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Original Article

Inhibitory effect of 5F on development of lung cancer in A/J mice

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Abstract: The purpose of the study is to investigate the effect of ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F) on the model of induced A/J mice lung cancer in A/J mice. The expressions of tumor-related molecules including P65 and Bcl-2 at protein level were examined using the immunohistochemical method (IHC). Side effects of 5F were also monitored. The results indicated that 5F significantly suppressed the development of B[a]P and NNK-induced lung cancer in vivo by facilitating cell apoptosis with minimal side effects. Compared to the expressions of P65 and Bcl-2 in model group, the levels were strongly attenuated both in blank and 5F injection groups. Moreover, P65 and Bcl-2 levels varied among different groups receiving 5F treatment. The expressions of P65 and Bcl-2 were much lower in groups receiving high-concentration 5F treatment than those with low-concentration 5F injection. Findings revealed that 5F inhibited the pathogenesis of lung cancer through accelerating apoptosis in a dose-dependent manner.

Keywords: 5F, A/J mice, induced lung cancer, suppression

Introduction

Lung cancer has become the main cause of death in China and accounts for 22.7% of death in all malignant tumors. Increasing morbidity and mortality of lung cancer will bring more and more threats to human life and property security. Smoking is closely associated with the development of lung cancer, which has been demonstrated by lots of epidemiological studies, and smokers are getting younger and younger. It is important for researchers both in the domestic and overseas to prevent and treat lung cancer using new drugs, to look for effective constituents of anti-cancer medicine and to explore in the fields of chemistry, pharmacology and clinic.

Previous studies find that 5F has good anti-tumor effects both in vivo and vitro. 5F, a compound possessing a structure of α, β-methylene cyclopentanone, manifests anti-tumor activity by binding and inactivating mercapto enzyme [1]. Studies demonstrate that 5F can promote the apoptosis of many malignancies in vitro including non-small cell lung cancer, nasopharynx cancer and gastric cancer [2]. Studies show that there are specific binding sites of NF-κB in the promoter of Bcl-2 gene. Bcl-2 is considered as an important gene at the downstream of NF-κB. The mechanism of NF-κB in facilitating tumorgenesis may be its functions in inhibiting cell apoptosis and accelerating oncogenesis by regulating the expression levels of anti-apoptosis-related indicators (Bcl-2).

Smoking is recognized as the main factor in lung cancer genesis. B[a]P and NNK are the major carcinogens in cigarette smog. A/J mice lung cancer model constructed through the abduction of B[a]P and NNK is the preferred pattern for lung cancer study in human (especially cancers caused by smoking) due to great similarities in forming mechanism of lung cancer between human and A/J mice [3, 4]. Therefore, our study is conducted to explore antineoplastic activity of 5F in vivo using induced lung cancer model in A/J mice, whose growth is much closer to that of human tumor. However, the mechanism of 5F for repressing

Table 1. Animal groups and drug dose

Groups	Doses
Blank	No treatment
Model	NNK+ B[a]P
Positive control	NNK + B[a]P + 5-Fu 25 mg/kg
5F-L	NNK + B[a]P + 5F 25 mg/kg
5F-M	NNK + B[a]P + 5F 50 mg/kg
5F-H	NNK + B[a]P + 5F 75 mg/kg
Positive + 5F-M	NNK ++ B[a]P + 5F 50 mg/kg + 5-Fu 25 mg/kg

induced tumor (especially lung cancer induced by smoking) is still unclear in vivo at present.

Materials and methods

Animals and experiment reagents

A/J mice (6 weeks old with a weight range from 18 to 20 g) were provided by Guangdong Medical College. Mice with related infectious diseases or any peromely were excluded from the present study. Benzo[a]pyrene (B[a]P) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were purchased from Sigma company (the United States), and dissolved in olive oil in advance. 5-Fu was obtained also from Sigma. 5F was purified by Guangdong Medical College according to the previous description [5, 6].

Construction of induced lung cancer model and treatment groups

A/J mice were randomly divided into seven groups. Intragastric administration was conducted on all mice using mixed revulsant of 3 μmol B[a]P and 3 μmol NNK. The aforementioned operation was performed once per week and continued for 8 weeks. 5F and 5-Fu was constantly injected into mice from the ninth week until the fourteenth week. Different treatments on seven groups were detailed in **Table 1**. The operations of our study all accorded with the ethical guidelines of Guangdong Medical College.

Determination of 5F side effects

The King Hawk Pharmaceutics were performed to measure blood urea nitrogen (BUN), serum creatinine (SCR), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) for showing the side effects of 5F. The reference ranges of BUN, SCR, ALT and AST were 1.8-7.1 mmol/L, <106 μmol/L, <40 U/L and <37 U/L respectively [7].

Immunohistochemical assay

The next day after all treatment, mice were killed and their blood were obtained from posterior vena cava using a direct venipuncture method. The immunohistochemical staining was performed in accordance with the following steps: fixing and incising lung into 4 μm sections which were deparaffinized, rehydrated and experienced the treatment of antigen retrieval, antibody incubation and staining according to the previous description [8].

Statistical analysis

The data were expressed as mean ± SD and statistically analyzed using SPSS 20.0 software. Immunohistochemistry scores were compared using Mann-Whitney *U* test. A one-way ANOVA was used to determine the significant differences among seven groups in the study. Student's *t* test was employed to analyze biochemical tests.

Results

Acute toxic effect

In the study, mice were injected with 5F, and expressed no abnormal behavior like self-quarantine, self-torture and activity decrements. Renal and liver function tests demonstrated that the concentrations of BUN, SCR, AST and ALT in all groups were within aforementioned normal ranges of reference. The result showed that 5F did not cause major side effects, such as liver dysfunction and renal insufficiency in lung tumor, revealing that 5F was not a toxicologically lethal factor.

