

Involvement of ROS in the inhibitory effect of thermotherapy combined with chemotherapy on A549 human lung adenocarcinoma cell growth through the Akt pathway

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Abstract. Mechanisms of synergistic effect of thermotherapy and chemotherapy on human lung adenocarcinoma cell growth are unclear. The purpose of this study was to investigate these effects and explore the function of ROS, Akt and caspase-3 in relation to these. A549 cells were subjected to different thermochemotherapy treatments: 43°C heat + 50 µg/l paclitaxel (thermochemotherapy group), 43°C heat + 50 µg/l paclitaxel + 1 µmol/l wortmannin (wortmannin group), 43°C heat + 50 µg/l paclitaxel + 30 µmol/l NAC (NAC group) and 50 µg/l paclitaxel (chemotherapy group). The cells without any treatment were regarded as controls. Cell proliferation rates were measured with MTT assay and intracellular ROS levels were detected with fluorescence labeling technologies. Phosphorylation of Akt and caspase-3 expression were determined by western blotting and the cell apoptosis rates were examined by flow cytometry. Tests *in vivo* were carried out at the same time. It was found that the cell proliferation rates of the thermochemotherapy group were significantly lower compared to those of the controls or the chemotherapy group ($P < 0.05$). The intracellular ROS levels of the thermochemotherapy group were elevated significantly compared with those of other groups, and these changes could be reversed using the ROS inhibitor NAC but not a PI3K inhibitor (wortmannin). Phosphorylation of Akt was significantly decreased in the thermochemotherapy group ($P < 0.05$), which could be blocked by wortmannin, but increased by NAC ($P < 0.05$). The caspase-3 expression levels and cell apoptosis rates of the thermochemotherapy group were higher compared to those of the other groups ($P < 0.05$). All results have been confirmed by *in vivo* tests. Thus, the combination of thermotherapy with chemotherapy showed a stronger inhibitory effect on A549

cell growth compared to chemotherapy alone, which may be able to cause additional cell apoptosis through inhibition of Akt phosphorylation and activation of caspases by increased intracellular ROS production.

Introduction

Lung cancer is one of the most common malignant tumors worldwide and non-small cell lung cancer accounts for 80% of all lung cancer cases. Thermotherapy is an approach taking advantage of biological effects of heat to cure tumors. Thermotherapy undergoes a systematic research with the medicine development and equipment advancement (1). Now, thermotherapy is regarded as an effective treatment such as surgery, radiotherapy, chemotherapy and biological treatment (2). In recent years, tumor thermotherapy has been widely used to treat cancer (3), including lung cancer (4). Tumor thermotherapy increased tumor cell sensitivity to drugs in the effective temperature range, reversed tumor cell drug-resistance and induce apoptosis (5).

Intracellular ROS could be induced and affected by many factors such as heat, cell cytokines or some chemicals. Its function is to signal to activate cellular apoptosis inducing cell apoptosis pathway (6-9). However, UV radiation was found to be able to lead to increased production of ROS and activation of mitogen activated protein kinase (MAPK) pathway (10). As a member of MAPK family, protein kinase B (PKB)/Akt plays key roles on regulation of cell growth and apoptosis. Phosphorylated Akt (p-Akt) takes part in many physiological and pathological processes directly or indirectly. Activation of Akt pathway showed an obvious promotion on cell growth and was necessary to normal cell growth (11,12). In addition, activated Akt (13), but inactivated cysteinyl aspartate-specific proteinases (caspase) simultaneously were observed in most tumor cells, which might be responsible for tumor occurrence and development (14). Caspase is the core regulatory element in the formation of cell apoptosis regulating network and could be activated by self-catalysis or caspase cascade catalysis. Oxidative stress was able to induce elevated caspase-3 activation and increased cell apoptosis (15) through degrading more target proteins (16).

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We observed that thermotherapy in combination with chemotherapy showed stronger inhibitory effect on lung tumor cell growth than thermotherapy or chemotherapy alone (17), the molecular mechanisms in the process remained unclear.

Materials and methods

Cell culture and cell treatments. Human lung adenocarcinoma A549 cell line was purchased from Shanghai Cell Biology Institute Cell Bank, (China) and was cultured in F-12k cell supernatant (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma) at 37°C in a 5% CO₂ saturated humidity atmosphere. Logarithmic phase cells were digested with 5% trypsin solution and the digestion was inactivated in cell supernatant, and then was separated into single cells. The cell density was diluted into 2.5x10⁷ cells/l and 6 ml of the cell suspension was added into each well of culture plates. Then cells were cultured at 37°C in a 5% CO₂ humidified incubator (Heraeus, Germany). After culturing for 24 h, the cells were subjected to different group schemes as follows: cells treated only by 50 µg/l paclitaxel (Taiji Group Sichuan Taiji Pharmacy Co., Ltd., China; the approval number: GY H19994040) were the chemotherapy group while cells treated with 50 µg/l paclitaxel and 43°C heat were the thermo-chemotherapy group. Cells in wortmannin (Alexis, USA) group were treated by thermo-chemotherapy combined with 1 µmol/l wortmannin and cells in NAC (Sigma) group were treated by thermo-chemotherapy combined with 30 µmol/l NAC. The cells without any treatment were regarded as controls. Hyperthermic treatments were performed by immersing either the culture flasks or parafilm-wrapped 96-well plates in a waterbath at 43°C for 90 min.

