Effects of Feiyan Nong Decoction on gene expression of nuclear factor-κB activated by tumor necrosis factor-α in lung adenocarcinoma cell line

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Objective: To study the effects of Feiyan Nong Decoction, a compound traditional Chinese herbal medicine, on gene expression of nuclear factor-κB (NF-κB) activated by tumor necrosis factor-α (TNF-α) in lung adenocarcinoma cell line (A549).

Methods: A549 cells were incubated with rat serum containing Feiyan Nong at different concentrations for 24 and 48 h, respectively. Morphology of cells was observed by an inverted microscope after treatment with reagents. The cell proliferation was examined by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assay. The expressions of NF-κB and inhibitor IκBα (IκBα) were studied by Western blotting. NF-κB-dependent luciferase reporter (3×κB-luc) was transfected for 24 h, and the cells were treated with the reagents for 48 h, and then the transcriptional activity of NF-κB promoter was detected by luciferase assay.

Results: TNF-α (1 μg/L) strongly induced the expression of NF-κB by approximately 1.75-fold compared with the control in the nuclei of A549 cells, and the induced NF-κB expression was significantly suppressed by addition of Feiyan Nong (P<0.01). In addition, Feiyan Nong inhibited the transcriptional activity of the NF-κB promoter. However, we observed no significant changes in IκBα expression (P>0.05).

Conclusion: Feiyan Nong Decoction can markedly inhibit human lung cancer A549 cell proliferation, which may be partly due to inhibition of NF-κB activation induced by TNF-α. It is therefore expected to be a new strategy for treating lung cancer.

Keywords: lung neoplasms; Feiyan Nong Decoction; NF-kappa B; tumor necrosis factor-α

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肺岩宁方对肿瘤坏死因子α诱导的人肺癌细胞系
核转录因子κB基因表达的影响

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目的: 探讨中药肺岩宁方含药血清对肿瘤坏死因子α(tumor necrosis factor-α, TNF-α)诱导的人肺癌细胞系A549 核转录因子κB(nuclear factor-κB, NF-κB)基因表达的影响。

方法: 制备肺岩宁方含药血清, 以肺癌细胞系A549为靶细胞, 确定肺岩宁方含药血清用药浓度。将A549细胞分为对照血清组、TNF-α组、肺岩宁方含药血清组和综合治疗组。观察用药前后A549细胞大体形态变

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Lung cancer is one of the most common cancer-related causes of death worldwide. Chemotherapy plays an important role in the treatment of advanced lung cancer. Major improvements have been achieved in the past decades, however, the effects are not satisfactory and response rate is 17% to 22%, and median survival time is 8 to 10 months. Traditional Chinese medicine (TCM) treats advanced lung cancer with safety and less side effects; it is an urgent need to study its anti-cancer mechanism. Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that is activated by a variety of cytokines and mitogens, and is a key regulator in the process of cell survival and deterioration. The purpose of the present study was to examine the effect of Feiyanning Decoction on gene expression of NF-κB activated by tumor necrosis factor-α (TNF-α) in lung adenocarcinoma cell line A549 through 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-[2,4-disulphonyl]-2H-tetrazoiium, monosodium salt (WST-8), Western blotting and luciferase assay methods.

1 Materials and methods

1.1 Materials Feiyanning Decoction [Radix Astragali Mongolici (Sheng Huangqi) 40 g, Rhizoma Atractylodis Macrocephalae (Baizhu) 15 g, Succus Bufo skin (Ganchanpi) 9 g, Nidus Vespeae (Fengfang) 9 g, Rhizoma Paridis (Qieye Yizhihua) 15 g, Rhizoma Polygonati Sibirici (Huangjing) 30 g, Herba Epimedi (Erhuihong) 15 g, Canoaradica Lucidum (Lingzhi) 30 g, etc.] was provided by Longhua Hospital Pharmacy, Shanghai University of TCM. The above drugs were soaked in 2000 mL distilled water (DW) for 2 h, boiled for 60 min for the first decoction; The second decoction was added DW to 1000 mL, boiled for 60 min. After mixture, the Feiyanning Decoction was condensed with water bath to crude drug content 2.0 g/mL and stored at 4℃ until use. Fetal bovine serum (FBS) was purchased from Shanghai Huamei Biotechnology Company. RPMI 1640 medium and 0.25% trypsin were purchased from Gibco Company, USA. Recombinant TNF-α was purchased from Calbiochem, Germany. Dimethyl sulfoxide (DMSO) and NF-κB p65 antibody were purchased from Santa Cruz, USA. Transfection factor II B (TF II B) antibody was purchased from BD Transduction Laboratories. Inhibitor α (100 μM) and α-tubulin antibodies were purchased from Cell Signaling Technology. Lipofectamine™ Plus reagent kit was purchased from Invitrogen. Dual-luciferase reporter gene assay system was purchased from Promega. Lumat 9507 fluorescence detector was purchased from Beijing Xinfeng Electromechanical Technology Company.

