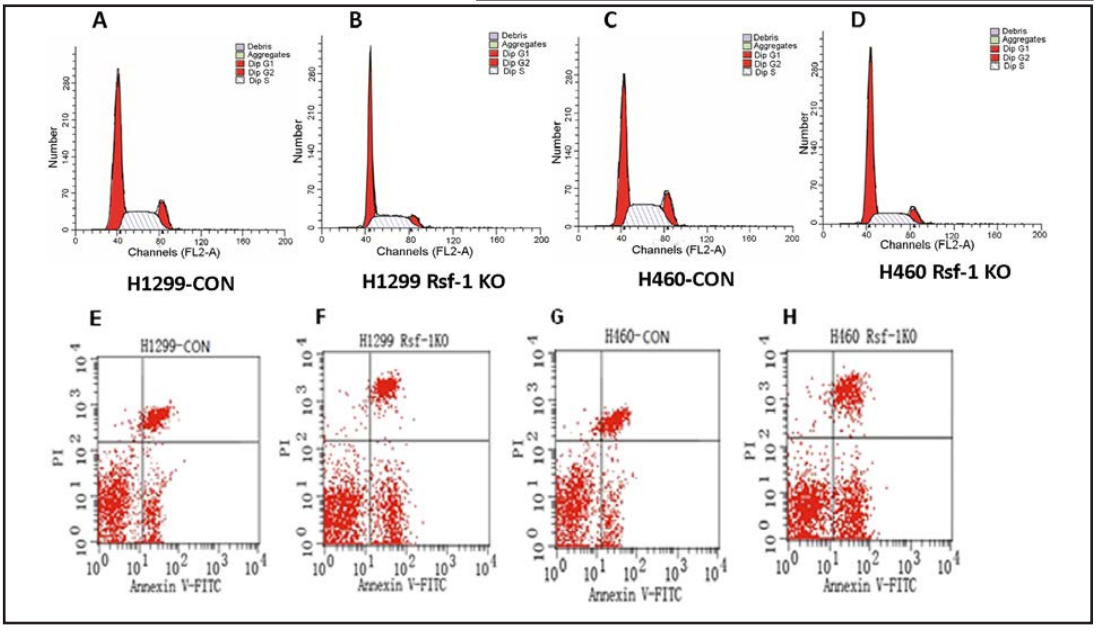

Fig. 3. Flow cytometry analysis of the impact of Rsf-1 knockout on the progression of cell cycle and apoptosis. Panel A-D: impact of Rsf-1 knockout on the progression of cell cycle; panel A: the number of cells at different cell cycle phases in the control H1299 cells; panel B: the number of cells at different cell cycle phases in Rsf-1 knockout H1299 cells; panel C: the number of cells at different cell cycle phases in the control H460 cells; panel D: the number of cells at different cell cycle phases in Rsf-1 knockout H460 cells. Panel E-H: impact of Rsf-1 knockout on the cell apoptosis; panel D: the numbers of living cells and dead cells in control H1299 cells; panel E: the numbers of living and dead cells in H1299 cells with Rsf-1 knockout; panel F: the numbers of living cells and dead cells in the control H460 cells; panel H: the numbers of living cells and dead cells in H460 cells with Rsf-1 knockout. After Rsf-1 being knocked out, cells in G1 phase significantly increased while cells in S phase significantly decreased ($p<0.05$); the transit from G1 phase to S phase was significantly inhibited ($p<0.05$); and cell apoptosis was significantly increased ($p<0.05$), in both H1299 cells and H460 cells.

