EXPERIMENTAL STUDY

Enhancement of radiosensitivity of lung adenocarcinoma using a decoction from the Fuzhengzengxiao formula

Zhang Qiaoli, Wu Tong, Lei Yong, Li Bo, Liu Weiyi, Tian Yehong, Zhao Weipeng, Huang Jingchang

Zhang Qiaoli, Department of Traditional Chinese Medicine, Beijing Hepingli Hospital, Beijing 100013, China
Wu Tong, Department of Traditional Chinese Medicine, Beijing Ditan Hospital, Capital Medical University, Beijing, 100015, China
Lei Yong, Liu Weiyi, Tian Yehong, Zhao Weipeng, Graduate School of Beijing University of Traditional Chinese Medicine, Beijing 100029, China
Li Bo, Health Science Center, Peking University, Beijing 100191, China
Huang Jingchang, Department of Acupuncture and Minimally Invasive Oncology, Beijing University of Chinese Medicine Third Affiliated Hospital, Beijing, 100029, China

Supported by a Grant from the National Natural Science Foundation of China: the Effect of TCM Combined Radiotherapy and RNAi Suppression on the Protein Expression Changes of S100A9 & Cyclophilin A and Radiosensitivity in Lung Adenocarcinoma (No. 81072925)

Correspondence to: Prof. Huang Jingchang, Department of Acupuncture and Minimally Invasive Oncology, Beijing University of Chinese Medicine Third Affiliated Hospital, Beijing, 100029, China zryhuang@163.com
Telephone: +86-10-84205733
Accepted: April 27, 2015

Abstract

OBJECTIVE: To study the effects of a decoction of Fuzhengzengxiao formula on lung adenocarcinoma regarding the inflammatory protein S100A9 known to enhance cancer cell sensitivity.

METHODS: A nude mouse model of human lung adenocarcinoma was established. The mice were randomly divided into four groups using the random number table method: Group I, control; Group II, treatment with a decoction of the Fuzhengzengxiao formula alone; Group III, treatment with radiotherapy alone; and Group IV, treatment with radiotherapy plus a decoction of Fuzhengzengxiao formula. When the tumor body was 1 cm³ in diameter, the tumor bearing mice in Groups III and IV were irradiated at a single dose of 10 Gy and the tumor inhibition rate was evaluated. The expression of S100A9 was determined using Western blotting and q-PCR (Real-time Quantitative PCR Detecting System). The sensitivity of cells containing RNAi S100A9 to radiotherapy was evaluated using the Click multiple target model, and the cell cycle was analyzed using flow cytometry.

RESULTS: Relative to the control group, the expression of S100A9 in the tumors in each treatment group was decreased, especially in Group IV. The sensitizing enhancement ratio (SER) Dq was >1 after RNAi S100A9; it decreased the surviving fraction after a 2 Gy dose exposure, and also the D₀ and Dq of the tumor cells; in addition, the radiosensitivity of G₂/M cells was significantly increased.

CONCLUSION: The decoction of the Fuzhengzengxiao formula downregulated the expression of S100A9 in lung adenocarcinoma cells.

© 2015 JTCM. All rights reserved.

Key words: Calgranulin B; RNA interference; Radiation tolerance; Adenocarcinoma of lung; Fuzhengzengxiao formula

INTRODUCTION

Lung cancer is one of the most fatal tumors worldwide. Eighty percent of non-small cell lung cancer patients
have a metastasis rate of ≤ 70%; because of aging and other associated diseases, most patients cannot tolerate surgical treatment and consequently radiotherapy is the main treatment method. However, the treatment failure rate ranges from 60% to 70%.

Previous studies have reported that the Fuzhengzengxiao formula significantly improved the efficacy and reduced the side effects of radiotherapy in lung cancer patients; however, the pathogenesis regarding its enhancement of the efficacy of radiotherapy remains unclear. It has been found that in mice that were treated with the Fuzhengzengxiao formula before irradiation, S100A9 expression in tumor cells was obviously abnormal (when assayed using protein microarray technology), relative to mice that received treatment with radiation alone. We hypothesized that S100A9 may be involved in enhancing tumor radiosensitivity after treatment with the Fuzhengzengxiao formula. The objective of the present study was to investigate how S100A9 influenced cancer cell sensitivity to radiotherapy while the cancer was being treated with a decoction of the Fuzhengzengxiao formula.

