Fangchinoline inhibits the proliferation of SPC-A-1 lung cancer cells by blocking cell cycle progression

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Abstract. Fangchinoline (Fan) is a bioactive compound isolated from the Chinese herb *Stephania tetrandra* S. Moore (Fen Fang Ji). The aim of the present study was to investigate the effect of Fan on the proliferation of SPC-A-1 lung cancer cells, and to define the associated molecular mechanisms. Following treatment with Fan, Cell Counting Kit-8, phase contrast imaging and Giemsa staining assays were used to detect cell viability; flow cytometry was performed to analyze the cell cycle distribution; and reverse transcription-quantitative polymerase chain reaction and western blot assays were used to investigate changes in the expression levels of cell cycle-associated genes and proteins. In the present study, treatment with Fan markedly inhibited the proliferation of SPC-A-1 lung cancer cells and significantly increased the percentage of cells in the G_0/G_1 phase of the cell cycle in a dose-dependent manner ($P<0.05$ for 2.5-5 μm; $P<0.01$ for 10 μm), whereas the percentage of cells in the S and G_2/M phases were significantly reduced following treatment ($P<0.05$ for 5 μm; $P<0.01$ for 10 μm). Mechanistically, Fan significantly reduced the mRNA expression levels of cyclin D1, cyclin-dependent kinase 4 (CDK4) and CDK6 ($P<0.05$ for 2.5-5 μm; $P<0.01$ for 10 μm), which are key genes in the regulation of the G_0/G_1 phase of the cell cycle. Furthermore, treatment with Fan also decreased the expression of phosphorylated retinoblastoma (Rb) and E2F transcription factor-1 (E2F-1) proteins ($P<0.05$ for 5 μm; $P<0.01$ for 10 μm). In summary, the present study demonstrated that Fan inhibited the proliferation of SPC-A-1 lung cancer cells and induced cell cycle arrest at the G_0/G_1 phase. These effects may be mediated by the downregulation of cellular CDK4, CDK6 and cyclin D1 levels, thus leading to hypophosphorylation of Rb and subsequent suppression of E2F-1 activity. Therefore, the present results suggest that Fan may be a potential drug candidate for the prevention of lung cancer.

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Introduction

Non-small cell lung cancer (NSCLC) is among the most malignant types of tumor, with the highest incidence and mortality rates of any cancer variety worldwide. It has been reported that there were 239,320 new cases of lung cancer and 161,250 cases of mortality from lung cancer in the USA in 2010 (1). Although surgical excision, chemotherapy, radiation and targeted therapy have been applied to the treatment of lung cancer, the five-year survival rate remains at ~15.6%; thus, improved therapies for the treatment of NSCLC are urgently required (1,2). Traditional Chinese herbs are considered to be a good source for the identification of novel anti-cancer agents (3).

Fangchinoline (Fan) is a bioactive compound isolated from the *Stephania tetrandra* S. Moore (Fen Fang Ji) Chinese herb. Various studies have demonstrated that Fan possesses a wide range of biological activities, including: Blood pressure lowering activity (4), histamine release inhibition (4), aortic vascular smooth muscle cell proliferation suppression (5), anti-oxidative stress (6) and antihypertensive activity (7). Furthermore, the anti-cancer activity of Fan has been indicated in various tumor cell models, including in cancer of the prostate (8), breast (9,10) and liver (11), as well as leukemia (12). The molecular mechanisms of its anti-cancer activity include the induction of apoptosis, autophagy and cell cycle arrest; however, there is little information regarding the effect of Fan on NSCLC cells. In the present study, the antitumor effects of Fan and the associated molecular mechanisms were explored in NSCLC cells. Treatment with Fan stimulated cell cycle arrest at the G_0/G_1 phase in SPC-A-1 NSCLC cells via downregulation of cyclin-dependent kinase 4 (CDK4), CDK6 and cyclin D1, which subsequently repressed the expression of phosphorylated retinoblastoma protein (pRB) and E2F transcription factor-1 (E2F-1) . Therefore, the results of the present study suggest that Fan may potentially be useful in the prevention and treatment of NSCLC.