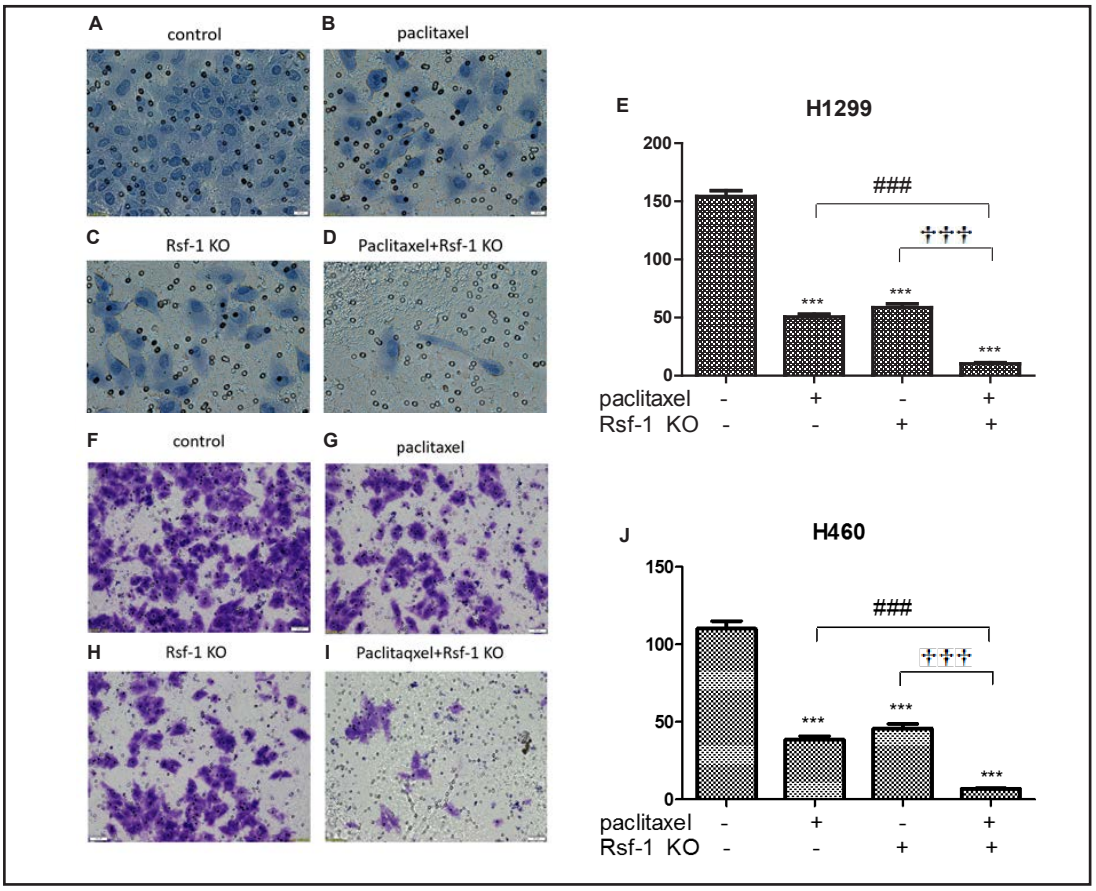

Fig. 4. Migration of H1299 and H460 cells after paclitaxel treatment and Rsf-1 knockout. A, control H1299 cells; B, H1299 cells treated with paclitaxel; C, Rsf-1 knockout H1299 cells; D, Rsf-1 knockout H1299 treated with paclitaxel; E; relative levels of cell migration in H1299 cell; F, control H460 cells; G, H460 cells treated with paclitaxel; H, Rsf-1 knockout H460 cells; I, Rsf-1 knockout H460 treated with paclitaxel; J, relative levels of cell migration in H1299 cell (***: significantly lower than the control cells, $p<0.001$; ###: significantly different between cells treated with paclitaxel and the Rsf-1 knockout cells treated with paclitaxel, $p <0.001$; †††: significantly different between the Rfs-1 knockout cells and the Rsf-1 knockout cells treated with paclitaxel, $p<0.001$).

($p < 0.001$) decreased the cell migration in paclitaxel treated cells to even lower levels; and there was no significant ($p > 0.05$) difference in the cell migration ability between the cells treated with paclitaxel alone and the Rsf-1 knockout cells without any other treatment (Fig. 4A to J).

After the cells were treated with paclitaxel at 0.5 μg/ml, 1 μg/ml, 2 μg/ml and 4 μg/ml for 48 hours, the inhibition of cell proliferation was significantly higher in H1299 Rsf-1 KO and H460 Rsf-1 KO cells than the corresponding control cell (Fig. 5A and B). After cells were treated with 4 μg/ml paclitaxel for 24, 48 and 72 h, respectively, the inhibition of proliferation was also significantly higher in H1299 Rsf-1 KO and H460 Rsf-1 KO cells than the corresponding control cells at all time points, respectively (Fig. 5C and D). The inhibition of paclitaxel on cell proliferation increased as the time and dosage increased in all cell lines (Fig. 5A, B, C, D).

Caspase3 and cleaved-caspase 3 (C-caspase3) expression

After H1299 CON, H1299 Rsf-1 KO, H460 CON and H460 Rsf-1 KO cells were treated with 4 μg/ml paclitaxel for 48 h, the levels of caspase3 and C-caspase 3 in the cells were detected with Western blotting (Fig. 6A, Fig. 7A,C).