MATERIALS AND METHODS

Drugs and reagents

The Fuzhengzengxiao formula is composed of Huangqi (Radix Astragali Mongolici), Shihu (Herba Dendrobii Nobilis), Beishashen (Radix Glehniae), Jinyinhua (Flos Lonicerae), Honghua (Flos Carthami), Sumu (Lignum sappan), Baizhu (Rhizoma Atractylodis Macrocephalae), Taizishen (Radix Pseudostellariae), Gouqizi (Fructus Lycii), Jixuqin (Caulis Spatholobi), Fuling (Rhizoma Atractylodis Macrocephalae), Baimutong (Rhizoma Atractylodis Macrocephalae), and Beishan (Radix Pseudostellariae), which were decocted twice. The decoctions were mixed and concentrated into 2.2 g of crude drugs/mL, and then disinfected by boiling. RNAi S100A9 was provided by Cyagen Biosciences Inc., (Guangzhou, China); it was subcultured in RPMI1640 containing 10% fetal calf serum. Calf serum and diethyl pyrocarbonate (DEPC) were purchased from the Sigma Company (St. Louis, Missouri, USA). Rabbit anti-S100A9 antibody was purchased from the Proterintech Company (Chicago, IL, USA).

Animals and the PAa cell line

The experimental animals were 6-week-old male BALB/C-nu/nu nude mice. They were purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences, and raised in specified pathogen free conditions (certificate of quality No. SCXKJ 2009-004). The low transfer human pulmonary adenocarcinoma was purchased from the Pathological Section of the Medical Department, Peking University (Beijing, China).

Establishment of animal models

Pulmonary adenocarcinoma cells in the logarithmic growth phase were taken. They were prepared at a concentration of 5 × 10⁶ cells/mL with RPMI1640 culture medium; 0.3 mL of these cells was injected into the armpit of the forelimb of each nude mouse. The mouse were randomly divided into four groups (each group have 28 nude mouse) using the random number table method: Group I, control; Group II, treated with the decoction of the Fuzhengzengxiao formula alone; Group III, treated with radiotherapy alone; and Group IV, treated with radiotherapy plus the decoction of the Fuzhengzengxiao formula. From the day of tumor inoculation, each nude mouse in Groups II and IV was intragastrically administered 0.5 mL (1.1 g crude drug) of the decoction of the Fuzhengzengxiao formula daily, and the other two groups were administered an equal volume of distilled water. When the tumor had reached a size of 1 cm³ (at about 28 days after inoculation) the mouse in Groups III and IV were irradiated at a single dose of 10 Gy. In 18 nude mouse from each group, the tumors were removed and homogenized at 6, 12 and 24 h after irradiation. Changes in the tumor growth rate were monitored in the 10 remaining mouse in each group.

Tumor inhibition rate

To observe changes in tumor volume, this was measured 1 day before radiotherapy and every 2 days after radiotherapy for a total of 10 times; mouse were killed after the last measurement, and the weight of the tumor was measured and the growth inhibition rate calculated. The tumor inhibition rate = (C − T)/C × 100%, where C is the mean weight of the tumors in the negative control group, and T is the mean weight of the tumors in a given experimental group.

Detection of S100A9 protein expression

First, 200 μL of protein lysate was added into a centrifuge tube holding 20 mg of tumor, which was homogenated and placed on ice for 15 min, and centrifuged at 4 °C (9000 ×g for 10 min); the supernatant was drawn to determine the protein content. Protein loads were 20 μg/lane. It was separated using 12% separation gel and 5% stacking gel. Then the protein was semi dryly transferred to PVDF film, 8-mL anti-S100A9 was diluted using closed liquid (1:500 dilution; acetin, 1:1500 dilution) and reacted at 4 °C overnight; the second antibody (1:10000) was reacted for 4 h. The ECL chemiluminescence method, exposure to X-rays and scanning film JX330 (Sharp Electronics, Tokyo, Japan) involving a transmission scanner were used for analysis. Finally, LabWorks software was used to evaluate the image grayscale.