Materials and methods

Cell culture and agents. Human SPC-A-1 lung cancer cells (Cell Bank of the Chinese Academy of Sciences, Shanghai,

China) were cultured in Dulbecco's modified Eagle medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Sangon Biotech Co., Ltd., Shanghai, China). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C. Fan (purity, >98.0%; Nature Standard Ltd., Shanghai, China) was prepared as a 50 mM stock solution in dimethyl sulfoxide (DMSO), prior to supplementation into the medium at various concentrations, for 48 or 72 h.

Cell Counting Kit-8 (CCK-8) assay. Cells were grown in 96-well culture plates and treated with various dosages of Fan (1.25, 2.5, 5, 10, 20 and 40 μM), as required, prior to incubation with 10 μl CCK-8 for 2 h. Following this, a Model 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure the optical density (OD) of the samples at a wavelength of 450 nm. The cell inhibitory rate (IR) was calculated, as follows: $IR = [1 - (OD_{experiment} - OD_{blank}) / (OD_{control} - OD_{blank})] \times 100\%$.

Cell imaging. Following treatment with Fan, phase contrast imaging and Giemsa staining assays were used to analyze the proliferation of SPC-A-1 lung cancer cells. SPC-A-1 cells were treated with various concentrations of Fan (0, 2.5, 5 and 10 μM) and, after 48 h, the cells were visualized under an inverted microscope (CKX41; Olympus, Tokyo, Japan) prior to staining with a Giemsa assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions. In brief, the cells were fixed with the included solution I for 1 min and then solution II was added to stain the cells for another 5 min. Subsequently, the solution was removed and the images of cells were obtained using the Olympus CKX41 microscope.

Flow cytometry analysis. SPC-A-1 cells were cultivated in a 6-well plate for 24 h, prior to treatment with Fan (0, 2.5, 5 or 10 μM) or equal volumes of DMSO. Following 48 h incubation, the cells were collected, fixed in 70% ice-cold ethanol (Sangon Biotech Co., Ltd.) and maintained at 4˚C overnight. Cells were then washed in phosphate-buffered saline and the resultant pellet was re-suspended in 200 μg/ml RNase (Sangon Biotech, Co. Ltd.) for 1 h at 37˚C. Cells were subsequently stained with 50 μg/ml propidium iodide, and analyzed using a flow cytometer (FACSCalibur; Beckman Coulter, Inc., Fullerton, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. SPC-A-1 cells were treated with various concentrations of Fan for 48 h and the mRNA expression levels of genes that regulate the cell cycle were examined. Cells were collected and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using a RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) with 3 μg total RNA, random hexamers (Fermentas; Thermo Fisher Scientific, Inc.) and specific oligonucleotide primers to detect the expression levels of cyclin D1, CDK4 and CDK6 mRNA. The sequences of the primer pairs were as follows: Cyclin D1, forward 5'-ATGCTGGAGGTCTGCGAGGA-3' and reverse 5'-TTCGATCTGCTCCTGGCAGG-3'; CDK4, forward 5'-TGGCTTTACTGAGGCGACTG-3' and reverse 5'-ACGGGTGTAGTGCCATCTG-3'; CDK6, forward 5'-GGAGTGCCCACTGAAACCAT-3' and reverse 5'-GTGAGACAGGGCACTGTAGG-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-GAGAAGGCTGGGGCTCATTT-3' and reverse 5'-GTCAGGTCCACCACTGACAC-3'. GAPDH was used as an internal control. PCR was performed at a final reaction volume of 25 μl, containing 1 μl cDNA, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, 0.2 mM dNTP and 20 pM of each gene-specific oligonucleotide primer. The PCR reaction conditions were as follows: Denaturation at 94˚C for 30 sec, annealing at 52-56˚C for 30 sec, and extension at 72˚C for 45 sec. The amplified products were run on 1.5% agarose gel and documented using a Gel Doc XR+ system (Bio-Rad Laboratories, Inc.). The densitometric analysis of the RT-qPCR results was performed using Quantity One software, version 4.6.0 (Bio-Rad Laboratories, Inc.) using GAPDH for normalization.