MTT assay. Single cell suspension was prepared with the cells in logarithmic phase and cell density was adjusted to 2.5x10⁷ cells/l. Then this cell suspension was seeded into 96-well plates, 200 µl per well, and cultured at 37°C in a 5% CO₂ humidified incubator. After 24 h, cells were treated according to different schemes described above (6-wells were assayed for each group and repeated three times). The treated cells were incubated for 4 h and 20 µl of the solution containing 5 g/l MTT (Sigma) was added to each well and incubation for another 4 h was performed. MTT was discarded and 150 µl dimethylsulfoxide (DMSO) (Takara, China) was added to each well, and then vortexed for 10 min to dissolve the crystal. DMSO was used as negative control. Absorbance (A) value was measured at 492 nm with the microplate reader (Sunrise China). Cell proliferation rates were calculated according to the following formula: cell proliferation rate (%) = (average A value of experimental group/average A value of control group) x 100%.

Intracellular ROS detection. After the treatment was performed, about 1x10⁶ cells in the logarithmic growth phase were collected, washed by PBS and centrifuged at 1200 r/min for 5 min. Then reagent was added and cultured at 37°C for 20 min. Intracellular ROS was detected using Reactive Oxygen Species Testing kit (Shanghai Jiemei Gene Medicine Technology Co., Ltd., China). Preservative medium was

added and detected at 490 nm (excitation) and 530 nm (emission) qualitatively by fluorescence microscope (Olympus, Japan) and quantitatively by F-4500 fluorospectrophotometer (Hitachi, Japan).

Western blotting. Cells in logarithmic phase (1x10⁶) (*in vitro*) or 50 mg tissue (*in vivo*) from each group were harvested and suspended in cell lysis buffer containing 30 mmol/l NaHCO₃, 0.1 mmol/l EDTA, 0.1 mmol/l DTT, 0.5 mmol/l PMSF and 2 mg/l aprotinin. Cells were lysed on ice and centrifuged at 10,000 r/min and 4°C for 10 min. The supernatant and loading buffer was mixed. Equal amount of total protein dissolved in the mixture of each sample was loaded and separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto nitrocellulose membrane (Beijing Applygen Technology Ltd., Beijing, China) for antibody blotting. The membrane was incubated with a rabbit monoclonal antibody against Akt (Cell Signaling Technology, Inc., Danvers, MA), a rabbit monoclonal antibody against p-Akt (Ser473) (587F11) (Cell Signaling Technology, Inc.), a rabbit monoclonal antibody against caspase-3 (Cell Signaling Technology, Inc.) or a rabbit monoclonal antibody against β-actin (Cell Signaling Technology, Inc.) overnight at 4°C, respectively. After being incubated with goat anti-rabbit IgG antibodies (1:300 dilution) (Beijing Zhongshan Jinqiao Biology Technology Co., Ltd., Beijing, China) for 2 h. The membrane was subjected to immunoblot analysis and the enhanced chemiluminescence method (ECL kit, China) and HRP-DAB kit (Tiangen, Biotech Co., Ltd., Beijing, China) were used to visualize the proteins. β-actin was used as an internal control. Gel imaging system (Gene Genius, USA) was used to capture images. Semi-quantitative values of p-Akt and caspase-3 were measured by the ratio of the bands of t-Akt and β-actin.

Cell apoptosis detection. After cell concentration was adjusted, cells were seeded into 50-ml dishes to culture, and then they were treated according to schemes mentioned in cell culture and cell treatments. Cells in each group were suspended as single cells. A total of 1x10⁶ cells were collected to tubes, and fixed in ice-cold 70% ethanol for 12 h at 4°C. The ethanol was discarded and cells were digested by 1 ml 0.5% trypsin (pH 1.5-2.0) for 10 min and washed with PBS, labeled with propidium iodide (PI) and filtered by 500-mesh screen to obtain single cell suspension and analyzed directly by FACS 420 flow cytometry (B&D Systems, USA).

