·Basic Research·

Involvement of Akt in synergistic effects of thermo-chemotherapy on human small cell lung cancer H446 cell apoptosis

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[Abstract] Background and Objective: Akt pathway plays an important role in cell growth and apoptosis. This study was to characterize the role of Akt in the synergistic effects of thermo-chemotherapy on lung cancer cell growth and its underlying mechanisms. **Methods:** H446 cells were subjected to different thermo-chemotherapy schemes: 43℃ + paclitaxel (120 μg/L) (thermo-chemotherapy group), 43℃ + paclitaxel (120 μg/L) + Wortmannin (1 μmol/L, PI3K/Akt pathway inhibitor) (Wortmannin group), 43℃ + paclitaxel (120 μg/L) + N-acety-L-cysteine (NAC) (30 μmol/L, reactive oxygen species, ROS inhibitor) (NAC group), and paclitaxel (120 μg/L) group. The cells without any treatment were used as the control. MTT assay was conducted to measure the cell proliferation rate. Cell apoptosis was analyzed by flow cytometry (FCM). ROS was detected with fluorescence. Phosphorylation of Akt and the expressions of Caspase-3 were determined by Western blot. **Results:** The cell proliferation rate was significantly lower in the thermo-chemotherapy group than in the control and the chemotherapy groups ((59.83 ± 3.36)% vs. (100.00 ± 0.00)% and (69.16 ± 2.95)%, $P < 0.05$). The rate of cell apoptosis was the highest in the thermo-chemotherapy group (27.59 ± 5.47)% ($P < 0.05$). The ROS expression level was higher in the cells of thermo-chemotherapy group (102.14 ± 18.34) than in the other groups ($P < 0.05$), which could be inhibited by NAC (28.01 ± 1.19), but not by the PI3K inhibitor Wortmannin (99.87 ± 8.35). Phosphorylation of Akt significantly decreased in the thermo-chemotherapy group (0.69±0.03) ($P < 0.05$), which could be blocked by Wortmannin (0.00 ± 0.00), but increased by NAC (1.05 ± 0.29) ($P < 0.05$). The expression level of Caspase-3 was higher in the thermo-chemotherapy group (1.07 ± 0.08) than in other groups ($P < 0.05$). **Conclusion:** Thermo-chemotherapy has a stronger inhibitory effect than chemotherapy alone in lung tumor cell growth, probably through induction of ROS production and subsequent inhibition of Akt pathway activation and Caspase pathway-induced cancer cell apoptosis.

Key words: Akt pathway, thermotherapy, lung tumor H446, apoptosis

The incidence and mortality of lung cancer are the highest in the world, and it is also a malignant tumor with a very low cure rate. Thermotherapy is a new cancer therapy, which has become the fifth therapy for cancer after surgery, radiotherapy, chemotherapy and biological therapy[1]. Thermotherapy increases the sensitivity of tumor cells to the chemotherapeutic drugs at an effective temperature range, reverses drug resistance of tumor cells[2], and induces cell apoptosis[3]. Therefore, thermotherapy could induce the cell apoptosis and increase chemotherapy sensitivity to tumor cells. However, the theoretical basis of thermotherapy is still not very clear and its molecular mechanism needs in-depth studies.

Protein kinase B (PKB/Akt) is an important intracellular signaling pathway, which closely relates to cell growth, apoptosis and other events in cells[4]. Phosphorylated Akt takes part in lots of physiological and pathological processes directly or indirectly. Akt signaling pathway promotes cell growth[5], proliferation and survival, which is highly activated in most of tumor cells[6], and closely associated with tumor initiation and development[7]. Akt is also essential for normal cell proliferation[8]. In this study, we combined thermotherapy with chemotherapy using paclitaxel

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injection (TAX) in an attempt to explore the synergistic effect of combined thermotherapy and chemotherapy in H446 cell proliferation and the function of Akt pathway in this process.

