

Fangchinoline suppresses growth and metastasis of melanoma cells by inhibiting the phosphorylation of FAK

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Received December 12, 2016; Accepted May 22, 2017

DOI: 10.3892/or.2017.5678

Abstract. Melanoma is a malignant tumor with high degree of malignancy, metastasis and high mortality. The etiology of melanoma has not been fully elucidated, and there is no effective drug for the complete treatment of melanoma. In recent years, many traditional Chinese herbal medicines have played an important role in clinical treatment and experimental research on cancer. As a natural product, fangchinoline has the characteristics of enhancing immune function, low toxicity and good liver protection features, so it is considered to be a new type of anticancer drug. In the present study, we found that fangchinoline has inhibitory effects on the proliferation and metastasis of A375 and A875 cells in a concentration-dependent manner. Fangchinoline inhibited the proliferation of A375 and A875 cell activity with IC_{50} values of 12.41 and 16.20 μ M. We also found that fangchinoline could significantly reduce the phosphorylation of Focal adhesion kinase (FAK). In summary, we demonstrated that fangchinoline inhibits the proliferation and metastasis of melanoma cells by suppressing FAK and its downstream signaling pathway. More importantly, we provide a novel mechanism that fangchinoline could be an effective candidate for the treatment of melanoma.

Introduction

Melanoma is a malignant tumor which originates from nerve endoderm. The malignant degree of melanoma is generally high, and the early stage of blood channel metastasis is especially prone to occur (1). The main features of melanoma

are: early onset age, tumor growth rate is rapid, easy to occur lymphatic or blood channel metastasis. In general, melanoma is not sensitive to treatments such as chemotherapy, radiotherapy and immunotherapy. The prognosis is usually poor, and statistics show that the average survival time of melanoma is usually less than a year (2).

Focal adhesion kinase (FAK) is a cytoplasmic no receptor protein-tyrosine kinase, belonging to the protein tyrosine kinase superfamily, also called PTK II, FAK is essential for the cell signal transduction, participates in many 'outside-in' and 'inside-out' pathways. FAK can integrate signals from intrigrin, growth factors and mechanical stress, activate the PI3K/Akt and Ras/MAPK pathways, and regulate cell proliferation, apoptosis and metastasis. Besides, FAK is tightly linked to the embryonic development and the occurrence and development of cancer.

Recent studies showed that as a tumor proto-oncogene, the expression and the activity of FAK was increased in many types of tumors, such as colon, breast, gastric cancer and melanoma. When tumor cells were treated with specific inhibitor of FAK, the growth and invasion of the cells could be significantly inhibited. Phosphorylation of FAK can activate or inhibit a number of downstream pathways, including PI3K/Akt pathway, RIP pathway and Erk pathway (3-5). With the function of FAK on pathways, FAK participates in the occurrence and development of tumors. Therefore, FAK is being investigated as potential targets for treatment strategies in various cancers.

Fangchinoline are widely distributed in nature. It has extensive biological activities, such as enhancing immunity, anti-inflammatory sterilization and anti-atherosclerosis. In recent years, researchers found that fangchinoline has a certain role in the resistance on variety of tumor promoters, but also can inhibit the growth of malignant tumor cells (6,7). In addition, fangchinoline can also induce apoptosis of tumor cells. A variety of experimental studies show that fangchinoline has inhibitory effect on lung, breast, prostate cancer and other tumor cells (8-11). Thus, the antitumor activity of fangchinoline has gained increased attention from researchers. The underlying anticancer mechanism of fangchinoline still needs to be explored further, and the study of fangchinoline on melanoma has not been reported.

In the present study, we report that by inhibiting the phosphorylation of FAK and suppressing its downstream signaling

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Key words: fangchinoline, melanoma, focal adhesion kinase, signaling pathway

Table I. The primer sequences used in the present study.

Name	Forward primer (5'→3')	Reverse primer (5'→3')
FAK	GAGCGTCTAATCCGACAGCAA	GCCCGTCACATTCTCGTACAC
Cyclin D1	CCGAGGAGCTGCTGCAAATGGAG	TGAAATCGTGCGGGGTCATTGCG
Bcl-2	GGTGAAGTGGGGGAGGATTG	GGCAGGCATGTTGACTTCAC
Bax	AGCTGAGCGAGTGTCTCAAG	GTCCAATGTCCAGCCCATGA
Paxillin	GGAGTCTACCCCTCCACA	CCACTGGTCTAAGGGGTCAA
GAPDH	AGAAGGCTGGGGCTCATTG	AGGGGCCATCCACAGTCTTC

pathway, fangchinoline could suppress the growth and metastasis of melanoma cells, thus, we provide new strategies for therapies in melanoma.