Graft inhibition rates in nude mice. Thirty BALB/c-nu/nu nude mice (purchased from Shanghai Slack Experimental Animals Co., Ltd., China) were treated according to the following schemes: chemotherapy group (50 µg/kg paclitaxel intraperitoneal injection), thermo-chemotherapy group (50 µg/kg paclitaxel intraperitoneal injection with 43°C waterbath), wortmannin group (thermo-chemotherapy combined with wortmannin intraperitoneal injection), NAC group (thermo-chemotherapy combined with NAC intraperitoneal injection) and controls (equal saline intraperitoneal injection without any other treatments). There were 6 nude mice in each group and every nude mouse was treated once a week for 3 weeks. Nude mice were sacrificed 2 days after the last treatment.

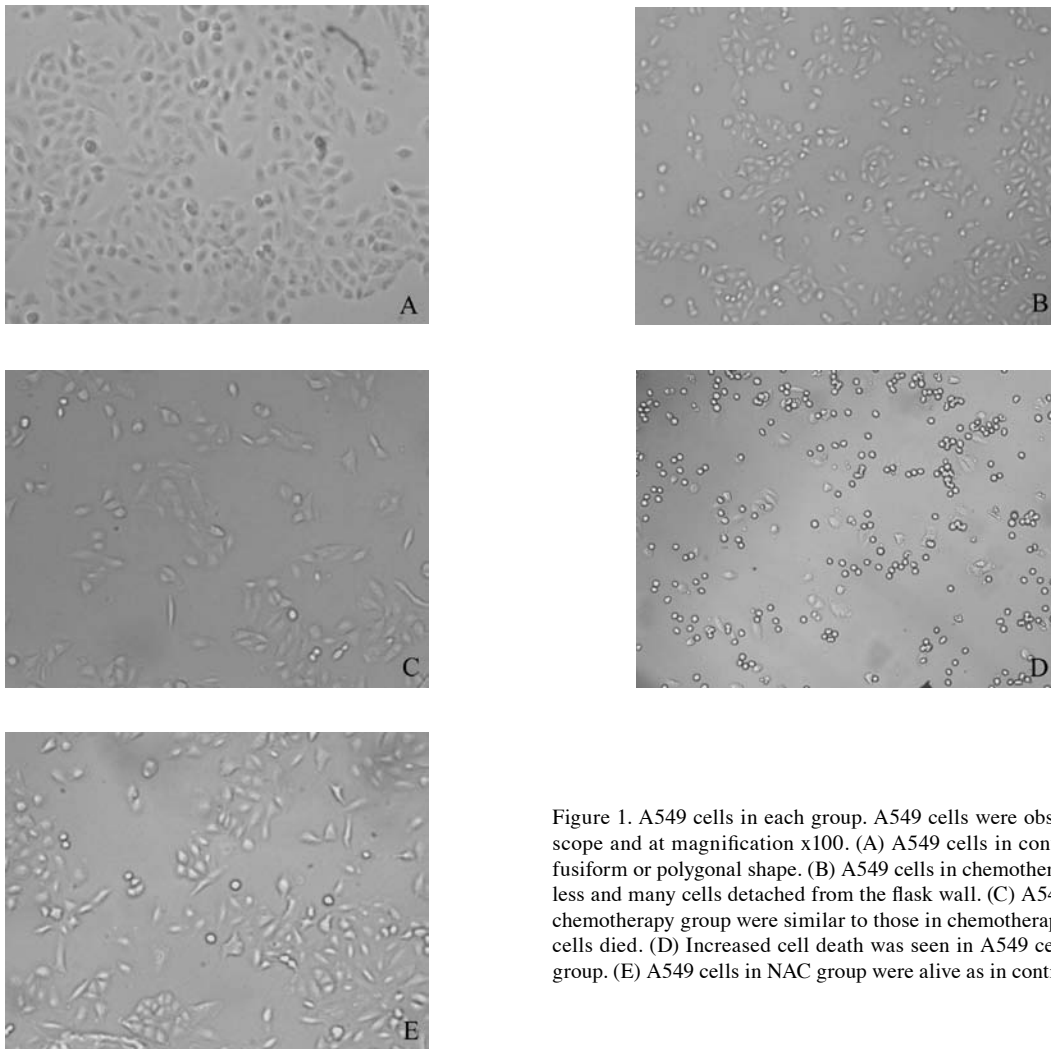


Figure 1. A549 cells in each group. A549 cells were observed by a microscope and at magnification x100. (A) A549 cells in control group were in fusiform or polygonal shape. (B) A549 cells in chemotherapy group became less and many cells detached from the flask wall. (C) A549 cells in thermo-chemotherapy group were similar to those in chemotherapy group, but more cells died. (D) Increased cell death was seen in A549 cells in wortmannin group. (E) A549 cells in NAC group were alive as in control.