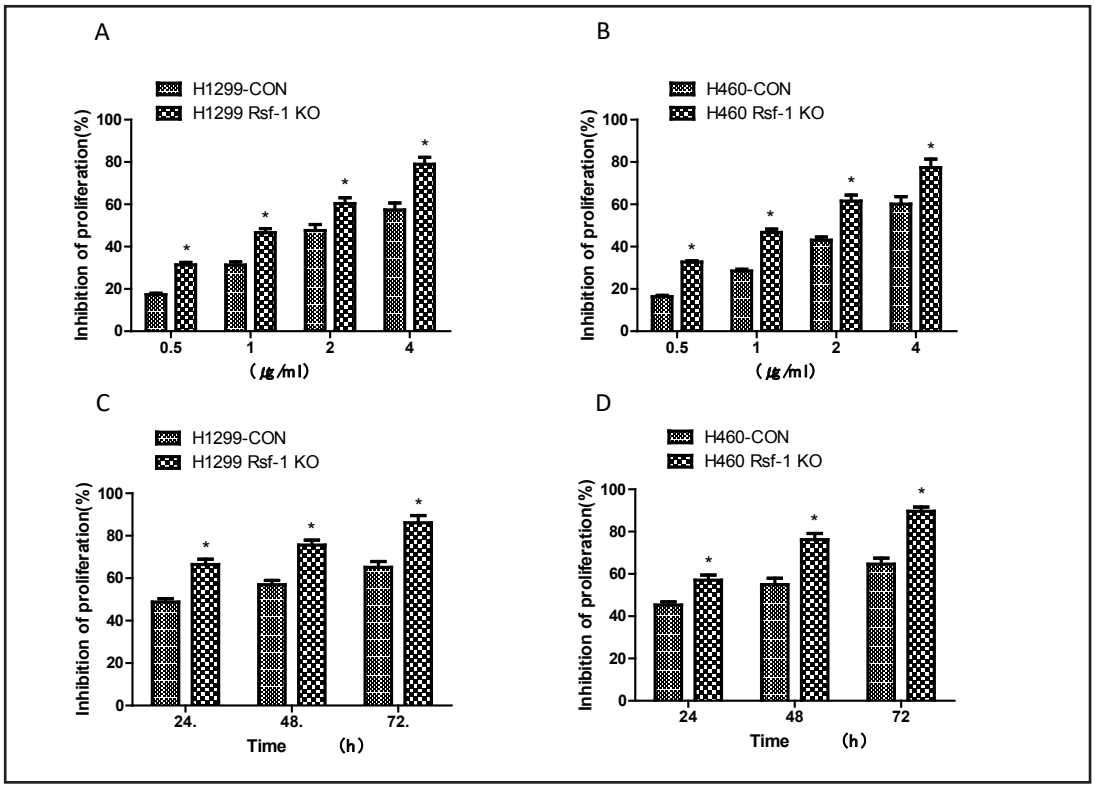

Fig. 5. Inhibition effect of paclitaxel on cell proliferation in vitro. Panel A: H1299 cells and H1299 cells with Rsf-1 knockout treated with different concentrations of paclitaxel; panel B: H460 cells and H460 cells with Rsf-1 knockout treated with different concentrations of paclitaxel; panel C: H1299 cells and H1299 cells with Rsf-1 knockout treated with 4μg/ml of paclitaxel; panel D, H460 cells and H460 cells with Rsf-1 knockout treated with 4μg/ml of paclitaxel; after cells were treated with paclitaxel at 0.5μg/ml, 1μg/ml, 2μg/ml and 4μg/ml for 48 hours, or 4μg/ml paclitaxel for 24h, 48h and72h, the inhibition of proliferation was tested with CCK8 test (*: $p<0.05$).

The levels of caspase3 protein were significantly higher in both H460 Rsf-1 KO and H1299 Rsf-1 KO cells than the corresponding control cells (Fig. 6B, C). The levels of caspase3 protein were significantly higher in H460 and H1299 cells treated with paclitaxel than the corresponding untreated cells (Fig. 6B, C). The level of caspase3 protein significantly increased to an even higher level in H460 Rsf-1 KO cells treated with paclitaxel compared with H460 cells treated with paclitaxel and H460 Rsf-1 KO cells, respectively (Fig. 6C). The level of caspase3 protein significantly increased to an even higher level in H1299 Rsf-1 KO cells treated with paclitaxel compared with H1299 cells treated with paclitaxel and H1299 Rsf-1 KO cells, respectively (Fig. 6B).

The level of C-caspase3 protein was significantly ($p < 0.05$) higher in both H460 Rsf-1 KO and H1299 Rsf-1 KO cells than the corresponding control cells (Fig. 7A to D). The level of C-caspase3 protein was significantly ($p < 0.05$) higher both H460 and H1299 cells treated with paclitaxel than the corresponding control cells (Fig. 7A to D). The level of C-caspase3 protein significantly ($p < 0.05$) increased to an even higher level in H1299 Rsf-1 KO cells treated with paclitaxel compared with H1299 cells treated with paclitaxel and H1299 Rsf-1 KO cells, respectively (Fig. 7A, B). The level of C-caspase3 protein significantly ($p < 0.05$) increased to an even higher level in H460 Rsf-1 KO cells treated with paclitaxel compared with H460 cells treated with paclitaxel and H460 Rsf-1 KO cells, respectively (Fig. 7C, D).

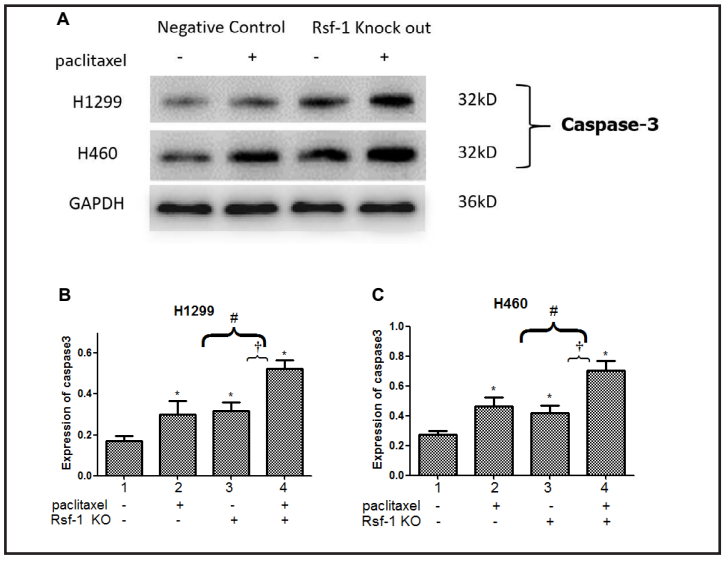

Fig. 6. Expression level of caspase3 in the cells treated with paclitaxel. Expression of caspase3 was detected after H1299 CON, H1299 Rsf-1 KO, H460 CON and H460 Rsf-1 KO cells were treated with 4μg/ml paclitaxel for 48. Panel A: expression of caspase3 detected with Western blotting; panel B: relatively quantitative level of caspase3 in H1299 cells; panel C: relatively quantitative level of caspase3 in H460 cell; 1) control cells without paclitaxel treatment; 2) control cells treated with paclitaxel; 3) Rsf-1 knocked out cells without paclitaxel treatment; 4) Rsf-1 knocked out cells treated with paclitaxel. GAPDH was used as an internal control (*: p<0.05; #: p<0.05; †: p< 0.05).