1.2 Methods

1.2.1 Preparation of serum containing Feiyanning

Sixteen male Wistar rats were obtained from Shanghai Slac Laboratory Animal Co., Ltd. with body weight at (300±20) g and the permit number was SCXX (Hu) 2007-0005. They were randomly divided into two groups (with 8 animals per group). Rats in the control group were administered equal volume of normal saline twice daily; rats in the treatment group were administered Feiyanning Decoction at a dose of 22 mL/kg daily (equivalent to the human clinical dose of 8 times) based on body weight. According to serum preparation program, rats were administered drugs daily by oral gavage for 3 days. The same serum was mixed, inactivated for 30 min in a 56℃ water bath, and then filter-sterilized through a 0.22 μm membrane filter. Serum was stored at −20℃ until use.

1.2.2 Cell culture

The human lung cancer cell line A549 was originally obtained from the American Type Culture Collection (ATCC) and maintained in 5% CO₂ at 37℃ in RPMI 1640 medium containing 10% FBS.

1.2.3 Cell morphology

After treatment with reagents, cells were observed under an inverted microscope.

1.2.4 WST-8 assay

The cell proliferation was evaluated by using WST-8 assay. A549 cells (1×10⁶ cells/well) were seeded into 96-well plates in 100 μL of culture medium overnight, and then treated with various concentrations of serum containing
Feiyanning (10%, 15% and 20%) or control serum. At the indicated times (24 h or 48 h), 10 μL of WST-8 reagent solution (cell counting kit, Dojindo Laboratories, Japan) was added and incubated for 2 h. The optical density (OD) value was detected at a test wavelength of 450 nm according to manufacturer’s instructions. Percentage of inhibition of proliferation was calculated as follows: Percentage of inhibition = (mean control OD - mean experimental OD) / mean control OD × 100%.

1.2.5 Extraction of nuclear and cytoplasm proteins Based on above results, the concentration of the serum containing Feiyanning was determined. The A549 cells were divided into four groups; control serum group (serum culture medium), TNF-α group (TNF-α 1 μg/L), serum containing Feiyanning group (15% serum containing Feiyanning) and integrated group (TNF-α 1 μg/L plus 15% serum containing Feiyanning). After 48-hour treatment as described above, nuclear and cytoplasm proteins of the A549 cells were extracted according to the methods described by Sugimori et al[25]. Briefly, the cells were harvested in 1 mL of ice-cold PBS and centrifuged for 1 min at 5000 × g for 4°C. The cell pellet was lysed with 0.4 mL of buffer A, containing 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 10 mmol/L potassium chloride (KCl), 0.1 mmol/L ethylenediamine tetaacetic acid (EDTA), 0.1 mmol/L ethyleneglycol bis (2-aminoethyl ether) tetaacetic acid (EGTA), 1 mmol/L dithiothreitol (DTT) and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), for 15 min on ice. Then, 25 μL of 10% Nonidet P-40 solution was added and the samples were vortexed for 15 s before centrifuging at 15,000 × g for 5 min at 4°C. The supernatant (cytoplasm protein) was stored at −80°C until use. The pellet was washed once with 0.5 mL of buffer A and resuspended in 50 μL of buffer B, which was composed of 20 mmol/L HEPES, pH 7.9, 0.4 mol/L sodium chloride (NaCl), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L PMSF. The lysed nuclei were left on ice for 30 min and then centrifuged at 15,000 × g for 5 min at 4°C. The nuclear protein concentration was determined by the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Nuclear extracts were stored at −80°C until use.

1.2.6 Western blotting TF II B and α-tubulin were used as controls. Aliquots of the protein extracts (20 μg of protein each) were separated by 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), then blocked with 5% nonfat milk in TBS-Tween buffer (20 mmol/L Tris-HCl, pH 7.4, 135 mmol/L NaCl, 0.1% Tween) for 1.5 h at room temperature, and incubated with the appropriate antibody (1:500) overnight at 4°C, then with horseradish peroxidase conjugated secondary antibody (1:5000) for 30 min at room temperature. After extensive washing, immunoreactive proteins were detected with an enhanced chemiluminescence detection system (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA).