Materials and Methods

Main reagents and instruments

Human small cell lung cancer cell line NCI-H466 is a kind gift from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Paclitaxel injection (the approval number: GY H19994040) was purchased from the Group of Sichuan Taiji Pharmaceutical Co., Ltd. Thiazole blue and reactive oxygen species (ROS) specific inhibitor N-acety-L-cysteine (NAC) were purchased from Sigma. ROS Testing Kit was purchased from Shanghai Genius America Pharmaceutical Technology Co., Ltd. Mouse anti-human Caspase-3 antibody and monoclonal β-actin antibody were purchased from the Santa Cruz Biotechnology. Rabbit anti-human Akt antibody and mouse anti-human p-Akt (Ser473) (587f11) antibody were purchased from Cell Signaling Inc. Horseradish peroxidase (HRP) goat anti-mouse IgG and HRP goat anti-rabbit IgG were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Akt specific inhibitor Wortmannin was purchased from the Alexis, Inc. Vertical electrophoresis tank was purchased from Beijing Liuyi Instrument Factory. Electroporation instrument was purchased from Bio-Rad Company. Sunrise microplate reader was purchased from Tianjin Kaiyuan Medical Equipment Co., Ltd. Constant temperature water bath was purchased from Shanghai Experimental Equipment Co., Ltd.

Experimental groups

Logarithmic phase cells were digested to single cell suspension, cell density was adjusted to 2.5×10^4 cells/mL and cultured in 100 mL dish, and each dish contained 6 mL. Cells were cultured at 37℃ in 5% CO_2 humidified incubator. After 24 h, cells were subjected to different schemes as follows: chemotherapy alone group (paclitaxel, 120 μg/L, cells were cultured at 37℃ for 48 h), thermo-chemotherapy group (paclitaxel, 120 μg/L, 43℃ for 40 min, cells were cultured at 37℃ for 48 h), thermo- chemotherapy and Akt inhibitor Wortmannin group (paclitaxel, 120 μg/L and Wortmannin, 1 μmol/L, 43℃ for 40 min, cells were cultured at 37℃ for 48 h), thermo-chemotherapy and ROS specific inhibitor NAC group (paclitaxel, 120 μg/L, Wortmannin, 1 μmol/L and NAC, 30 μmol/L, 43℃ for 40 min, cells were cultured at 37℃ for 48 h) and control group (cells were cultured at 37℃ for 48 h).

Detection of H466 cell survival rate by MTT assay

Logarithmic phase cells were digested to single cell suspension, cell density was adjusted to 2.5×10^4 cells/mL and seeded into 96-well plates, 200 μL per well, and cells were cultured at 37℃ in 5% CO_2 humidified incubator. After 24 h, the cells were treated as described above (six wells were assayed for each group and repeated three times). Twenty μL of 5 g/L MTT was added to each well and cells were incubated for another 4 h. MTT was discarded and 150 μL dimethylsulfoxide (DMSO) was added to each well, and vortexed for 10 min to dissolve the crystal. DMSO was used as negative control. Absorbance (*A*) values were measured at 492 nm with the microplate reader. Cell proliferation rate was calculated according to the following formula: cell proliferation rate = (average *A* value of experimental group / average *A* value of control group) × 100%.

Detection of ROS expression

Cells in the logarithmic growth phase (1×10^6) were collected, washed and centrifuged (1200 r/min, 5 min). Dyeing reagent was added and cultured at 37℃. Preservative media was added, and detected at 490 nm (excitation) and 530 nm (emission) qualitatively and quantitatively.

Detection of Akt phosphorylation and Caspase-3 expression by Western blot analysis

Cells from each group were collected and 1×10^6 cells were resuspended in cell lysis buffer (30 mmol/L $NaHCO_3$, 0.1 mmol/L EDTA, 0.1 mmol/L DTT, 0.5 mmol/L PMSF, 2 mg/L Aprotinin). Cells were lysed on ice and centrifuged (10 000 r/min) at 4℃ for 10 min, and supernatant was the cell lysate. The total protein concentration was determined and an equal amount of cell extract was subjected to electrophoresis in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane for antibody blotting. Mouse anti-human Caspase-3 antibody, rabbit anti-human Akt antibody and rabbit anti-human phosphorylate Akt were used as first antibody. HRP tagged goat anti-mouse or rabbit IgG was used as secondary antibody for blotting. After illustrating by ECL, GeneTool image analysis software was used for quality identification.