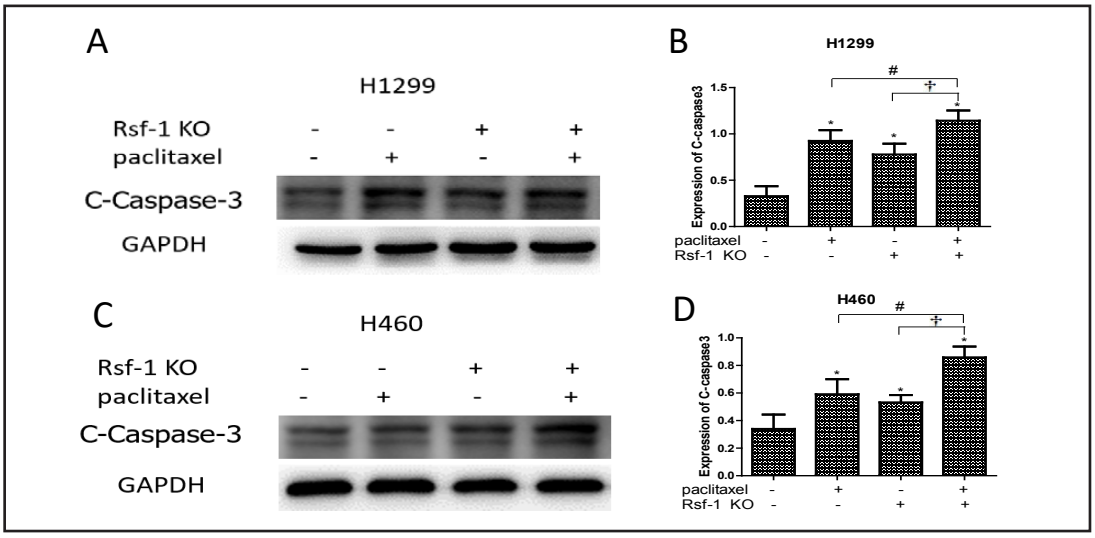

Fig. 7. Effects of paclitaxel on the level of C-caspase 3 in cells. Cells were treated with 4μg/ml of paclitaxel for 48 h, and the level of C-caspase 3 was tested with Western blotting using GAPDH as internal standard. A, Western blot of H1229 and H1299 Ref-1 KO cells; B, relative level of C-caspase 3 in H1229 and H1299 Rsf-1 KO cells; C, Western blot of H460 and H460 Ref-1 KO cells; D, relative level of C-caspase 3 in H460 and H460 Rsf-1 KO cells; E, Western blot of A549 and A549 Ref-1 KO cells; F, relative level of C-caspase 3 in A549 and A549 Rsf-1 KO cells; G, Western blot of SPC and SPC Ref-1 KO cells; F, relative level of C-caspase 3 in SPC and SPC Rsf-1 KO cells (*: compared to the control, P<0.05; #: comparison between cells treated with paclitaxel alone with the Resf-1 knockdown cells treated with paclitaxel, p<0.05; †: comparison between Rsf-1 knockdown cells with the Resf-1 knockdown cells treated with paclitaxel, p<0.05).

Effect of paclitaxel on tumor size, tumor weight and body weight in the mice with H460 cell or H460 Rsf-1 KO cell xenograft

From the fifteenth to the thirtieth day after the tumor cells were implanted into the nude mice, the tumors grew significantly ($p < 0.05$) faster in the mice with H460 cell xenograft