Changes in behavior, eating, defecation and weight were tracked. When grafts were removed, graft inhibition rates were calculated according to the following formula: graft inhibition rates (%) = (graft quality in control group - graft quality in experimental group)/graft quality in control group x 100%. The institutional committee for animal research approved these experiments.

Statistical analysis. The software of SPSS 13.0 was employed for data analysis and results are shown as mean \pm SD. Comparisons among groups were analyzed with a one-way ANOVA and LSD method was applied for multiple comparisons. $P < 0.05$ was regarded as significant.

Results

Cell proliferation rates. A549 cells in control group grew normally in fusiform or polygonal shape. In chemotherapy group, the number of A549 cells became less and many cells were detached from the flask wall. Many cells were in division phase and cell bodies became round and small. In thermo-chemotherapy group, cell morphology was in the shape described as in chemotherapy group, but more cells died. After wortmannin was added, the number of cells

became less but most cells were alive after NAC was added (Fig. 1).

Compared with control group ($100.00 \pm 0.00\%$), significantly decreased proliferation rate was detected in the groups treated with chemotherapy ($78.77 \pm 2.38\%$) and thermo-chemotherapy ($56.34 \pm 4.30\%$) ($P < 0.05$) and proliferation rate in thermo-chemotherapy group was significantly lower than in other groups ($P < 0.05$). Proliferation rate in wortmannin group ($47.40 \pm 0.01\%$) was significantly lower than that in thermo-chemotherapy group ($P < 0.05$) and there was no significant difference between NAC group ($92.65 \pm 0.09\%$) and control group ($P > 0.05$), which indicated that chemotherapy in combination with chemotherapy could inhibit A549 cells growth distinctly and ROS and Akt pathways played a role inhibited by their own inhibitors (Fig. 2).

Changes of intracellular ROS. Cells in control group were red, which showed there was no ROS in them (Fig. 3A); cells in chemotherapy group were green, which indicated ROS in them (Fig. 3B); cells in thermo-chemotherapy group were bright green showing increased ROS (Fig. 3C). Wortmannin had no effect on ROS produced by thermo-chemotherapy (Fig. 3D), but after NAC was added, cells became red (Fig. 3E).

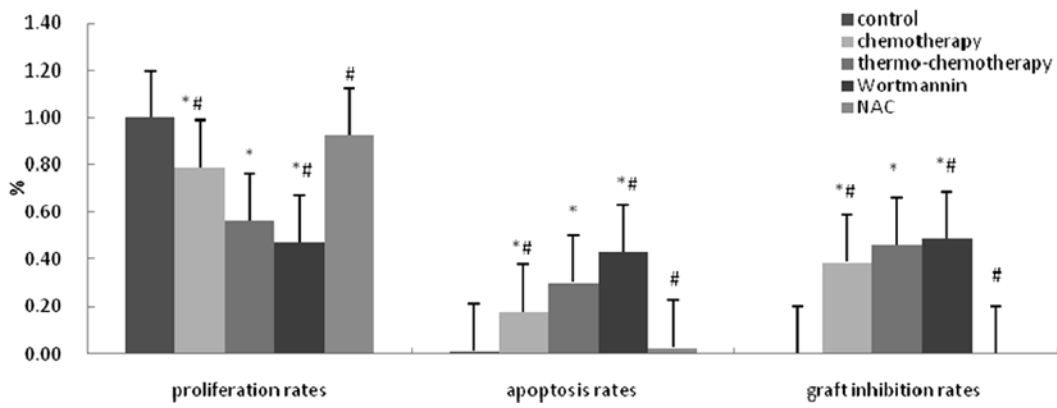


Figure 2. Proliferation rates, apoptosis rates and graft inhibition rates. Proliferation rates were measured by MTT and it decreased significantly in thermo-chemotherapy group compared with control and chemotherapy group. Proliferation rate in wortmannin group was significantly lower than that in thermo-chemotherapy group and there was no significant difference between NAC group and control group. Apoptosis rates were analyzed by FCM and the highest rate was in wortmannin group and that in thermo-chemotherapy group was higher than that in control and chemotherapy group. Graft inhibition rate was higher in thermo-chemotherapy group than in control and chemotherapy group. There was no significance difference between control group and NAC group. *P<0.05 vs. control group, #P<0.05 vs. thermo-chemotherapy group. Thermochemotherapy in combination with chemotherapy could inhibit A549 cell growth by leading to apoptosis and ROS and Akt pathway played a role, which was confirmed *in vivo*. Each column represents mean ± SD value for three repeated independent experiments.