Flow cytometry (FCM)

Cell concentration was adjusted and cells were seeded into 50-mL dishes, cultured for 72 h and suspended to single cell. A total of 1×10^6 cells were collected to tubes, and fixed in ice-cold 70% ethanol for 12 h at 4℃. The ethanol was discarded and cells were digested by 1 ml 0.5% typsin (pH = 1.5–2.0) for 10 min and washed with PBS once, labeled with propidum iodide (PI) and filtered by 500 mesh screen to get single cell suspension and analyzed directly.

Statistical analysis

Statistical analysis was performed using SPSS13.0, data were shown by mean ± standard deviation. Single-factor analysis of variance was used for statistical analysis and LSD method was applied for multiple comparison, $\alpha = 0.05$ as test standard.

Results

Effects of various treatments on cell growth

As shown by MTT assay, the cell proliferation rate was significantly lower in the chemotherapy group (69.16 ± 2.95)% and thermo-chemotherapy (59.83 ± 3.36)% as compared with the control group (100.00 ± 0.00)%, and cell proliferation rate in thermo-chemotherapy group was lower than in chemotherapy group, and the differences were statistically significant ($P < 0.05$). In addition, the cell proliferation rate in Wortmannin group (40.65 ±0.14)% and NAC group (98.65 ± 0.09)% was

significantly different as compared with thermo-chemotherapy group (P < 0.05). Thermotherapy combined with paclitaxel greatly inhibited cell proliferation. ROS and Akt signaling pathway played roles in this process, and the two pathways were suppressed by corresponding inhibitors.

Changes of cell apoptosis

We detected the cell apoptosis by FCM. Compared with control group (1.29 ± 0.27)% and chemotherapy group (19.43 ± 2.68)%, cell apoptosis in the thermo-chemotherapy group (27.59 ± 5.47)% was increased with significant difference (P < 0.05). Cell apoptosis was increased in Wortmannin group (48.92 ± 1.87)% , while decreased in NAC group (2.37 ± 0.72)% compared with thermo-chemotherapy, with significant difference (P < 0.05).

Changes of ROS expressions

We used fluorescence to determine the ROS expressions in cells. In the control group, cells appeared red, showing that no ROS was produced. In chemotherapy group, cells appeared green, indicating that ROS was present. In thermo-chemotherapy group, cells were stained light green, which meant that cells produced more ROS. Additionally, Wortmannin did not affect the ROS production in thermo-chemotherapy. After NAC was added, cells were turned red as shown in Figure 1. ROS in thermo-chemotherapy group (102.14 ± 18.34) was much higher than in the control group and (32.40 ± 1.14) chemotherapy group (49.69 ± 7.93), with significant difference (P < 0.05). Wortmannin had no effect on ROS production (99.87 ± 8.35) compared with thermo-chemotherapy, and no statistical significance was found (P > 0.05). Compared with thermo-chemotherapy, NAC suppressed ROS expression (28.01 ± 1.19), and the differences were statistically significant (P < 0.05).

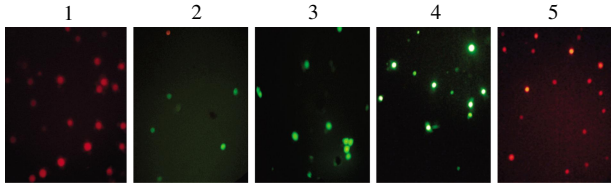

Figure 1 Changes of ROS under different conditions (×100)

Panel 1, control group (37℃, 0 μg/L TAX); panel 2, chemotherapy group (37℃, 120 μg/L TAX); panel 3, thermo-chemotherapy group (43℃, 120 μg/L TAX); panel 4, Wortmannin group(43℃, 120 μg/L TAX, 1 μmol/L Wortmannin); panel 5, NAC group (43℃, 120 μg/L TAX, 30 μmol/L NAC). Red cells show no ROS and the extent of the green light shows the amount of ROS.