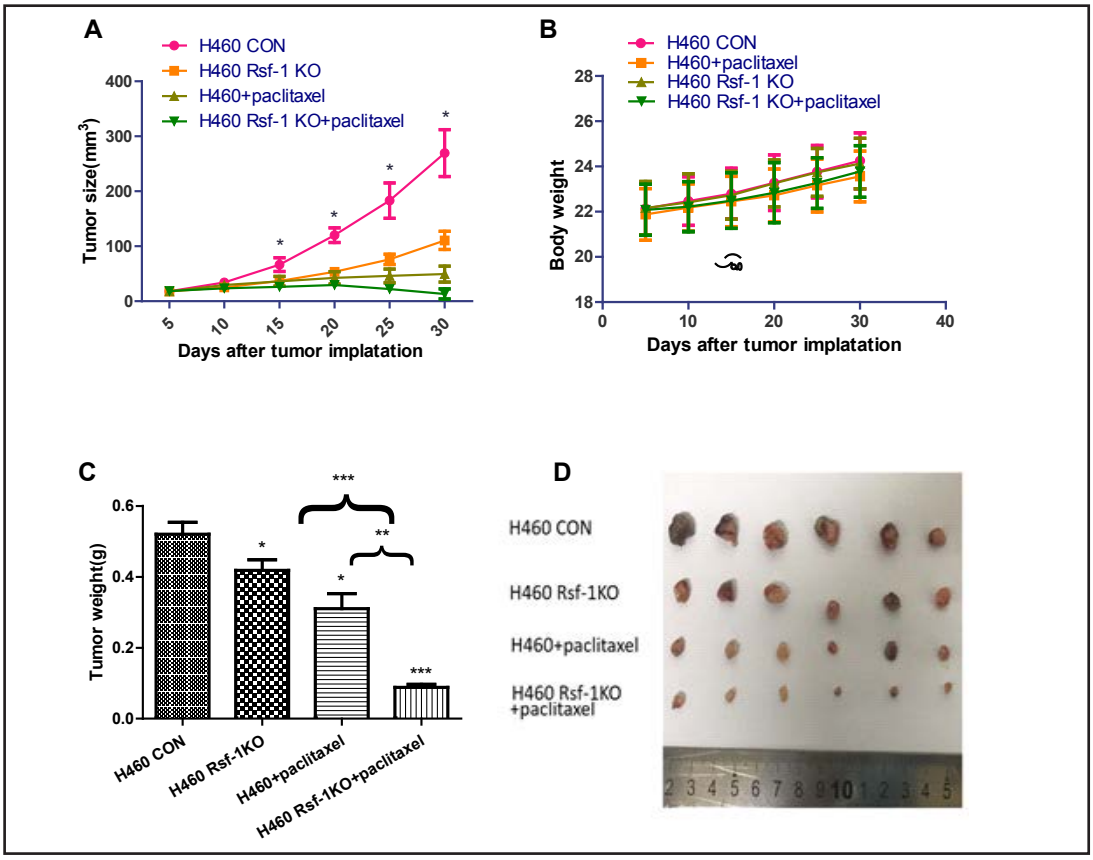

Fig. 8. Effect of paclitaxel on tumor size, tumor weight and body weight in the mice with H460 cell or H460 Rsf-1 KO cell xenograft. Panel A: growth curve of xenograft tumors; panel B: time course of the body weight of the animals; panel C: tumor weight; panel D: xenograft tumors. *: significant differences as compared to H460 control cells ($p<0.05$); **: significant difference between H460 cells treated with paclitaxel and H460 Rsf-1KO cells treated with paclitaxel ($p<0.01$); ***: significant difference between H460 Rsf-1KO cells and H460 Rsf-1KO cells treated with paclitaxel ($p<0.001$).

than in H460 cell xenograft mice treated with paclitaxel, H460 Rsf-1 KO cell xenograft mice, and H460 Rsf-1 KO cell xenograft mice treated with paclitaxel (Fig. 8A). On the thirtieth day after implanting tumor cells, the tumor volumes were significantly ($p < 0.05$) larger in the mice implanted with H460 cells (269.4 ± 42.6 mm^3) than in H460 cell xenograft mice treated with paclitaxel (49.4 ± 14.5 mm^3), H460 Rsf-1 KO cell xenograft mice (110.6 ± 16.6 mm^3), and H460 Rsf-1 KO cell xenograft mice treated with paclitaxel (13.0 ± 9.2 mm^3) (Fig. 8A). During the test period, the body weights increased at a similar rate across all animal groups. The average body weight was 24.2 ± 1.2g for the mice implanted with H460 cells, 24.1 ± 1.1g for H460 Rsf-1 KO cell xenograft mice, 23.6 ± 1.1g for H460 cell xenograft mice treated with paclitaxel, and 23.8 ± 1.1g for H460 Rsf-1 KO cell xenograft mice treated with paclitaxel on the thirtieth day after tumor cell implantation ($p > 0.05$) (Fig. 8B). Thirty days after tumor cell implantation, the tumor weight was significantly lower in H460 cell xenograft mice treated with paclitaxel (0.31 ± 0.10g, $p < 0.05$), H460 Rsf-1 KO cell xenograft mice (0.42 ± 0.07g, $p < 0.05$), and H460 Rsf-1 KO cell xenograft mice treated with paclitaxel (0.09 ± 0.02g, $p < 0.001$) than the mice implanted with H460 cells (0.52 ± 0.08g), where H460 Rsf-1 KO cell xenograft mice treated with paclitaxel demonstrated the greatest decrease (Fig. 8C, D). The decrease in the tumor weight in H460 Rsf-1 KO cell xenograft mice treated with paclitaxel was also significant ($p < 0.01$) compared to H460 cell xenograft mice treated with paclitaxel and H460 Rsf-1 KO cell xenograft mice (Fig. 8C).