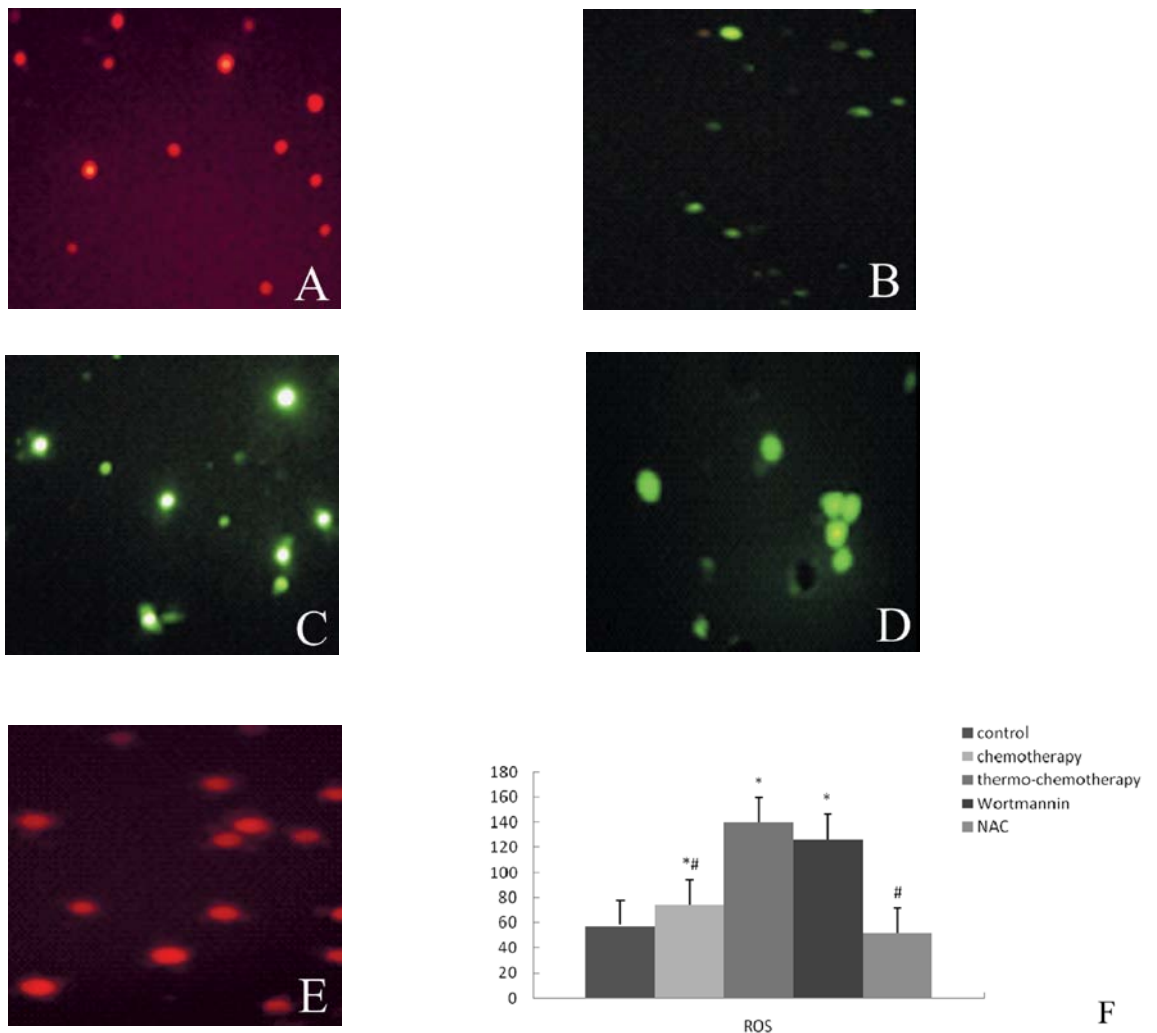


Figure 3. Changes of intracellular ROS. Intracellular ROS changes were detected qualitatively and quantitatively, respectively, by fluorescence microscope and F-4500 fluorospectrophotometer. (A) Cells in control group were red. (B) Cells in chemotherapy group were green. (C) Cells in thermo-chemotherapy group were bright green. (D) Wortmannin had no effect on ROS and (E) NAC inhibits ROS thoroughly to make cells red. (F) ROS production was the highest in thermo-chemotherapy group and NAC decreased its production significantly. Wortmannin has no effect on ROS production. Each column represents mean ± SD value for three repeated independent experiments. *P<0.05 vs. control group; #P<0.05 vs. thermo-chemotherapy group.