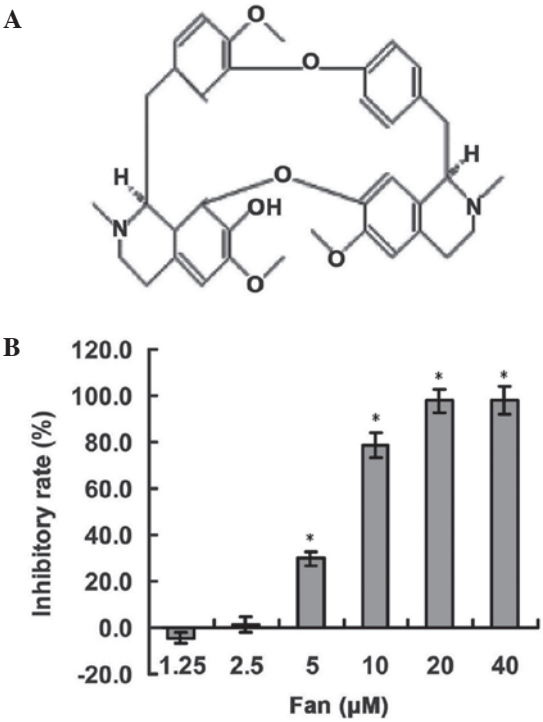

Figure 1. Growth inhibition effect of SPC-A-1 cells induced by Fan. (A) Chemical structure of Fan. (B) SPC-A-1 cells were treated with the indicated concentrations of Fan or an equal volume of the dimethylsulfonide drug vehicle for 72 h. Viable cells were evaluated by Cell Counting Kit-8 assay and the inhibitory rate was calculated. Error bars indicate the mean ± standard deviation (n=3). *P<0.01. Fan, fangchinoline.

Western blot analysis. SPC-A-1 cells were cultivated in 6-well plates for 24 h, prior to treatment with Fan (0, 2.5, 5 and 10 μM) or DMSO (0.02%) for 48 h. Protein expression was detected using 10% SDS-PAGE at 250 V for 90 min. Subsequently, 20-30 μg total protein was transferred to polyvinylidene difluoride membranes and the membranes were blocked for 60 min with freshly prepared 5% non-fat milk in Tris-buffered saline and Tween-20 (TBST). Following this, the membranes were incubated with rabbit monoclonal pRb (1:1,500; #8180), polyclonal E2F-1 (1:2,000; #3742)