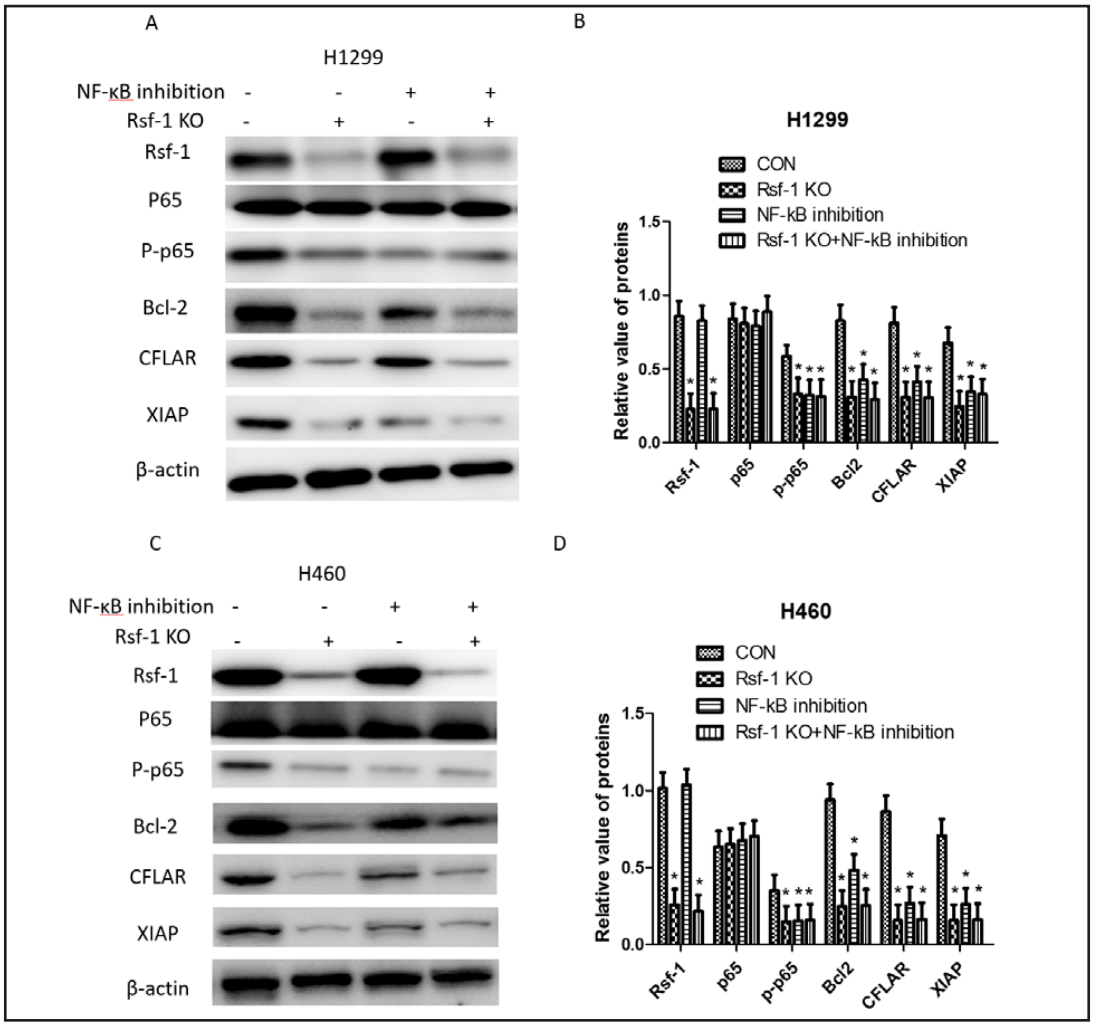

Fig. 9. Protein levels of the NF-κB pathway genes in H1299 and H460 cells. Both the control cells and Rsf-1 knockout cells were treated with NF-κB inhibitor. Protein levels of the NF-κB pathway genes were detected with Western blotting using β-actin as an internal standard. Panel A: proteins detected in H1299 cells with Western blotting; panel B: relative levels of the proteins detected in H1299 cells; panel C: proteins detected in H460 cells with Western blotting; panel D: relative levels of proteins detected in H460 cells (*: significant difference as compared to the control cells, $p<0.05$).

Protein expression of the genes involved in NF-κB pathway

Rsf-1 knockout significantly decreased the levels of Rsf-1 protein in both H1299 and H460 cells (Fig. 9A to D, Fig. 10A to D). The protein expression levels of p-P65, BcL2, CFLAR, and XIAP were significantly ($p<0.05$) decreased after Rsf-1 knockout, inhibition by NF-κB pathway inhibitor, and Rsf-1 knockout plus NF-κB inhibition in H1299 and H460 cells (Fig. 9A to D). NF-κB inhibitor did not change the protein level of Rsf-1 in H1299 cells or H460 cells (Fig. 9A to D). The level of p65 was not altered by Rsf-1 knockout, NF-κB inhibition, hSNF2H knockout, Rsf-1/hSNF2H double knockout, and Rsf-1 knockout plus NF-κB inhibition in H1299 cells or H460 cells ($p > 0.05$) (Fig. 9A to D, Fig.10A to D). There were no differences in the protein levels of Rsf-1 after hSNF2H was knocked down in H1299 cells (Fig. 10A, B) or H460 cells (Fig. 10C, D). Rsf-1 knockout alone and hSNF2H knockout alone significantly decreased the level of p-p65 in H1299 cells (Fig. 10A, B) or H460 cells (Fig. 10C, D) ($p<0.05$). Knocking out Rsf-1 from hSNF2H knockout cells further decreased the level of p-p65 down to the same as in Rsf-1 knockout H1299 cells (Fig. 10A, B) and Rsf-1 knockout H460 cells (Fig. 10C, D) ($p<0.05$).