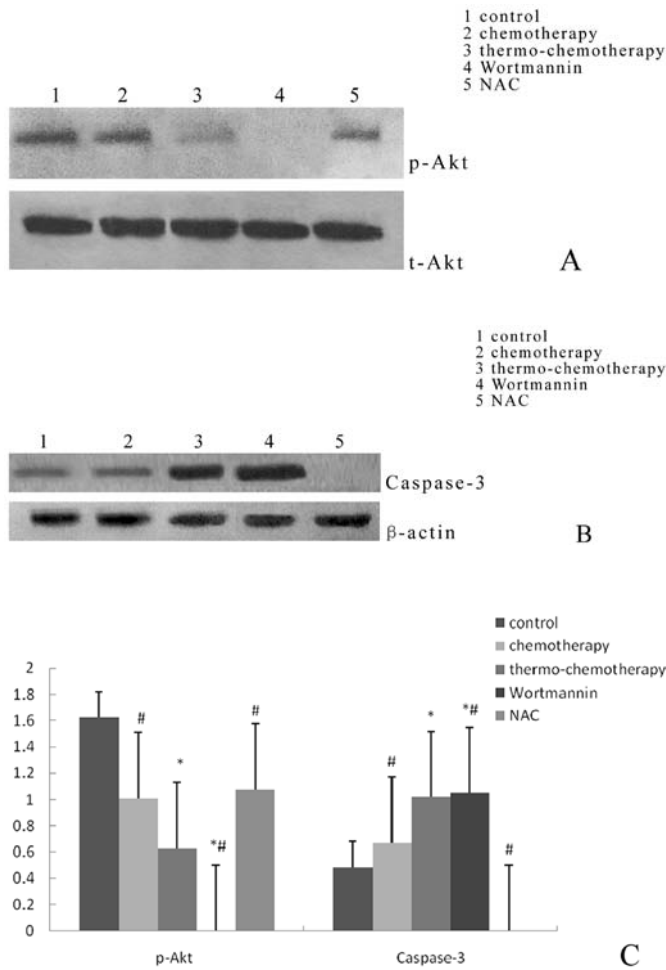


Figure 4. Phosphorylation of Akt and caspase-3 expression in cells. (A) Compared with control group and chemotherapy group, phosphorylation of Akt decreased significantly in thermo-chemotherapy group. NAC increased and wortmannin inhibited it thoroughly. (B) Caspase-3 expression increased in thermo-chemotherapy group compared with control group and chemotherapy group and NAC inhibited its expression completely while wortmannin increased it, which showed that ROS inhibited activation of Akt pathway and activated caspase pathway and Akt pathway was upstream of caspase pathway in thermo-chemotherapy. (C) Western blot analysis of phosphorylation of Akt and caspase-3 expression in cells following different treatment in A549 cells. *P<0.05 vs. control group; #P<0.05 vs. thermo-chemotherapy group. Semi-quantitative analyses were performed from three independent experiments for t-Akt / β -actin.

Compared with thermo-chemotherapy group (139.93 \pm 37.59%), significantly decreased ROS production was detected in control group (58.07 \pm 2%), chemotherapy group (74.40 \pm 3.01%) and NAC group (52.11 \pm 1.05%) (P<0.05) and wortmannin had no effect on ROS (126.48 \pm 8.76%) (P>0.05), indicating that thermo-chemotherapy induced production of ROS and ROS was upstream of the Akt pathway (Fig. 3F).

Effect of thermo-chemotherapy on phosphorylation of Akt and caspase-3 expression. Compared with thermo-chemotherapy group (0.63 \pm 0.00%), Akt phosphorylation in control group (1.62 \pm 0.03%) and chemotherapy group (1.01 \pm 0.41%) decreased significantly (P<0.05) and wortmanin completely inhibited Akt phosphorylation (0.00 \pm 0.00%) (P<0.05); but when NAC, the specific inhibitor of ROS, was added, Akt

phosphorylation (1.08 \pm 0.24%) was significantly higher than that in thermo-chemotherapy group (P<0.05) (Fig. 4A and C).

Caspase-3 expression in thermo-chemotherapy group (1.02 \pm 0.02%) was significantly higher than that in control group (0.48 \pm 0.11%) and chemotherapy group (0.67 \pm 0.02%) (P<0.05); after Akt specific inhibitor wortmannin pretreatment, caspase-3 expression (1.05 \pm 0.09%) was significantly higher than that in thermo-chemotherapy group (P<0.05); NAC could inhibited caspase-3 expression completely (0.00 \pm 0.00%) (P<0.05) (Fig. 4B and C) showing that ROS could inhibit activation of Akt pathway and activate the caspase pathway. The Akt pathway was upstream of caspase pathway in thermo-chemotherapy.

