Original Article

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Xanthii fructus inhibits malignant behaviors of lung cancer cells

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Abstract

Objective: This study aimed to investigate the influence of *Xanthii fructus* on the expression of small noncoding RNA (sncRNA) and the malignant behaviors of lung cancer cells.

Method: A549 cells were treated with *Xanthii fructus* extract. SncRNA expression was detected by real-time PCR. Proliferation, anchorage-independent growth, and invasion capacities were determined using Cell Counting Kit (CCK)-8, soft agar colony formation, and Matrigel assays, respectively.

Results: *Xanthii fructus* extract downregulated microRNA (miR)-21 expression and upregulated PIWI-interacting RNA (piRNA)55490 expression. The proliferation, anchorage-independent growth, and invasion capacities of A549 cells were strongly inhibited by the extract.

Conclusion: *Xanthii fructus* can inhibit the malignant behaviors of lung cancer cells.

Keywords: *Xanthii fructus*, lung carcinoma, noncoding RNA

1 Introduction

Lung cancer is the leading cause of cancer death globally [1,2]. The initiation of lung cancer is insidious, generally exhibiting no obvious symptoms during the early stages of development. Many patients are diagnosed at the mid-to-late stages, wherein the opportunity for surgery has already been lost [3,4]. Routine chemotherapy drugs aim to kill tumor cells. However, they are extremely toxic and are related with high recurrence and metastasis rates [5]. Therefore, it is imperative that we apply innovative ideas and approaches to develop effective and nontoxic drugs for combating lung cancer. These drugs will not only be representative of the Chinese society but also be a great contribution to the entire human society.

Traditional Chinese medicine (TCM) has accumulated extensive and valuable clinical experiences, particularly in tumor treatment, and it has formed unique and integral theoretical systems. The meridian tropism theory in TCM provides a convenient guideline for developing new drugs for lung cancer therapy. Among the many drugs distributing along the lung meridian, the *Xanthii fructus* is a commonly used herb in TCM for clearing heat and removing toxicity, as well as for eliminating carbuncles and scrofula. Therefore, it has considerable research value [6].

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Small noncoding RNAs (sncRNAs) are important regulatory molecules in organisms. The abnormal expression of some sncRNAs in lung cancer tissues and cells leads to inactivation of tumor suppressor genes, activation of oncogenes, and loss of genome stability, which promotes the malignant behaviors of cancer cells, including proliferation, invasion, and metastasis [7]. Correcting the abnormal expression of sncRNAs in lung cancer cells is a novel effective pathway for lung cancer therapy [8].

In this study, we selected the Chinese herb *Xanthii fructus* to treat human lung cancer cells and found that it can strongly inhibit the malignant behaviors of lung cancer cells by downregulating the expression of microRNA (miR)-21, an oncogenic sncRNA, and upregulating the expression of PIWI-interacting RNA (piRNA)55490, a tumor-suppressive sncRNA.

2 Materials and methods

2.1 Cells

The human lung cancer cell line A549 was purchased from the cell bank of Nanjing Kaiji Co. Ltd. (Nanjing, Jiangsu).

2.2 Herb

The crude drug of *Xanthii fructus* was purchased from the Inner Mongolia Hospital of Traditional Chinese Medicine (Huhhot, Inner Mongolia).

2.3 Reagents

Fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), RPMI-1640 medium (Gibco), trypsin (Gibco), PBS (Gibco), Cell Counting Kit (CCK)-8 (Amresco, Cleveland, Ohio, USA), Matrigel (BD Biosciences, San Jose, CA, USA), crystal violet (Guo Yao, Shanghai, China), low-melting-point agarose (Santacruz Biotechnology, SantaCruz, CA, USA), RNA EasyPure miRNA Extraction Kit (Transgen, Beijing, China), and fluorescence quantitative reverse transcription PCR TransScript Green miRNA Two-Step qRT-PCR SuperMix kit (Transgen, Beijing, China) were used in the study.

2.4 Instruments

Carbon dioxide incubator (Thermo Fisher Scientific), super clean bench (Suzhou Jin Yan), inverted microscope (TE2000; Nikon), real-Time PCR system (7500, Applied Biosystems), and 8 μ m Transwell device (Costar) were used.

2.5 Methods

2.5.1 Preparation of *Xanthii fructus* extract

The dried crude drug of *Xanthii fructus* was crushed into powder form and extracted using 75% ethanol three times, with each step lasting 30 min. The extracts were filtered and combined. The ethanol was recovered to obtain a thick paste, which was then freeze-dried to obtain the dry powder. The dry powder was dissolved in phosphate-buffered saline (PBS) to prepare a 80 mg/ml stock solution, which was sterilized using a 0.22- μ m filter and stored at 4°C until application.