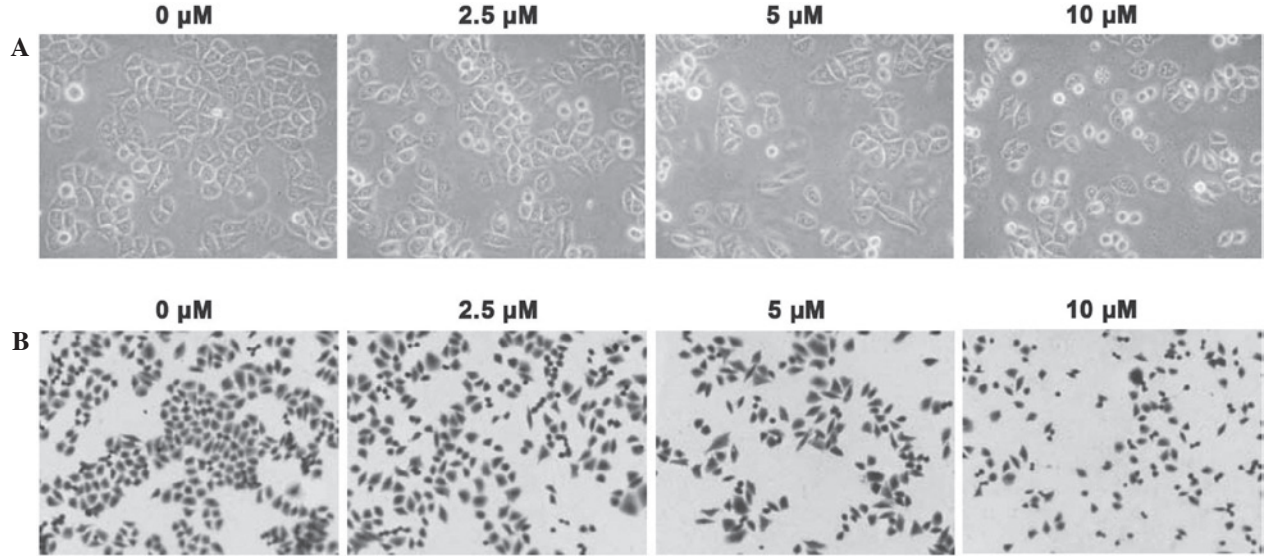

Figure 2. Proliferation of Fangchinoline (Fan)-inhibited SPC-A-1 cells (magnification, x200). (A) SPC-A-1 cells were treated with the indicated dosages of Fan for 48 h and visualized under a phase contrast microscope. (B) SPC-A-1 cells were incubated with Fan and stained with Giemsa staining, prior to visualization under a microscope.

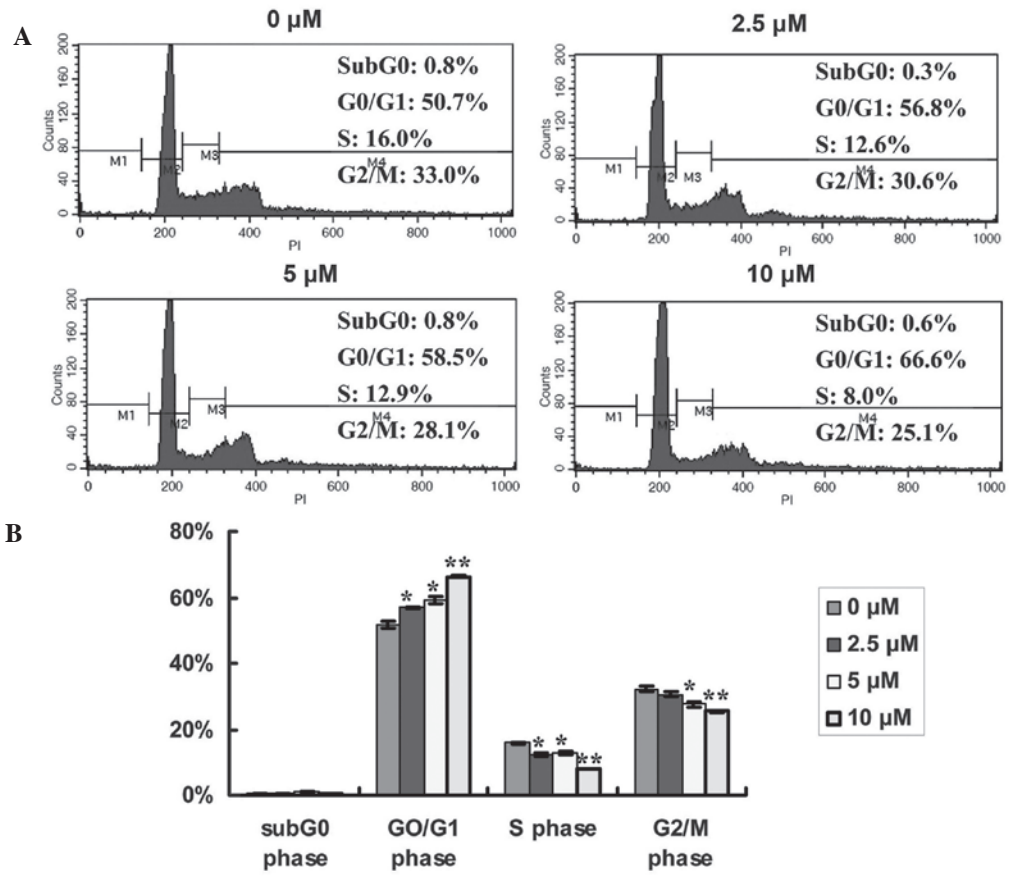

Figure 3. Fangchinoline (Fan)-induced SPC-A-1 cell cycle arrest. (A) SPC-A-1 cells were treated with the indicated concentrations of Fan for 48 h. Cell numbers were detected during the subG$_0$, G$_0$/G$_1$, S, and G$_2$/M cell cycle phases using PI staining and flow cytometry. (B) Histogram of the respective rates of subG$_0$, G$_0$/G$_1$, S and G$_2$/M phase SPC-A-1 cell proliferation, determined via fluorescence-activated cell sorting analysis (n=3). *P<0.05; **P<0.01 vs. control. PI, propidium iodide.