P65 and Bcl-2 analysis

A/J mice with induced lung cancer were injected with 5-Fu (25 mg/kg), 5F (densities of 25 mg/kg, 25 mg/kg and 25 mg/kg, respectively), and mixture (5F: 50 mg/kg and 5-Fu: 25 mg/kg) for 24 hours. Immunohistochemistry (IHC) was used to analyze the expressions of Bcl-2 and P65 at their protein levels. Our studies found that the quantities of Bcl-2 and P65 were significantly decreased in the groups receiving

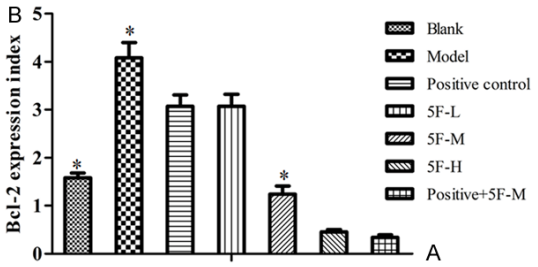

Figure 1. Expression of Bcl-2 at protein level in different groups with lung cancer. A. Immunohistochemical staining results showed that the stainings of Bcl-2 were strong, moderate and weak in model, low 5F dose and high 5F dose groups respectively (high magnification, ×400). B. The expression levels of Bcl-2 were lower in groups receiving 5F treatment than in model one.

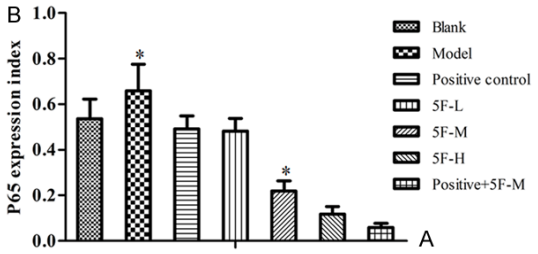

Figure 2. The expression levels of P65 protein were different among seven groups using immunohistochemical staining. A. Findings displayed that P65 staining in model, low 5F dose and high 5F dose groups presented to be strong, moderate and weak respectively (high magnification, ×400). B. In groups receiving 5F treatment, the expressions of P65 manifested a trend of decline.

5F treatment and blank group compared with model group with cancer, which revealed that 5F could obviously suppress the development of lung cancer via reducing the levels of Bcl-2 and P65 (**Figures 1** and **2**).

Dose-dependent effects of 5F on suppressing lung cancer

The results displayed that the expression levels of Bcl-2 and P65 were apparently higher in groups receiving 5F with concentration of 25 mg/kg, 50mg/kg and 75mg/kg respectively compared with the mice receiving NNK+ B[a]P only. The findings revealed that 5F could independently act as an inhibitor of lung cancer. It was also noted that higher concentration of 5F in mice treatment presented better tumoricidal effect. Therefore, the influence of 5F on the repression of lung cancer depended on its concentration (**Figures 1** and **2**).

Discussion

Lots of reports indicate that 5F is a strong anticancer agent in malignancies including hepatocellular cancer, lung cancer and thyroid cancer [9-11], because 5F can promote the apoptosis in vivo. It is regarded as the optimal strategy and focus point in malignancy treatment to kill cancer cells via apoptosis [12, 13].

NF-κB is a group of transcriptional regulation factors in almost all cells which can specifically bind with NF-κB site in the promoter or enhancer of genes to start gene transcription. NF-κB contains P50 and P65 which possesses transcriptional activity. Studies find that a variety of carcinogenic factors accelerate cells growth, resist apoptosis, make cells transform into malignancies and promote the transfer of tumor cells by activating NF-κB. Hence, it will be a new target in oncotherapy to inhibit the activity of NF-κB [14-16]. Results show that P65 is significantly reduced after 5F injection, which correspond to reports that the mechanism of 5F in abducting apoptosis is operated via inhibiting signal pathway of P65.

We measured the expression of Bcl-2, which might regulate the permeability of mitochondrial outer membrane, to further illuminate possible mechanism of 5F in inducing the apoptosis of lung cancer. Bcl-2 protein plays important roles in cell apoptosis mediated by mitochondrial pathway. Bcl-2 is an inhibitor of apoptosis and frequently used as a prognostic biomarker for cancers in clinical practice [17]. However, the up-regulation of Bcl-2 is associated with the poison tolerance of cells [18, 19]. In our present study, the expression levels of Bcl-2 were obviously reduced in the groups receiving 5F treatment and blank group compared to model group with lung cancer, which demonstrated that 5F could inhibit the development of lung cancer.

In the study, 5F had a dose-dependent effect on the inhibition of induced lung cancer. Our findings exhibited that the expressions of Bcl-2 and P65 were decreased in groups receiving 5F injection with doses of 25 mg/kg, 50 mg/kg and 75 mg/kg respectively compared with ones receiving NNK+ B[a]P only. The findings suggested that 5F could be independently considered as a suppressor for lung cancer. It was also noted that higher dose of 5F treatment in mice presented better tumoricidal effect.

In conclusion, lower expression of P65 indicate that 5F can inhibit the pathogenesis of induced lung cancer in A/J mice. Attenuated Bcl-2 levels reveal that 5F can facilitate the apoptosis of internal cells in lung cancer by mitochnodria pathway. Consequently, 5F plays a part in the repression of induced lung cancer.

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Disclosure of conflict of interest

None.

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