Materials and methods

Drugs. Fangchinoline was purchased from the Shenyang Pharmaceutical University (Shenyang, China). Dimethyl sulfoxide (DMSO) was used to dissolve configurations into different concentrations. PF-562271 (Selleck Chemicals LLC, Houston, TX, USA) is a potent ATP-competitive reversible inhibitor selective for recombinant FAK and PYK2 kinase.

Cell culture. HEK 293 (Human embryonic kidney cells, purchased from Shanghai Maisha Biotech Corp., Shanghai, China), A375 (melanoma cells, purchased from ATCC, Manassas, VA, USA; CRL-1619) and A875 (melanoma cells, from Shanghai Maisha Biotech) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 IU/ml penicillin and 100 mg/ml streptomycin (Baomanbio, Shanghai, China), at 37°C in a humidified atmosphere containing 5% CO₂.

MTT assays. Cells were plated 96-well plates at a density of 1x10⁴ cells/well; 24 h later, the medium was replaced with medium containing DMSO or the indicated concentrations of fangchinoline or 1.5 nM PF-562271 then incubated for 12, 24, 36 and 48 h. At the end of the incubation, the capability of cellular proliferation was measured by MTT assay. The optical densities at 490 nm were measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometry. After incubating with the indicated concentrations of fangchinoline for 24 h, at the end of the incubation cells were suspended in a staining buffer. The cells were then analyzed by a FACS Vantage flow cytometer with the CellQuest acquisition and analysis software program (Becton-Dickinson, Franklin Lakes, NJ, USA). Gating was set to exclude cell debris, doublets and clumps.

Metastasis assay. Cells (1x10⁵) were seeded onto the upper part of a Transwell chamber (BD Biosciences, San Jose, CA, USA) containing a gelatin-coated polycarbonate membrane filter (pore size, 8 mm) for the migration and invasion assays, respectively. Medium without serum was supplemented into the upper well and medium containing 10% FBS was

supplemented into the lower well. After 24-h incubation at 37°C with 5% CO₂, the filters were stained with crystal violet (Amersco, Solon, OH, USA). Five random fields were counted per chamber by using an inverted microscope.

Hoechst 33258 staining. Cells were incubated with DMSO or fangchinoline for 24 h. After incubation, cells were washed then incubated with 10 µg/ml Hoechst 33258 for 5 min at room temperature. Five random fields were counted per chamber by using an inverted microscope.

Annexin V-FITC/PI staining. Following the Annexin V-FITC/PI staining apoptosis detection kit instructions, the specific steps were as follows: cells were washed twice with cold phosphate-buffered saline (PBS), then re-suspended with binding buffer at a concentration of 1x10⁶ cells/ml, adding 5 µl of Annexin V-FITC and 10 µl of PI. The cells were incubated in the dark, at room temperature for 15 min. Finally, adding 400 µl binding buffer to each tube and the apoptosis rate was measured by flow cytometry within 1 h.

Reverse transcription and quantitative real-time PCR. Total RNA of A375 cells after treating with different factors for 24 h were isolated by TRIzol (Invitrogen) according to the manufacturer's protocol. Complementary DNA was synthesized by reverse transcription of total RNA using an RT reaction kit (Promega, Madison, WI, USA). Real-time PCR was performed according to the manufacturer's instruction. SYBR Premix Ex Taq (Takara Bio, Tokyo, Japan) was used as a DNA-specific fluorescent dye. The primer sequences were synthesized as shown in Table I.

All the reactions were repeated at least three times. Gene expression levels were calculated relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) by using Stratagene Mx3000P software (Stratagene, La Jolla, CA, USA).

Western blot analyses. Whole cell extracts (lysate) were prepared from 1x10⁶ cells in lysis buffer, to determine the expression of protein. A total of 30 µg of proteins from each sample were subjected to 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Target proteins were probed with specific antibodies-FAK^{p-Tyr397} (sc-81493), FAK (sc-271195), cyclin D1 (sc-70899), bcl-2 (sc-56015), bax (sc-20067), paxillin (sc-136297) and GAPDH (sc-367714) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Gels were stripped and reprobed with antibodies against GAPDH to assure equal loading.