and GAPDH (1:4,000; #5174; Cell Signaling Technology, Inc., Danvers, MA, USA) primary antibodies, washed three times with TBST, and incubated with goat anti-mouse or goat anti-rabbit IgG-horseradish peroxidase-conjugated antibodies (1:4,000; #32260; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h. Protein bands were revealed using a ECL Plus Western Blotting Detection System kit (GE Healthcare Life Sciences, Roosendaal, The Netherlands), with GAPDH used

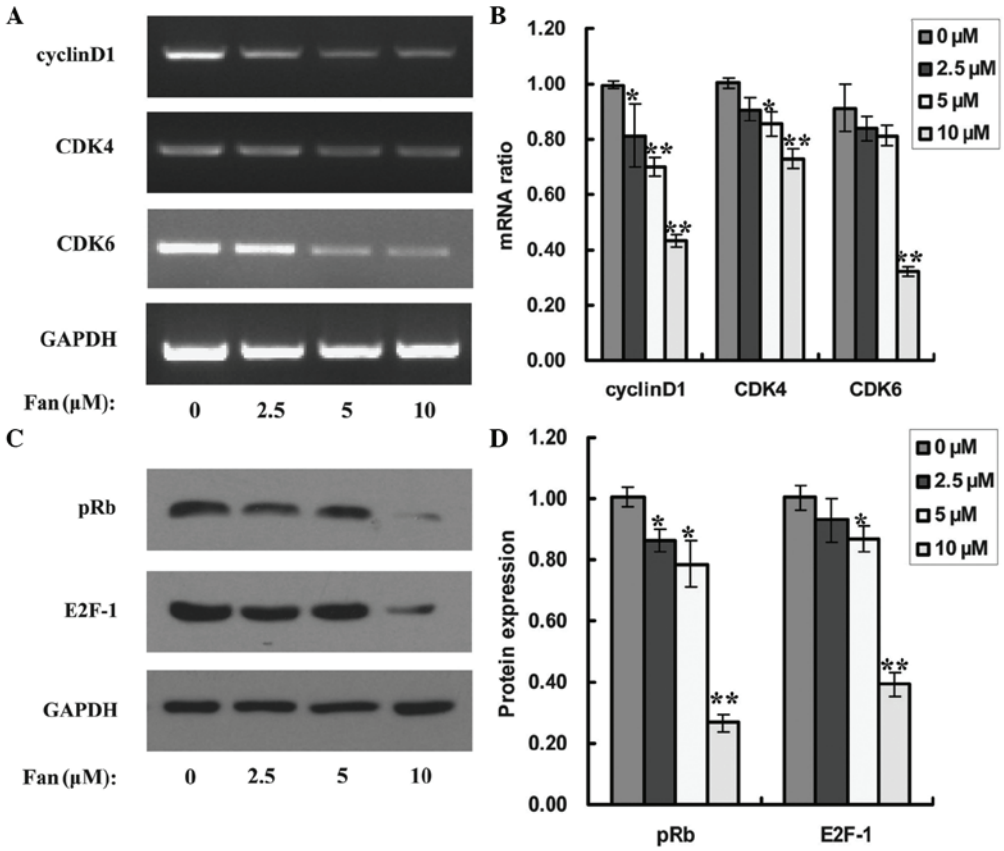

Figure 4. Expression levels of cell cycle-related genes and proteins affected by Fan in SPC-A-1 cells. (A) SPC-A-1 cells were treated with the indicated concentrations of Fan for 48 h and the mRNA expression levels of cyclin D1, CDK4, and CDK6 were detected using a reverse transcription-quantitative polymerase chain reaction. (B) The ratios of cyclin D1, CDK4 and CDK6 mRNA expression levels were calculated. (C) SPC-A-1 cells were treated by the indicated concentrations of Fan for 48 h and the expression levels of pRb and E2F-1 proteins were detected via western blot analysis. (D) The ratio of pRb and E2F-1 proteins was calculated. Error bars denote the mean ± standard deviation (n=3). *P<0.05; **P<0.01 vs. control. CDK4, cyclin-dependent kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Fan, fangchinoline; pRb, phosphorylated retinoblastoma protein; E2F-1, E2F transcription factor-1.

as a loading control. Densitometric analysis of the western blot was performed using Quantity One software, version 4.6.0 (Bio-Rad, Laboratories, Inc., USA), with GAPDH used for normalization.