2.5.2 Culture of lung cancer cells

A549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 0.1% penicillin-and-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The cells in logarithmic growth phase were used.

2.5.3 Grouping

In the extract-treated group, the stock solution was added to cell culture medium or cell suspension to treat the cells at drug concentrations of 5 mg/ml, 10 mg/ml, and 15 mg/ml. In the control group, the same volume of PBS was added to the cell culture medium or cell suspension.

2.5.4 Detection of sncRNA expression

The A549 cells were treated with the extract for 24 h. Then, the cells were collected and total RNA was isolated using the EasyPure miRNA Kit. Reverse transcription and real-time PCR were performed with the TransScript Green miRNA Two-Step qRT-PCR SuperMix according to the operation instructions in the kit. The cycling conditions for real-time PCR were as follows: 94°C for 30 s, and 40 cycles of 94°C for 5 s and 60°C for 34 s. Relative expression levels of each small RNA were calculated with the $2^{-\Delta\Delta C_t}$ method using U6 as the endogenous control.

2.5.5 Determination of proliferation capacity

Cells were suspended in RPMI-1640 medium at a concentration of 1.5×10^4 cells/ml, and 0.2 ml of the suspension was added into each well of 96-well plates. After 12 h culture, the cells were treated with different concentrations of *Xanthii fructus* extracts (5 mg/ml, 10 mg/ml, and 15 mg/ml) or PBS. After treatment for 48 h, 20 µl CCK-8 solution was added to each well. Then, the plates were continuously incubated for 2 h in a humidified CO₂ incubator at 37°C. Finally, the absorbance of the sample taken from each well was measured using a microplate reader at 450 nm.

2.5.6 Determination of anchorage-independent growth capacity

A 0.6% (wt/vol) solution of low-melting-point agar was prepared in normal medium, placed in six-well culture plates, and allowed to solidify. Cells were suspended in RPMI-1640 medium containing 0.3% low-melting-point agar (10⁴/0.5 ml), and the extract was added into the suspension. Then, the suspension was covered on the lower agar layer. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 3 weeks. Colonies of sizes >100 µm were counted under a microscope.

2.5.7 Determination of invasion capacity

Matrigel was diluted with a precooled serum-free RPMI-1640 medium. The upper surfaces of the Transwell membranes were precoated with 100 µl diluted Matrigel, which was allowed to solidify at 37°C for 4 h. Cells were serum-starved for 24 h and suspended in serum-free RPMI-1640 medium (10⁶/ml). Every 150 µl of the cell suspension was seeded into each upper chamber, and 600 µl of RPMI-1640 containing 20% FBS was added into the lower chamber. The *Xanthii fructus* extract was added into the upper chambers. The plates were incubated for 24 h at 37°C, and then the media were removed from the Transwell chambers, and the cells on the upper surface of the Transwell membrane were wiped off. Cells that had migrated to the lower

surface of the Transwell membrane were fixed and stained with crystal violet, and the number of cells in five randomly selected fields at $\times 200$ magnification was counted.

2.5.8 Statistical analysis

Statistical analysis was performed with the SPSS19.0 software. One-way analysis of variance was used to analyze the data, and the significance level was set at $P < 0.05$.

3 Results

3.1 *Xanthii fructus* extract changed the expression of miR-21 and piRNA55490 in lung cancer cells

Figure 1 shows that the expression of miR-21 was higher and that of piRNA55490 was lower in the extract-treated A549 cells than in the control-treated A549 cells. These differences were particularly significant in the 10 mg/ml and 15 mg/ml groups. These results indicate that *Xanthii fructus* extract can effectively downregulate the expression of miR-21 and up-regulate the expression of piRNA55490 in the lung cancer cells.

3.2 *Xanthii fructus* extract inhibited the anchorage-independent growth capacity of lung cancer cells

Figure 2 shows that the extract-treated cells proliferated more slowly than the control-treated cells. This result indicates that *Xanthii fructus* extract can effectively inhibit the proliferation capacity of lung cancer cells.

3.3 *Xanthii fructus* extract inhibited the anchorage-independent growth capacity of lung cancer cells

Table 1 shows that the extract-treated cells formed considerably less colonies in soft agar medium than the control-treated cells. This decrease was particularly significant in the 10 mg/ml and 15 mg/ml groups.

Fig. 1: Effect of *Xanthii fructus* extract on the sncRNA expression in lung cancer cells. ** $P < 0.01$ vs. the control group.