Cell apoptosis. Cell apoptosis rate in control group was (1.22 \pm 0.18%) and significantly increased cell apoptosis rates were observed in chemotherapy group (17.70 \pm 3.23%) and thermo-chemotherapy group (30.39 \pm 2.89%) (P<0.05). Cell apoptosis rate in thermo-chemotherapy group was significantly higher than that in chemotherapy group (P<0.05). Compared with thermo-chemotherapy group, cell apoptosis rate in wortmannin group (43.23 \pm 9.26%) increased significantly and decreased significantly in NAC group (2.80 \pm 0.99%) (P<0.05) (Fig. 2). Thermo-chemotherapy in combination with chemotherapy inhibited A549 cell growth by leading to apoptosis through induction of ROS production and subsequent inhibition of Akt phosphorylation and activation of caspase.

Results of tests in vivo. A549 cells were injected subcutaneously into nude mice and grafts occurred about 1 week later, with round or oval shape. Compared with thermo-chemotherapy group (46.34 \pm 1.03%), graft inhibition rate in wortmannin group (48.97 \pm 0.11%) increased significantly, and decreased significantly in the control group (0.00 \pm 0.00%), chemotherapy group (38.76 \pm 1.83%) and NAC group (0.05 \pm 0.01%) (P<0.05), but there was no significant difference between control group and NAC groups (P>0.05) (Fig. 2).

Akt phosphorylation in thermo-chemotherapy group (0.66 \pm 0.06%) decreased significantly when compared with control group (1.66 \pm 0.06%) (P<0.05) and increased significantly in chemotherapy group (1.06 \pm 0.04%) and NAC group (1.07 \pm 0.02%) when compared with thermo-chemotherapy group (P<0.05), but was inhibited completely in wortmannin group (0.00 \pm 0.00%) (P<0.05) (Fig. 5A and C).

Caspase-3 expression in thermo-chemotherapy group (1.01 \pm 0.01%) was significantly higher than that in control group (0.49 \pm 0.10%) and in chemotherapy group (0.66 \pm 0.03%) (P<0.05); Caspase-3 expression in wortmannin group (1.06 \pm 0.07%) increased significantly (P<0.05) and caspase-3 expression in NAC group (0.00 \pm 0.00%) was inhibited completely (P<0.05) (Fig. 5B and C). All these results in cells were authenticated *in vivo*.

Discussion

Thermo-chemotherapy is a new treatment of tumors, which plays an important role in comprehensive treatment of tumors. Thermo-chemotherapy in combination with chemotherapy is able to improve tumor treatment efficiency (18). Initially thermo-

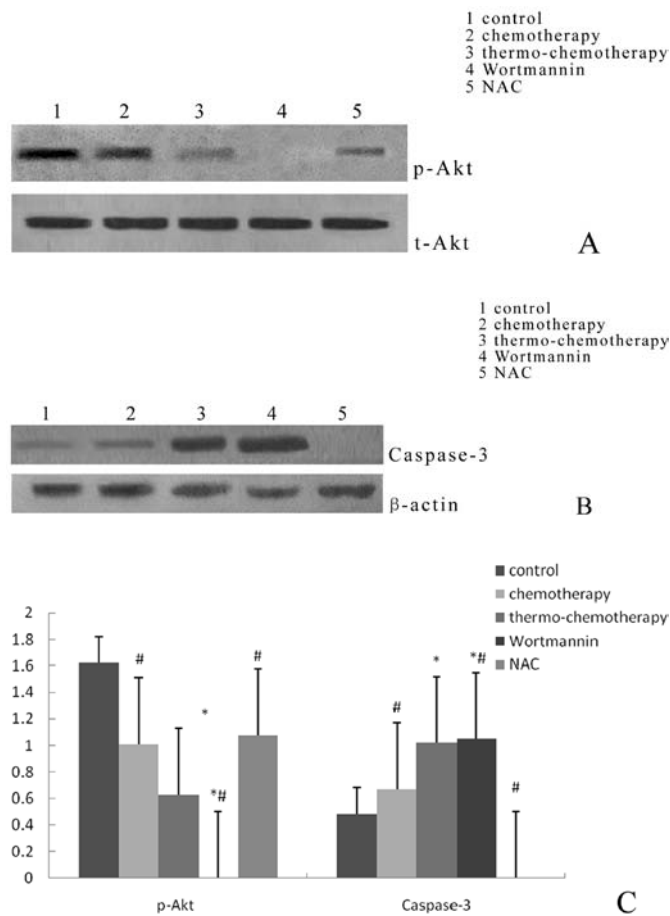


Figure 5. Phosphorylation of Akt and caspase-3 expression *in vivo*. This figure was confirmed *in vivo*. (A) Phosphorylation of Akt in thermo-chemotherapy group decreased significantly compared with control group and chemotherapy group. Wortmannin inhibited it thoroughly. (B) Compared with control group and chemotherapy group, caspase-3 expression increased in thermo-chemotherapy group. NAC could inhibit it completely and it increased in wortmannin group. (C) Western blotting analysis phosphorylation of Akt and caspase-3 expression in cells following different treatment *in vivo*. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. thermo-chemotherapy group. Semi-quantitative analyses were performed from three independent experiments for t-Akt/ β -actin.

therapy was regarded as 'green treatment' by the clinicians (19). In 1990s, some investigators paid attention to the phenomenon of cell apoptosis induced by heat. This study shows that thermotherapy in combination with chemotherapy could increase injury of drugs on tumor cells by increasing ROS production, which leads to Akt activation, and apoptosis of A549 cells through caspase cascade, which was consistent with observation *in vivo*.