Changes of Akt phosphorylation

After treatment, Western blot analysis was used to demonstrate the Akt phosphorylation. As shown in Figure 2, Akt phosphorylation level was lower in thermo-chemotherapy group (0.69 ± 0.03) than in control group (1.52 ± 0.01) and chemotherapy group (1.04 ± 0.42) by multiple comparisons. There were statistically significant differences by pair comparisons (P < 0.05). Thermo-chemotherapy combined with Akt specific inhibitor Wortmannin completely blocked Akt phosphoryaltion (0.00 ± 0.00). Compared with thermo-chemotherapy group, Akt phosphorylation level (1.05 ± 0.29) was higher in ROS specific inhibitor NAC treated group, with significant difference (P < 0.05).

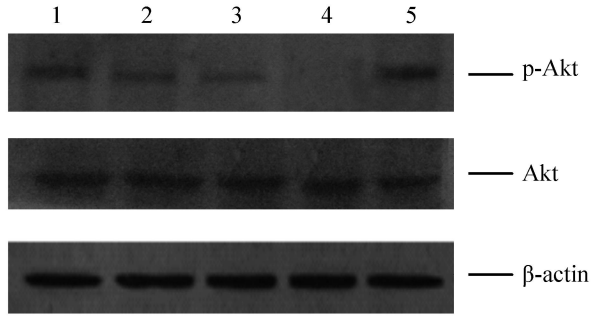

Figure 2 Phosphorylation of Akt under different conditions

Lane 1, control group (37℃, 0 μg/L TAX); lane 2, chemotherapy group (37℃, 120 μg/L TAX); lane 3, thermo-chemotherapy group (43℃, 120 μg/L TAX); lane 4, Wortmannin group (43℃, 120 μg/L TAX, 1 μmol/L Wortmannin); lane 5, NAC group (43℃, 120 μg/L TAX, 30 μmol/L NAC).

Expression of Caspase-3

Caspase-3 expression was higher in thermo-chemotherapy group (1.07 ± 0.08) than in control group (0.44 ± 0.25) and chemotherapy alone group (0.68 ± 0.06), with statistically significant difference (P < 0.05). After addition of Wortmannin, Caspase-3 expression was higher than thermo-chemotherapy group (1.09 ± 0.11), with statistically significant difference (P < 0.05). Caspase-3 expression was blocked totally (0.00 ± 0.00) by NAC, as shown in Figure 3, with statistically significance (P < 0.05).

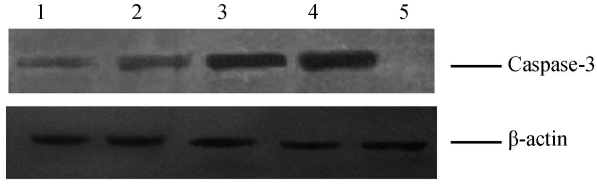

Figure 3 Expression of Caspase-3 under different conditions

Lane 1, control group (37℃, 0 μg/L TAX); lane 2, chemotherapy group (37℃, 120 μg/L TAX); lane 3, thermo-chemotherapy group (43℃, 120 μg/L TAX); lane 4, Wortmannin group (43℃, 120 μg/L TAX, 1 μmol/L Wortmannin); lane 5, NAC group (43℃, 120 μg/L TAX, 30 μmol/L NAC).

Discussion

Thermotherapy is a new cancer therapy re-emerged in the last 20 years, and plays an important role in tumor comprehensive treatment. Combined thermotherapy and chemotherapy can improve the efficacy of cancer treatment [9], which is known as a green therapy [10], has become one of the important auxiliary therapies. Around the 1990s, experimental studies noticed the phenomenon of heat-induced cell apoptosis. Thermotherapy can acidulate the environment within the cells, and promote tumor cell apoptosis induced by chemotherapeutic drugs. In addition, thermotherapy increases the tumor cell membrane permeability to promote the penetration and

absorption of chemot herapeutic drugs; enhances intracellular drug accumulation by changing the metabolism of drugs in the body; and promotes cell apoptosis induced by chemotherapeutic drugs, thus sensitizing the cancer chemotherapy. However, how and by which pathway thermotherapy induces tumor cell apoptosis still need to be explored in further studies.