Detection of the mRNA expression of S100A9

Extraction of total RNA and first strand synthesis of cDNA were carried out according to the instructions of the manufacturer. Real time PCR was performed in a 25 μL reaction system. It included: 10 μL of 2 ×
SYBR mixture (Synergy Brands, TIANGEN) 1 μL PCR forward primer (0 μM), 1 μL PCR reverse primer (10 μM), 0.3 μL Taq DNA polymerase and 25 μL ddH2O. The forward primer was S100A9:5′-AGATGGGAGCAGCATACAACC-3′. The reverse primer was S100A9: 5′-GCTAGGTGTCAGGGTGCTCCTT-3′, and the length of the amplified product was 80 bp. The PCR reaction was carried out in an ABI 7500 PCR instrument. The amplification reaction conditions for S100A9 were as follows: denaturation at 95 °C for 2 min and then denaturation for 20 s at 94 °C; denaturation for 20 s at 59 °C; denaturation for 30 s at 72 °C; 40 cycles; and elongation at 72 °C for 30 s. The mRNA expression of S100A9 was analyzed by the 2(-Delta Delta C[T]) method.

Colony forming experiment
Regarding the mice in the control group, the RNAi S100A9 in PAa cells and the cell suspension was prepared by centrifugation. For counting, cells were inoculated into 25 cm² cell culture bottles. The cell inoculation numbers were 600, 1000, 4000, 8000 and 10 000, with medium supplied to 4 mL. There were three groups: blank; control; and RNAi S100A9. Each group had three parallel experimental bottle sets. The cells were maintained in culture medium until for 10 days after irradiation, and the medium was changed every 3 days. Cells were fixed in 100% methanol, and stained using crystal violet. They were then counted using a Gel-Pro Analyzer and the radiation sensitivity of the cells containing RNAi S100A9 was evaluated using the Click multiple target model.

Determination of the phases of the cell cycle
The lung PAa cells were harvested in the logarithmic growth phase, digested using pancreatic enzymes, and suspended at a density of 1.5 × 10⁷/mL in 10% FCS+RPMI1640 culture medium in culture bottles. Each treatment group was irradiated at a single dose of 0 or 2 Gy after adherence and growth for 24 h. Each group had three parallel experimental bottle sets. The cells were collected after irradiation. They were centrifuged at 1000 rpm for 8 min, the supernatant was discarded and they were washed twice with PBS. Cells were fixed in anhydrous ethanol for 12 h at 4 °C. They were filtered using a 400 mesh nylon net, and then centrifuged. The supernatant was discarded and the cells were mixed with 500 μL PBS, pulsed with 10-μL RNaseA (10 mg/mL) in a water bath at 37 °C; 50-μL propidium iodide was added before detection and staining in the dark for 30 min at 4 °C. The cell cycle phases were determined using flow cytometry. Changes in the cell cycle were analyzed using CELLQUEST software.

Statistical analysis
Quantitative data were expressed as the mean ± standard deviation (x ± s). One-way analysis of variance and least significant difference-t were performed using SPSS 10.0 software (SAS Institute, Chicago, IL, USA) for the differences among the groups. A P-value < 0.05 was considered as being statistically significant.

RESULTS
Tumor inhibition rate
The average tumor weight in the Group I was (1.82 ± 0.10) g, and the average tumor weight in Group II was (1.69 ± 0.13) g; the tumor inhibition rate was 7.2%. The average tumor weight in Group III was (1.40 ± 0.15) g and the tumor inhibition rate was 22.9%. The average tumor weight in Group IV was (0.83 ± 0.12) g and the tumor inhibition rate was 54.3%. Relative to Group II, the tumor inhibition rate in Group III increased significantly (P < 0.05). As compared with the control group, the tumor inhibition rate increased in Group II, but the difference was not significant. Relative to all the other groups, the tumor inhibition rate in Group IV increased significantly (P < 0.05) (Figure 1).

Figure 1 Tumor inhibition rate
I : control group, treated intragastrically administrated with 0.5 mL distilled water; II : Fuzhengzengxiao formula group, treated intragastrically administrated with 0.5 mL (1.1 g crude drug) Fuzhengzengxiao formula; III : simple radiotherapy group, treated with radiated once 10 Gy dose the tumor body was 1 cm³ in diameter; IV : radiotherapy plus Fuzhengzengxiao formula group, treated intragastrically administrated with 0.5 mL (1.1 g crude drug) Fuzhengzengxiao formula and radiated once 10 Gy dose when the tumor body was 1 cm³ in diameter. 'P < 0.05, compared with control group; 'P < 0.05, compared with Fuzhengzengxiao formula group; 'P < 0.05, compared with simple radiotherapy group.