Normally, production and clearance of ROS is in a dynamic balance (20). Cell growth needs some ROS and ROS causes tissue cell apoptosis or necrosis (21). When ROS balance was broken, more ROS would be produced and oxidation stress occurred, which would induce internal Ca^{2+} flow, unregulating the expression of bax and activating caspase, then leading to cell apoptosis (22,23). ROS was induced in the process of tumor thermotherapy (24). The results of this study show that thermotherapy in combination with drugs would change induction and effect of ROS on tumor cells, which provides a new approach to treat tumors. This study finds that thermotherapy could increase production of intracellular ROS, which

is an important factor of apoptosis. It is also found that this induction of ROS caused by thermotherapy could be inhibited by the ROS inhibitor NAC, but not by the PI3K inhibitor, wortmannin (Fig. 3). Therefore, ROS is likely to be upstream of inhibition on Akt pathway activation.

PI3K (phosphoinositide 3 kinase) is a phosphatidyl kinase and induced tumor cell hyperplasia by activating apoptosis-related Akt pathway (25). Its anti-apoptosis function may have related to caspase activation. Phosphorylation of Akt could resist apoptosis in chemotherapy and radiotherapy (26). Previous studies (27) have showed that phosphorylation of Akt was increased from normal cells to atypia then to malignant transformation with molecular markers related to loss of apoptosis indicating that Akt extended survival of tumor cells and inhibited apoptosis. Noske *et al* (28) found that there was overexpression of Akt in 58% primary ovarian cancer and RNA interference inhibited phosphorylation of Akt to inhibit ovarian cancer cell proliferation. In this study, p-Akt is highly-expressed in A549 cells and heat can decrease levels of Akt phosphorylation. Also, p-Akt could be inhibited by NAC and wortmannin (Figs. 4 and 5). Results of MTT and FCM show that cell proliferation rates decreased and apoptosis rates increased in thermo-chemotherapy group. Therefore, inhibition of thermotherapy on lung tumor cell growth was associated with Akt pathway activation.

In practice, wortmannin could inhibit phosphorylation of Akt both *in vivo* and *in vitro* (29). In our study, levels of p-Akt in thermo-chemotherapy group were significantly lower than those in control group and chemotherapy group. Wortmannin completely inhibited phosphorylation of Akt (Figs. 4 and 5). Combined with results of MTT and FCM, cell apoptosis rates in wortmannin group are higher than those in thermo-chemotherapy group, which shows that thermotherapy inhibited Akt pathway activation and NAC activated the Akt pathway. Wortmannin can increase antitumor effects of thermotherapy at the same time.

Caspases are a group of cysteine proteases, which have similar amino acid sequences, and play an important role in cell apoptosis. Caspase family enzymes regulate cell apoptosis. Many apoptosis factors induce apoptosis by caspase-3 mediated signal pathway (30). In this study, heat could induce caspase-3 activation in A549 cells and NAC could inhibit caspase-3 expression in heated A549 cells (Figs. 4 and 5), which suggests that production of ROS was upstream of caspase-3 activation in A549 cell apoptosis induced by heat. When heat inhibited phosphorylation of Akt, the expression of caspase-3 increased, which shows that inhibition of Akt pathway activation was upstream of caspase-3 activation.

In summary, thermotherapy could block the cell cycle and improved induction of cell apoptosis (31). Cell apoptosis is one characteristic of cell life and an active death form conducted by genes. Inhibition of PI3K/Akt pathway activation could increase tumor cell apoptosis induced by drugs (32). Cell apoptosis rate in wortmannin group increased. But level of p-Akt increased while cell apoptosis rate decreases after NAC addition, which suggests that ROS inhibited activation of Akt pathway and induced cell apoptosis. Inhibition of caspase-3 activity or antagonism of its function may inhibit cell apoptosis, indicating that caspase-3 is essential to cell apoptosis (33). Thus, thermotherapy in combination

with chemotherapy showed a stronger inhibitory effect than chemotherapy alone on A549 cell growth, probably through induction of ROS production and subsequent inhibition of Akt phosphorylation to activate caspase cascade, which would lead to lung cancer cell apoptosis. All results were confirmed *in vivo*. These results provide a theoretical basis for thermo-therapy clinical practice.

Acknowledgements

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