Under normal conditions, production and removal system of ROS is in a dynamic equilibrium state[11]. Various reasons may lead to the increased ROS production or deceased capacity of ROS removal, causing the occurrence of oxidative stress in the body. If the oxidative stress was beyond the digestion capacity of anti-oxidant system, ROS would be accumulated, and Ca^{2+} influx was promoted. Expression of Bax was up-regulated, mitochondrial permeability transition pore (MPTP) was opened and Caspase was activated, causing cell apoptosis. In the present study, we have found that thermotherapy greatly promoted ROS production, which could be inhibited by NAC, but was not affected by Wortmannin, indicating that ROS production is an upstream event of inhibiting Akt pathway activation.

PI3K is an intracellular phosphatidylinositol kinase, which activates Akt, causing tumor cell proliferation[12]. Akt pathway is a common pathway for a great variety of signal transductions. After activated by phosphorylating, Akt regulates the activity of downstream factors and inhibits the cell apoptosis finally. The anti-apoptosis function of Akt pathway may be associated with inhibiting the activation of Caspase family members. Expression of active Akt suppressed cell apoptosis caused by growth factor removal, promoted cell growth and proliferation[5], increased cell invasion, metastasis and angiogenesis, and resisted cell apoptosis produced by chemotherapy and radiotherapy[13]. Studies have shown that[14], from normal cell to atypical hyperplasia and to malignant transformation, the expression of Akt increased gradually and companied with the loss of apoptosis related molecular markers, suggesting that Akt enhanced the ability of tumor survival and suppressed tumor cell apoptosis during tumor initiation and development. As a kinase, Akt is activated by phosphorylation, phosphorylated Akt is therefore, a marker for its kinase activity. It has been proved that Akt is activated in a number of cancers such as breast cancer, lung cancer, liver cancer, pancreas cancer and ovarian cancer. In our study, phosphorylated Akt was overexpressed in H466 cells, thermotherapy decreased Akt phosphorylation, additionally, NAC and Wortmannin could suppress the Akt phosphorylation level more significantly (Figure 2). MTT and FCM showed that cell proliferation was decreased and cell apoptosis was increased, which proved that thermotherapy inhibited the activation of Akt pathway. Thermotherapy inhibited lung cancer cell proliferation which may be related to Akt pathway inhibition.

In fact, cell apoptosis process is achieved by orderly activating Caspase proteins. Caspase family is the key downstream kinase in cell apoptosis, Caspase-3 is a main effecter in cell apoptosis of Caspase family. Inhibition of the activity of Caspase or antagonism of Caspase function may suppress cell apoptosis, suggesting that Caspase is required for cell apoptosis[15]. Most of the cell apoptosis-inducing factors eventually caused cell apoptosis through Caspase mediated signal transduction pathway[16]. Caspase-3 and other Caspases act on cell apoptosis by cascade reaction mode. The activity of Caspase can reflect the cell apoptosis induced by treatment. Our results confirmed that thermotherapy promoted Caspase-3 production, inhibited H466 cell proliferation and accelerated cell apoptosis. NAC and Wortmannin inhibited Caspase-3 expression, increased cell proliferation rate and decreased cell apoptosis, indicating that thermotherapy inhibited H466 cell proliferation by inducing ROS production and suppressing Akt pathway activation and through Caspase pathway-induced cancer cell apoptosis.

In summary, thermotherapy promoted the inhibitory effect on tumor cells by chemotherapeutic drugs, and Akt pathway played a role in this process, which has laid a basis for further exploring the mechanism of thermotherapy and shown an evidence for clinical application.

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