Protein and mRNA expression of S100A9
The Fuzhengzengxiao decoction increased sensitivity of lung cancer cells to radiotherapy through down-regulation of S100A9 protein and mRNA expression (Figures 2-4).
Results of the colony forming assay

The surviving fraction after irradiation at a dose of 2 Gy (SF$_{2Gy}$) was determined. By experiment the average SF$_{2Gy}$ of the blank was 0.401 ± 0.064, the average SF$_{2Gy}$ of the control was 0.442 ± 0.037 and the average SF$_{2Gy}$ of RNAi S100A9 was 0.350 ± 0.057. The radiosensitivity of lung adenocarcinoma was increased after RNAi S100A9. (Table 1, Figure 5)

Cell cycle analysis using flow cytometry

Silence S100A9 expression increased the radiosensitivity of lung adenocarcinoma through G2/M cell cycle arrest. (Tables 2, 3; Figures 6, 7).
DISCUSSION

S100A9 protein (calgranulin B, MRP14) belongs to the S100 protein family; it is a calcium binding protein related to trauma and tumor inflammation. Calcium binding protein is an important medium in which calcium ions are produced regarding physiological activity. S100A9 participates in multiple physiological processes, such as the inflammatory response, mediation of growth inhibition, and regulation of cell growth and differentiation. Studies have shown that S100A8/S100A9 positive inflammatory cells can be found in malignant stromal epithelial tumors, such as skin, colorectal and prostate tumors. Cell inflammation plays an important role in tumor stroma; it can result in the production of cytokines, growth hormones, increased levels of active oxygen and nitric oxide, and other factors. They are involved in promoting cell proliferation, inhibition of apoptosis and can induce changes in and damage to cell morphology, resulting in tumorigenesis. Abnormal expression of S100 protein in malignant tumors has been reported to be closely as-

Table 1 Radiobiological parameter values obtained using Click multiple target model fitting (x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>N</th>
<th>D0 (Gy)</th>
<th>Dq (Gy)</th>
<th>SER&lt;sub&gt;Dq&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>3</td>
<td>2.677±0.934</td>
<td>1.166±0.077</td>
<td>1.088±0.210</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>2.930±0.499</td>
<td>1.173±0.031</td>
<td>1.246±0.075</td>
<td>-</td>
</tr>
<tr>
<td>RNAi S100A9</td>
<td>3</td>
<td>2.228±0.560</td>
<td>1.159±0.026</td>
<td>0.902±0.141</td>
<td>1.382±0.022</td>
</tr>
</tbody>
</table>

Notes: blank group: pulmonary adenocarcinoma cell group; control group: pulmonary adenocarcinoma cells from the RNAi control group; RNAi S100A9 group: pulmonary adenocarcinoma cells from the RNAi S100A9 group. N: the extrapolation number, a parameter used to measure the width of the shoulder of the survival curve; D0, the mean lethal dose; Dq, the quasi-threshold dose; SER: sensitization enhancement ratio.

Table 2 Cell cycle analysis of non-irradiated blank, control and RNAiS100A9 groups (% , x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>3</td>
<td>49.9±0.4</td>
<td>38.9±0.9</td>
<td>11.2±0.9</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>51.3±3.0</td>
<td>38.8±3.4</td>
<td>9.9±0.6</td>
</tr>
<tr>
<td>RNAi S100A9</td>
<td>3</td>
<td>52.9±2.6</td>
<td>35.0±2.0</td>
<td>12. 1±0.6</td>
</tr>
</tbody>
</table>

Notes: blank group: pulmonary adenocarcinoma cell group; control group: pulmonary adenocarcinoma cells from the RNAi control group; RNAi S100A9 group: pulmonary adenocarcinoma cells from the RNAi S100A9 group. *P < 0. 05, compared with the control group.

Figure 5 Cell survival curves for the Blank, Control and S100A9 groups
Blank group: pulmonary adenocarcinoma cell group; control group: pulmonary adenocarcinoma cells from the RNAi control group; RNAi S100A9 group: pulmonary adenocarcinoma cells from the RNAi S100A9 group.