Statistical analysis. All cellular experiments were performed at least three times. Data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS 14.0 for Windows (SPSS, Inc., Chicago, IL, USA). Experimental and control groups were compared using the unpaired Student's t-test and one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Fan inhibits the proliferation of SPC-A-1 lung cancer cells. To assess the inhibitory effect of Fan (Fig. 1A) on the growth and survival of lung cancer cells, human SPC-A-1 lung cancer cells were treated with Fan at concentrations of 1.25, 2.5, 5, 10, 20 and 40 μM for 72 h, using a CCK-8 assay. As shown in Fig. 1B, the proliferative inhibitory effect of Fan was observed in a concentration-dependent manner, with statistical significance (P<0.01 for 5-40 μm). The half-maximal inhibitory concentration value of Fan in SPC-A-1 cells at 72 h was 7.19 μM. Furthermore, phase contrast imaging and Giemsa staining assays were also performed to measure the inhibitory function of Fan treatment (Fig. 2A and B, respectively). Following treatment with 2.5, 5 or 10 μM Fan for 48 h, the total cell number and cell volume of the SPC-A-1 cells decreased in a dose-dependent manner, and morphological changes, such as membrane blebbing, were detected. Thus, Fan appears to inhibit the proliferation of SPC-A-1 lung cancer cells.

Fan induces cell cycle arrest of SPC-A-1 cells at the G_0/G_1 phase. To determine whether Fan-induced suppression of cell proliferation was associated with an alteration in cell cycle distribution, the dose-dependent effects of Fan on the cell cycle distribution of lung cancer cells were measured (Fig.3A and B). Following treatment with 2.5, 5 or 10 μM Fan for 48 h, the proportion of SPC-A-1 cells in the G_0/G_1 phase (56.86±0.19, P<0.05; 59.12±1.00, P<0.05; and 66.22±0.32%, P<0.01; respectively) significantly increased, compared with the control (51.84±1.06%); whereas the percentage of cells in the S phase significantly decreased from 15.78±0.17% (control), to 12.42±0.52 (P<0.05), 12.83±0.65 (P<0.05) and 7.96±0.05% (P<0.01), respectively. Furthermore, the proportion of SPC-A-1 cells in the G_2/M phase decreased in a dose-dependent manner from 32.16±0.81% (control) to 30.67±0.70 (P<0.05), 27.52±0.60 (P<0.05) and 25.38±0.29% (P<0.01), respectively. These results indicated

that Fan-induced inhibition of SPC-A-1 cell proliferation is cell cycle-dependent, and may result in the enhanced accumulation of cells in the G_0/G1 phase. A representative profile of the cell cycle distribution is outlined in Fig. 3.

Fan affects cell cycle-related gene and protein expression in SPC-A-1 cells. D-type cyclins, such as cyclin D1, and its partner kinases CDK4 and CDK6, are central mediators of the G1 phase transition (13). To examine whether the enhancement of G_0/G_1 phase arrest in Fan-treated SPC-A-1 cells was a result of the dysregulation of cell cycle-related genes, the mRNA expression levels of cyclin D1, CDK4 and CDK6 were analyzed. The administration of Fan repressed the expression of cyclin D1, CDK4 and CDK6 mRNAs (Fig. 4A). Fan concentrations of 2.5, 5 and 10 μM significantly inhibited cyclin D1 levels by 19 ($P<0.05$), 30 ($P<0.01$) and 57% ($P<0.01$), respectively, compared with no treatment (Fig. 4B); whereas CDK4 expression levels were inhibited by 9 ($P>0.05$), 14 ($P<0.05$) and 27% ($P<0.01$), respectively (Fig. 4B), and CDK6 expression levels were inhibited by 16 ($P>0.05$), 19 ($P>0.05$) and 68% ($P<0.01$), respectively (Fig. 4B).