1.2.7 Recombinant human-specific NF-κB-luciferase reporter plasmid activity detection The human 3 × κB promoter constructs; NF-κB-dependent luciferase reporter (3 × κB-luc) was kindly provided by Dr. Shigeki Miyamoto[4]. A549 cells were seeded onto six-well plates and cotransfected with 1.0 μg of firefly-luciferase reporter plasmid and 0.1 μg of renilla-luciferase transfection control (pHR-CMV; Promega, Southampton, UK) using Lipofectamine™ Plus reagents. After 24-hour transfection, the cells were treated with reagents for 24 h. The cells were harvested, added cell lysate and then centrifuged at 15,000 × g for 5 min at 4°C. Luciferase activity was measured with the dual-luciferase assay system according to the manufacturer’s instructions by Lumat 9507.

1.3 Statistical analysis Data are presented as x ± s. Statistical analysis was performed with SPSS 10.0 statistical software. Comparisons among groups were performed with one way analysis of variance or factorial design analysis of variance, and comparisons between groups were performed by using t test.

2 Results

2.1 Morphology We observed the morphologic changes by inverted microscopy. In the control serum group, monolayer cells were adherently growing and full of transparent cytoplasm, oval-shaped nucleus. However, A549 cells became smaller and fell off, with particle-shaped and deep-colored nuclear after treatment with 15% or 20% serum containing Feiyanning (Figure 1).

2.2 Growth inhibition of A549 cell line Compared with the control serum, the proliferation of the A549 cells was inhibited after treated with sera containing Feiyanning (15%, 20%). The difference was not significant between 15% and 20% serum containing Feiyanning groups. Therefore, the NF-κB effects in A549 cells were detected at 15% serum containing Feiyanning (Table 1).

2.3 Effects of serum containing Feiyanning on expressions of NF-κB and IκB mRNAs There was not significant influence on NF-κB expression with 15% of serum containing Feiyanning (F = 2.425, P = 0.326), while TNF-α strongly induced NF-κB expression (F = 18.152, P = 0.004), however, the induced NF-κB expression was significantly suppressed by the addition of serum containing Feiyanning (15%) (F = 14.325, P = 0.005, Figure 2). To assess whether the down-regulation of NF-κB expression was mediated by the degradation of IκBα expression by 15% of serum containing Feiyanning, IκBα expression was determined in the cytosolic protein of A549 cells. We observed no sig-
significant change on IκBα expression level (P > 0.05, Figure 3). These results suggest that the inactivation of NF-κB by 15% of serum containing Feiyaning was not dependent on IκBα degradation.

2.4 serum containing Feiyaning decreases NF-κB promoter transcriptional activity induced by TNF-α

We performed the dual-luciferase assay with a reporter vector containing the NF-κB promoter in A549 cells. As shown in Figure 4, there was not significant influence on luciferase reporter activity with 15% of serum containing Feiyaning (F = 3.439, P = 0.401), and upon stimulation with TNF-α, the luciferase reporter activity was increased by approximately 2.5-fold as compared with the control serum (F = 23.724, P = 0.0032). 15% serum containing Feiyaning significantly inhibited the NF-κB promoter transcriptional activity induced by TNF-α (F = 18.236, P = 0.005, Figure 4).

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**Figure 1** Effects of serum containing Feiyaning on morphology of A549 cells (Inverted microscopy, × 400)

A. Control serum group, B. 10% serum containing Feiyaning group, C. 15% serum containing Feiyaning group, D. 20% serum containing Feiyaning group.

**Table 1** Effects of serum containing Feiyaning on proliferation of A549 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>OD value (24 h)</th>
<th>OD value (48 h)</th>
<th>Rate of inhibition after 48 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control serum</td>
<td>4</td>
<td>0.325±0.014</td>
<td>0.497±0.021</td>
<td>0</td>
</tr>
<tr>
<td>10% serum containing Feiyaning</td>
<td>4</td>
<td>0.37±0.042</td>
<td>0.424±0.018</td>
<td>14.69±1.38</td>
</tr>
<tr>
<td>15% serum containing Feiyaning</td>
<td>4</td>
<td>0.344±0.028</td>
<td>0.359±0.020</td>
<td>30.85±1.62*</td>
</tr>
<tr>
<td>20% serum containing Feiyaning</td>
<td>4</td>
<td>0.331±0.017</td>
<td>0.323±0.016</td>
<td>36.21±2.01**</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, vs control serum group.