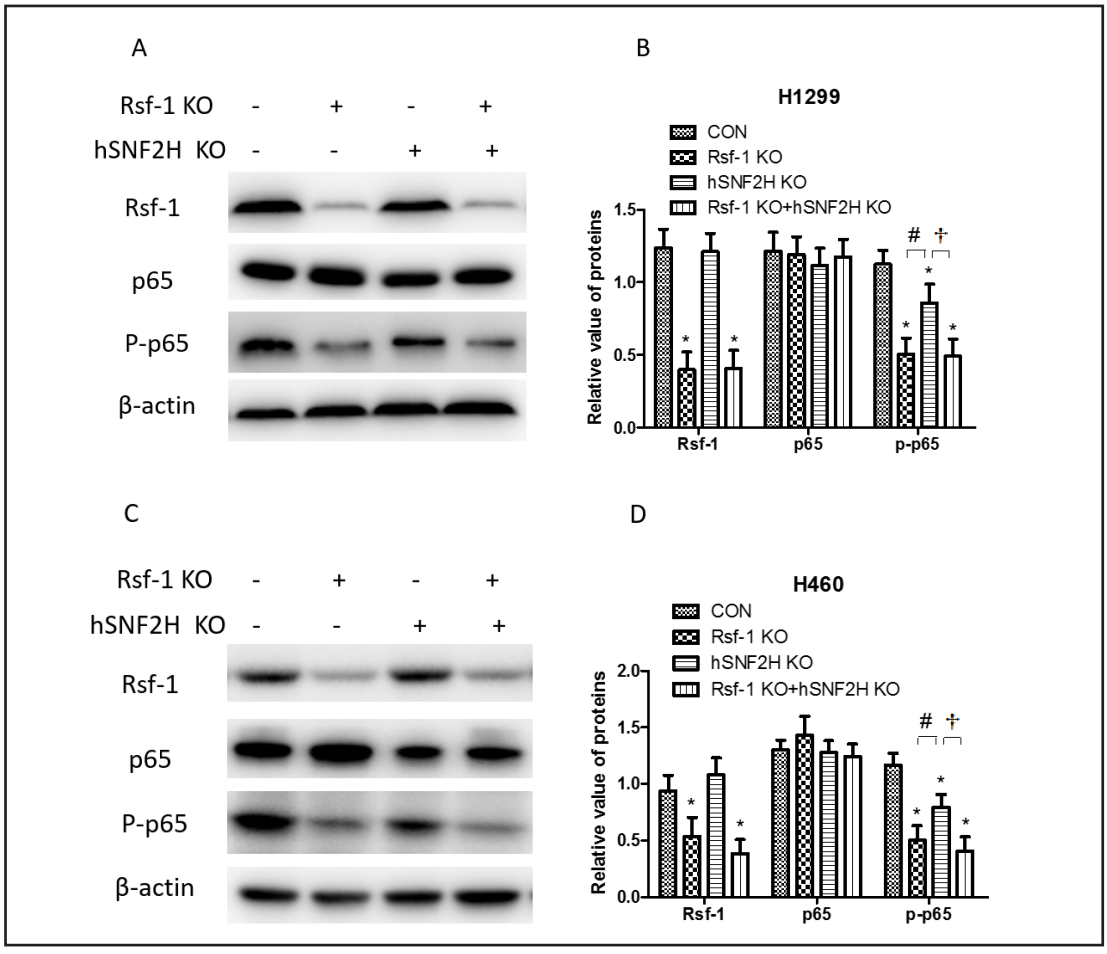

Fig. 10. Protein levels of p65 and p-p65 in H1299 cells and H460 cells. With Rsf-1 and hSNF2H single knockout and double knockout from the cells, the protein levels of p65 and p-p65 were tested with Western blotting using β-actin as an internal standard. A, Western blot of H1299 cells; B, relative levels of P65 and p-p65 in H1299 cells; C, Western blot of H460 cells; D, relative levels of P65 and p-p65 in H460 cells (*: significantly lower as compared to control, $p<0.05$; #: significantly higher in hSNF2H KO cells than Rsf-1KO cells, $p<0.05$; †: significantly higher in hSNF2H KO cells than Rsf-1 and hSNF2H double knockout cells, $p<0.05$).

Discussion

In this study, it was found that Rsf-1 knockout and paclitaxel treatment increased apoptosis and decreased cell proliferation in cultured NSCLC cells, and inhibited the growth of tumors in the nude mice. Rsf-1 knockout enhanced the inhibition effect of paclitaxel on NSCLC *in vivo* and *in vitro* to even higher levels. Both helenalin inhibition of NF-κB pathway and the strategies of Rsf-1 and hSNF2H knockout demonstrated that Rsf-1 influenced the sensitivity of NSCLC to paclitaxel via regulation of the NF-κB pathway.