Figure 6 Cell cycle distribution of blank, control and RNAiS1000A9 groups with non-irradiated
Blank group: pulmonary adenocarcinoma cell group; control group: pulmonary adenocarcinoma cells from the RNAi control group; RNAi S100A9 group: pulmonary adenocarcinoma cells from the RNAi S100A9 group. *P < 0. 05, compared with the control group.
Table 3 Cell cycle analysis after irradiation at a dose of 2 Gy in the blank, control and RNAi S100A9 groups (% ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>3</td>
<td>50.7±1.9</td>
<td>32.8±3.0</td>
<td>16.9±1.4</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>50.8±1.6</td>
<td>33.4±2.4</td>
<td>16.5±1.1</td>
</tr>
<tr>
<td>RNAi S100A9</td>
<td>3</td>
<td>42.6±3.6</td>
<td>28.9±3.6</td>
<td>28.5±0.9</td>
</tr>
</tbody>
</table>

Notes: blank group: pulmonary adenocarcinoma cell group; control group: pulmonary adenocarcinoma cells from the RNAi control group; RNAi S100A9 group: pulmonary adenocarcinoma cells from the RNAi S100A9 group. *P < 0.05, compared with the blank group; **P < 0.05, compared with the control group.

In the present study, we found that the radiation sensitizing effect of Fuzhengzengxiao formula was related to the expression of S100A9. The expression of S100A9 was decreased in the group treated with Fuzhengzengxiao formula and irradiation. The expression of S100A9 was detected using Western blotting and q-PCR; it was found that the expression of S100A9 was decreased. With increasing time after irradiation there was a significant difference in the tumor inhibition rate between the irradiated group and the control group (P < 0.05); this finding confirmed that S100A9 had a radiation protection effect regarding lung adenocarcinoma cells. In addition, with increasing time after irradiation, the level S100A9 expression decreased, and the radiation protection effect should gradually decrease resulting in enhancement of radiosensitivity. After irradiation, the expression of S100A9 was significantly decreased (P < 0.05) and the tumor inhibition rate increased (P < 0.05) in the group treated with Fuzhengzengxiao formula relative to the group that was not; this confirmed that the Fuzhengzengxiao formula enhanced tumor cell radiosensitization by inhibiting the expression of S100A9. The expression of S100A9 has been reported to be downregulated in esophageal squamous cell carcinoma, head and neck squamous cell cancer and other epithelial tumors, and was positively correlated with the degree of tumor differentiation, suggesting that S100A9 plays an important role in the pathogenesis of squamous cell carcinoma. This may be related to different mechanisms regarding S100A9 in different tumors.

In the current study it was found that determining tumor cell radiosensitivity in vitro was closely related to the effects of clinical radiotherapy on the tumor. Determination of tumor cell radiosensitivity would contribute to the formulation of more reasonable and effective radiotherapy plans in a clinical setting. Regarding the parameters reflecting cell radiosensitivity, SF2, DS, N are commonly used in evaluations. The higher the values of SF2, DS, N are, the lower the radiosensitivity, and the lower these values are the higher the radiosensitivity. To further confirm that the level of expression of S100A9 is related to the radiosensitivity of lung adenocarcinoma, we found using the colony formation assay that the value of SF2 was decreased after the administration of RNAi S100A9. In addition, colony survival analysis showed that before and after RNAi S100A9 administration the DS was 1.159 and 1.112, respectively, and the extrapolated values for N were 2.228 and 2.636, respectively; the SERDq was >1. Thus, comparison of the values of SF2, DS, N suggested that the radiosensitivity of lung adenocarcinoma was enhanced after RNAi S100A9 administration.

The resistance of tumor cells to radiotherapy is related to cell radiosensitivity, repair of sublethal damage, redistribution in the cell cycle, and the levels of oxygenation and hypoxia. Tumor cells are often in different phases of the cell cycle, and cell radiosensitivity varies greatly.
in these different phases. Among the cell cycle phases, G2/M has highest radiosensitivity, followed by the G1/S phase, and the late S phase has strong radioresistance. 16 The study found that the SF2Gy of the tumor cell was reduced by RNAi S100A9, and the SERDq was >1; RNAi S100A9 increased the percentage of cells in the G2/M phase, and further promoted tumor cells to enter and arrest in the G2/M phase from the more radiation resistant G1/S phase after irradiation. Consequently, we considered that the Fuzhengzengxiao formula improved the radiation sensitivity of tumor cells by lowering the expression of S100A9 and raising the ratio of cells arrested in the G2/M phase, thus improving the efficacy of radiotherapy. In conclusion, the decoction of the Fuzhengzengxiao formula increased the sensitivity of lung cancer cells to radiotherapy and reduced the side effects of the radiation. The mechanism underlying the effects requires further study.

REFERENCES
15 Mul Ian PB, Quinn JE, Harkin DP. The role of BRCA1 in transcriptional regulation and cell cycle control. Oncogene 2006; 25(43): 5854-5863.