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**Figure 2** Effects of serum containing Feiyaning on NF-κB expression induced by TNF-α in A549 cells

Confluent cells were incubated for 48 h with or without 15% serum containing Feiyaning, or TNF-α (1 μg/L). The two panels showed the protein expression levels of NF-κB and TF. Bar graphs showed the expression levels of NF-κB relative to those of TF. The data were represented as x ± s (n = 3). Compared with TNF-α, F = 14.325, P = 0.0054, **P < 0.01.

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**Figure 3** Effects of serum containing Feiyaning on IκBα expression in A549 cells

Confluent cells were incubated for 48 h with or without 15% serum containing Feiyaning, or TNF-α (1 μg/L). The two panels showed the protein expression levels of IκBα and α-tubulin. Bar graphs showed the expression level of IκBα relative to those of α-tubulin. The data were represented as x ± s (n = 3).
3 Discussion

NF-κB signaling pathway exists in a variety of cell differentiation and proliferation. The pivotal role of the NF-κB signaling pathway in tumor promotion and progression, together with the occurrence of constitutively activated NF-κB in various solid and hematopoietic malignancies, strongly suggests that NF-κB inhibitors would be useful in cancer therapy. A close relationship between NF-κB and cancer was proposed and recently reviewed. It was reported that NF-κB p65 expression was significantly increased and associated with disease progression in 394 cases of lung cancer patients by immunohistochemistry, compared with the control group.

Feiyanning Decoction was studied by Professor Xu Zhenye. Feiyanning Decoction is a compound nourishing qi and yin and diminishing stagnation by detoxification functions, which can prevent invasion and metastasis of lung cancer. Clinical studies show that Feiyanning Decoction has effects in stabilizing advanced lung cancer tumor, preventing tumor invasion and metastasis and improving the quality of life and survival time of patients. Chinese medicine serum pharmacology methods provided a reliable guarantee for studying complex components of Chinese medicine in vitro. It has been widely used. Our data showed that 15% of serum containing Feiyanning could markedly inhibit A549 cell proliferation, which may be related to drug formulations, animal health and so on. In addition, inactivation of NF-κB by 15% of serum containing Feiyanning is not dependent on IkBa degradation.

IkB activity is controlled by IkB kinase (IKK) complex. IKK, including IKK-α, IKK-β and IKK-γ, is also known as NF-κB essential modifier (NEMO). When IKK was activated, IKK complex caused rapid phosphorylation, ubiquitination and hydrolysis IkB, and released NF-κB from cytoplasm to nucleus.

These results show that 15% of serum containing Feiyanning can markedly inhibit A549 cell proliferation, which is not dependent on IkBa degradation. It provides an important basis and further mechanistic studies for inhibition of NF-κB activity are needed.

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第十次全国中西医结合虚证与老年病学术研讨会第一轮通知

中国中西医结合学会决定于2009年10月在安徽省召开“第十次全国中西医结合虚证与老年病学术会议”，并同时召开专业委员会工作会议和成立青年学组。现将会议征文通知如下。

1 征文内容 (1) 中西医结合老年医学的临床与基础研究，重点征文领域为老年心脑血管、肿瘤、呼吸系统疾病，老年认知功能障碍的中西医结合诊断研究；(2) 中西医结合虚证临床与基础研究；(3) 中西医结合虚证辨证标准研究。

2 论文要求 (1) 所投稿件为尚未公开发表的论文，要求科学性强，论点鲜明；(2) 来稿请寄全文（5000字以内）。请附400字以内的结构式摘要(目的、方法、结果、结论)及关键词；(3) 稿件一律用Word文档，标准A4版面，标题用3号宋体字，作者及单位用4号楷体字，简化单位部门、地址、邮政编码、E-mail地址、联系电话。摘要与关键词用5号宋体字。页边距上：3cm；左、右、下：2.5cm。稿件须提交打印稿和电子版两种形式，电子版请发送至yg1623402@163.com，打印稿请按上述联系地点寄送。(4) 打印稿须加盖单位公章，论文如属省部级以上科研基金课题，请予注明。(5) 论文截止日期：2009年8月31日(以邮戳日期为准)。经评审录用的论文将收入大会学术论文集，会议期间颁发论文证书，参会者可获得国家继续教育Ⅰ类学分。(6) 会议进行青年优秀论文竞赛活动，设一、二、三等奖，并对获奖的论文颁发证书以资鼓励。

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