The cyclin D1-CDK4/6 complexes formed during the G1 phase may phosphorylate Rb protein and activate a transcriptional factor, E2F-1 (14). Therefore, to determine whether Fan suppressed the expression of cyclin D1, CDK4 and CDK6 via inhibition of the pRB/E2F-1 signaling pathway, the expression levels of pRB and E2F-1 in Fan-treated SPC-A-1 cells were examined, using a western blot assay. As demonstrated in Fig. 4C, treatment with Fan significantly inhibited the expression of pRB protein, and at 2.5, 5 and 10 μM, the suppression rates were 14 ($P<0.05$), 21 ($P<0.05$) and 73% ($P<0.01$), respectively (Fig. 4D). Furthermore, Fan also significantly repressed the expression of E2F-1 protein, and the suppression rates were determined to be 7, 13 ($P<0.05$) and 61% ($P<0.01$) at 2.5, 5 and 10 μM, respectively (Fig. 4C and D).

Discussion

Previous studies have demonstrated that Fan is associated with various functions, including: Blood pressure lowering activity (4), the inhibition of histamine release (4), anti-oxidative stress (6) and anti-cancer activity (9-11). However, little is known about the effect of Fan on cell cycle arrest in cancerous cells. Various studies have shown that Fan induces cell cycle arrest at the G_0/G_1 phase in breast cancer and leukemia cells, by decreasing the expression levels of CDK4 and cyclin D1 (8,10,12). The present study demonstrated that, in SPC-A-1 lung cancer cells, Fan stimulated cell cycle arrest at the G_0/G_1 phase by downregulating the cellular levels of CDK4, CDK6 and cyclin D1, leading to the hypophosphorylation of Rb and the subsequent suppression of E2F-1 activity.

Cell proliferation is dependent on the progression of the cell cycle, which is composed of the G_1, S, G_2 and M phases. The transition from the G1 to S phase is critical, as it controls the subsequent progress of the cell cycle. In the present study, Fan inhibited the proliferation of SPC-A-1 lung cancer cells in a dose-dependent manner, with G_0/G_1 phase accumulation, and a decrease in S and G_2/M phase, demonstrating that Fan may have suppressed SPC-A-1 cell cycle initiation and blocked DNA synthesis. The G1 to S phase transition is tightly regulated by the activation of CDKs, which act consecutively in G1 to initiate the S phase, and in the G2 phase to initiate mitosis (15,16). Therefore, it is unsurprising that the G1 checkpoint is the most conspicuous target for various anti-cancer agents. D-type cyclins, cyclin E and CDK4/6, CDK inhibitors and pRB are the central players of G1 phase transition (15,17). Upon mitogenic stimulation, D-type cyclins, such as cyclin D1, are induced, and subsequently bind to and activate CDK4 and CDK6. These cyclin D-dependent kinases then initiate the phosphorylation of Rb, relieving the inhibition of E2F-1 and allowing for the expression of specific E2F-1 target genes (18). In the present study, Fan suppressed the expression of cyclin D1, CDK4 and CDK6, suggesting that Fan successfully blocked the cell cycle progression of SPC-A-1 lung cancer cells. Considering that previous studies have determined that the CDK4/6 complex phosphorylates Rb protein (18-20), it is logical that the administration of Fan may also have suppressed the phosphorylation of Rb. As a tumor suppressor protein, Rb may inhibit cancer cell proliferation via cell cycle arrest, as it is the hyperphosphorylation of Rb that induces Rb to dissociate from E2F-1 and subsequently promotes the G1 to S phase transition (19,20). In the present study, Fan inhibited the phosphorylation of Rb protein and E2F-1, which may have resulted from the Fan-induced inhibition of CDK4, CDK6 and cyclin D1.

In conclusion, the present study suggested that Fan promotes the cell cycle arrest of SPC-A-1 lung cancer cells at the G_0/G_1 phase by downregulating the cellular levels of CDK4, CDK6 and cyclin D1, leading to hypophosphorylation of Rb and subsequent suppression of the E2F-1 activity. Thus, the present results suggest that Fan may be a potential drug candidate for the prevention of lung cancer and have clinical applications in the future, and E2F-1 may be an effective target for consideration in anti-lung cancer drugs.

Acknowledgements

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