Rsf-1 influences the sensitivity of non-small cell lung cancer to paclitaxel

RSF1, a histone-binding protein, plays an important role in chromatin remodeling and transcriptional regulation [9]. RSF1 gene is overexpressed and increases proliferation in various cancers, including ovarian [7], breast [10], bladder [11], esophageal [12], colon [14], and head and neck cancers [15], and correlates with poor prognosis [16]. In this study, we

also observed that Rsf-1 knockout mediated arrest of cells at G1 phase, elevated caspase3 and C-caspase3 protein levels and cell apoptosis, reduced cell migration ability and cell proliferation in H460 and H1299 cells, and decreased the volume, weight, and the growth of tumors in H460 cell xenograft mice. These results confirm that Rsf-1 contributes to malignant cell growth, invasion and poor overall survival in NSCLC [20, 23]. Rsf-1 knockout increased the inhibition effect of cell proliferation, the reduction of cell migration ability and the augmentation of caspase3 and C-caspase3 protein levels by paclitaxel in H460 and H1299 cells. Rsf-1 knockout further decreased the volume, weight, and the growth of tumors by paclitaxel without influencing the body weight in H460 cell xenograft mice. These observations indicate that Rsf-1 knockout increases the sensitivity of NSCLC to paclitaxel, which is in agreement with the enhanced paclitaxel resistance by Rsf-1 in ovarian cancer [17, 18] and nasopharyngeal carcinoma [19]. It is proposed that an inhibitor of Rsf-1 would reverse Rsf-1 enhanced paclitaxel resistance, and if used in combination with paclitaxel, might be able to potentiate the chemotherapeutic efficacy of paclitaxel to treat NSCLC, just as observed in the YAP overexpression colon cancer HCT-8 cells treated by verteporfin [24].

NF-kB pathway is activated by Rsf-1

It is well defined that Rsf-1 contributes to chemoresistance in many cancers through activation of the NF-κB pathway [18, 19, 23, 25]. Rsf-1 knockout decreased the protein levels of p-P65, BcL2, CFLAR, and XIAP in H1299 and H460 cells, confirming that Rsf-1 regulates the activation of NF-κB and the expression of the genes downstream of NF-κB complex [23, 25]. Inhibition of the NF-κB pathway by helenalin decreased the protein levels of p-P65, BcL2, CFLAR, and XIAP in these cells without altering Rsf-1 protein levels, suggesting that Rsf-1 acts on the NF-κB pathway at a point no further downstream from the site of helenalin inhibition. In the classical (or canonical) pathway, NF-κB, composed of a p50 and a p65 subunit, is retained in an inactive cytoplasmic complex by binding to a third, inhibitory subunit IκB. Activation of IKK triggers the phosphorylation and proteolytic degradation of the IκB subunit, which then releases active NF-κB (p-P65) from the cytoplasmic complex. The activated factor then translocates to the nucleus, where it stimulates the transcription of its target genes. Helenalin, the sesquiterpene lactone parthenolide, was reported to inhibit NF-κB activation by preventing the degradation of inhibitive IκB subunits (IκB-α and IκB-β) [26], or selectively alkylating the p65 subunit of the transcripition factor, thereby inhibiting its DNA binding [27]. The unaltered level of p65 and the reduced level of p-P65 by helenalin inhibition indicated that helenalin inhibited NF-κB activation through reducing the phosphorylation of p65 in H1299 and H460 cells.

hSNF2H and Rsf-1 were co-upregulated in several human cancers, such as ovarian cancer [8] and glioma [25]. hSNF2H translocates into nuclei and interacts with Rsf-1 to form a chromatin remodeling complex (Rsf complex) that regulates gene expression and participates in several biological processes [8]. Formation of the Rsf-1/hSNF2H complex, as coactivators [18], is required for Rsf-1 overexpression to enhance paclitaxel resistance in ovarian cancer [17] and glioma [25] through regulating NF-κB [18, 25]. In the present study, hSNF2H knockout decreased the protein levels of NF-κB p-P65 without altering the protein levels of Rsf-1 or p65, and Rsf-1 and hSNF2H double knockout led to an even more decrease in the levels of NF-kB p-P65 in H1299 Rsf-1 KO cells and H460 Rsf-1 KO cells. These results support that Rsf-1 interacts with hSNF2H to activate the NF-κB pathway in NSCLCs.

The NF-κB pathway is found to cross talk with other signal transduction pathways through transcription factors, such as STAT3, p53 and AKT [28]. It is expected that chemicals, such as CB-PIC [29] and SI113 [30], which act on STAT3 [29], AKT [29], and p53 [30], may further influence the activation of NF-κB and its downstream genes, and compensate for the decreased paclitaxel sensitivity by Rsf-1 in NSCLCs. The cross talk between YAP and NF-κB [31] suggests that verteporfin should have the potential to reverse paclitaxel enhanced resistance in NSCLCS [24].

Conclusion

It was concluded that Rsf-1 was highly expressed in H1299 and H460 cells. Paclitaxel inhibited the invasion and growth of NSCLC by reducing migration ability, increasing cell apoptosis, and reducing cell proliferation. Rsf-1 knockout mediated arrest of cells at G1 phase, elevated cell apoptosis, reduced cell migration ability and reduced cell proliferation in H460 and H1299 cells, and decreased the volume, weight, and the growth of tumors in H460 cell xenograft mice. Rsf-1 knockout also enhanced the anticancer effects of paclitaxel on NSCLC both *in vitro* and *in vivo*. Rsf-1 influenced the sensitivity of NSCLCs to paclitaxel by interacting with hSNF2H and regulating the activation of NF-κB and the expression of its downstream genes.

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Disclosure Statement

The authors declare that they have no competing interests.

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