This result indicates that *Xanthii fructus* extract can effectively inhibit the anchorage-independent growth capacity of lung cancer cells.

3.4 *Xanthii fructus* extract inhibited the invasion capacity of lung cancer cells

Table 2 shows that the cells that had penetrated through the Matrigel were substantially less in the extract-treated groups than in the control group. This decrease was particularly significant in the 10 mg/ml and 15 mg/ml groups. This result indicates that *Xanthii fructus* extract can effectively inhibit the invasion capacity of lung cancer cells.

Fig. 2: Effect of *Xanthii fructus* extract on the proliferation capacity of lung cancer cells. * $P < 0.05$, ** $P < 0.01$ vs. the control group.

Tab. 1: Effect of *Xanthii fructus* extract on the anchorage-independent growth capacity of A549 cells

Groups	Number of colonies
Control	166±13
Extract concentration	
5 mg/ml	126±9**
10 mg/ml	83±8**
15 mg/ml	33±8**

** $P < 0.01$ vs. control group.

Tab. 2: Effect of *Xanthii fructus* extract on the invasion capacity of A549 cells

Groups	Invasive cells
Control	177±12
Extract concentration	
5 mg/ml	153±8**
10 mg/ml	111±4**
15 mg/ml	67±7**

* $P < 0.05$ vs. control group; ** $P < 0.01$ vs. control group.

4 Discussion

miRNAs constitute an important class of sncRNAs, with lengths of about 22 nucleotides. They can recognize the 3'-untranslated regions of the mRNA of their target genes and downregulate target gene expression by inhibiting target mRNA translation or inducing target mRNA degradation through the action of RNA-induced silencing complex [9]. The target genes of some miRNAs are key tumor suppressor genes; the abnormal upregulation of these miRNAs can silence the expression of key tumor suppressors, greatly accelerating the initiation and progression of malignant tumors [10]. MiR-21 has been reported to be one of the most prominent miRNAs implicated in the genesis and development of many human malignancies, including lung carcinoma [11]. The overexpression of miR-21 in lung cancer cells promotes their migration, invasion, and chemoresistance by inhibiting the tumor suppressor PTEN or enhancing the activity of the Ras/MEK/ERK pathway [11].

PiRNA is a class of sncRNAs discovered in recent years, with a length of 25–31 nucleotides. They play important roles in regulating transposable element activities, chromatin integrity, DNA methylation, and mRNA stability [12]. It has been found in multiple cancers, including lung cancer [13], gastric carcinoma [14], and breast cancer [15], that the expression of some piRNAs was deregulated, which either silences the expression of tumor suppressor genes by inducing hypermethylation at their promoters or enhances the translation of oncogene mRNAs by considerably increasing their stability [16]. These effects seriously promote the initiation and progression of tumors. Moreover, piRNA55490 has been found to be downregulated in lung cancer tissues, and the downregulation results in accelerated growth and proliferation of lung cancer cells [17].

Xanthii fructus is a commonly used herb distributing along the lung meridian and it is often used to treat lung-related illnesses. In this study, we selected *Xanthii fructus* to treat human lung cancer cells and found that it effectively reduced the expressions of the oncogenic sncRNA miR-21 and increased the expression of the tumor-suppressive sncRNA piRNA932. Consequently, the proliferation, anchorage-independent growth, and invasion capacities of the lung cancer cells were effectively suppressed. These results demonstrated the excellent anti-lung cancer activity of *Xanthii fructus* at the molecular and cellular levels.

So far, no drugs that regulate the expression of tumor-suppressive piRNAs have been reported. This is the first study to validate the effectiveness of *Xanthii fructus*, a traditional Chinese herb, in upregulating the expression of a tumor-suppressive piRNA in lung cancer cells. This finding not only shows the wide prospect of developing antitumor drugs from traditional Chinese herbs but also provides a new class of molecular targets for exploring the action mechanisms of antitumor drugs.

"TCM is a vast treasure trove that needs to be explored and exploited." This was the principal ideology and foresight adopted by the team of Tu You-You, who later achieved huge breakthroughs that benefited mankind for many years. These achievements indicate the expansiveness and extensiveness of TCM, in addition to validating the correctness of the theoretical systems of TCM. Inspired by Tu, we adopted the theoretical framework of drug meridian tropism of TCM to analyze the effects of *Xanthii fructus* on lung cancer cells. The analysis results obtained in this study were consistent with our speculations, confirming that TCM is indeed a vast treasure trove of knowledge and a valuable source of wisdom for Chinese people.

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