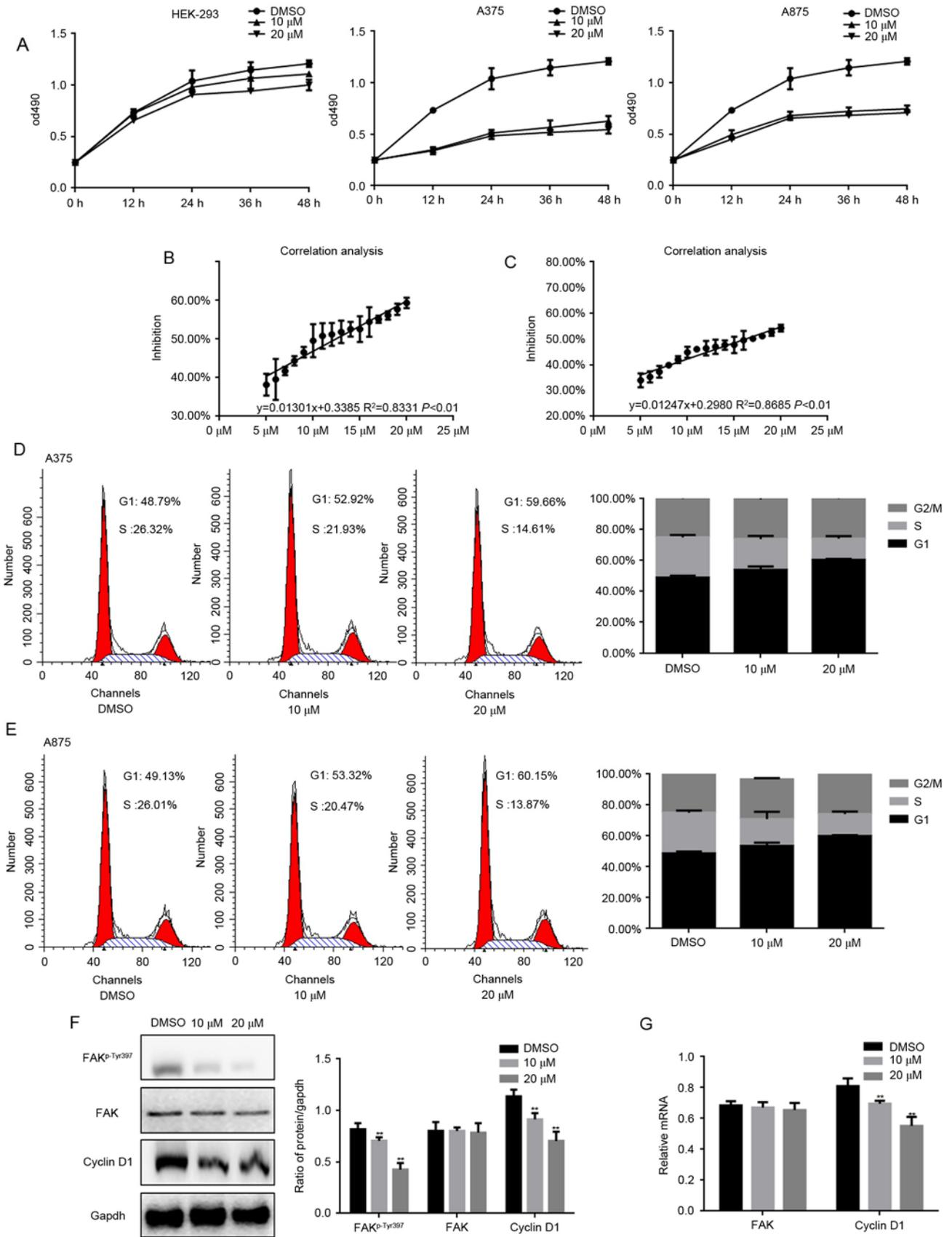


Figure 1. Fangchinoline inhibits the proliferation of melanoma cells. (A) HEK293, A375 and A875 cells were treated with indicated concentrations of fangchinoline, cells growth was detected by MTT assay. Data are shown as mean ± SEM. (B and C) The inhibition rate of fangchinoline on A375 and A875 cells were detected by MTT, IC₅₀ values were calculated and linear fit curve was drawn with an equation of $y=0.01301x+0.3385$ $R^2=0.8331$ and $y=0.01247x+0.2980$ $R^2=0.8685$. (D) A375 and (E) A875 cells were analyzed by a FACS Vantage flow cytometer with the CellQuest acquisition and analysis software program, the experiment was repeated three times. (F) A375 cells were treated with the indicated concentration of fangchinoline for 24 h, the indicated proteins were detected by western blot analysis. Data are shown as mean ± SEM. ** $P<0.01$ vs. DMSO treated group. (G) A375 cells were treated with the indicated concentration of fangchinoline for 24 h, the indicated mRNA levels were detected by real-time PCR. Data are shown as mean ± SEM. ** $P<0.01$ vs. DMSO treated group.

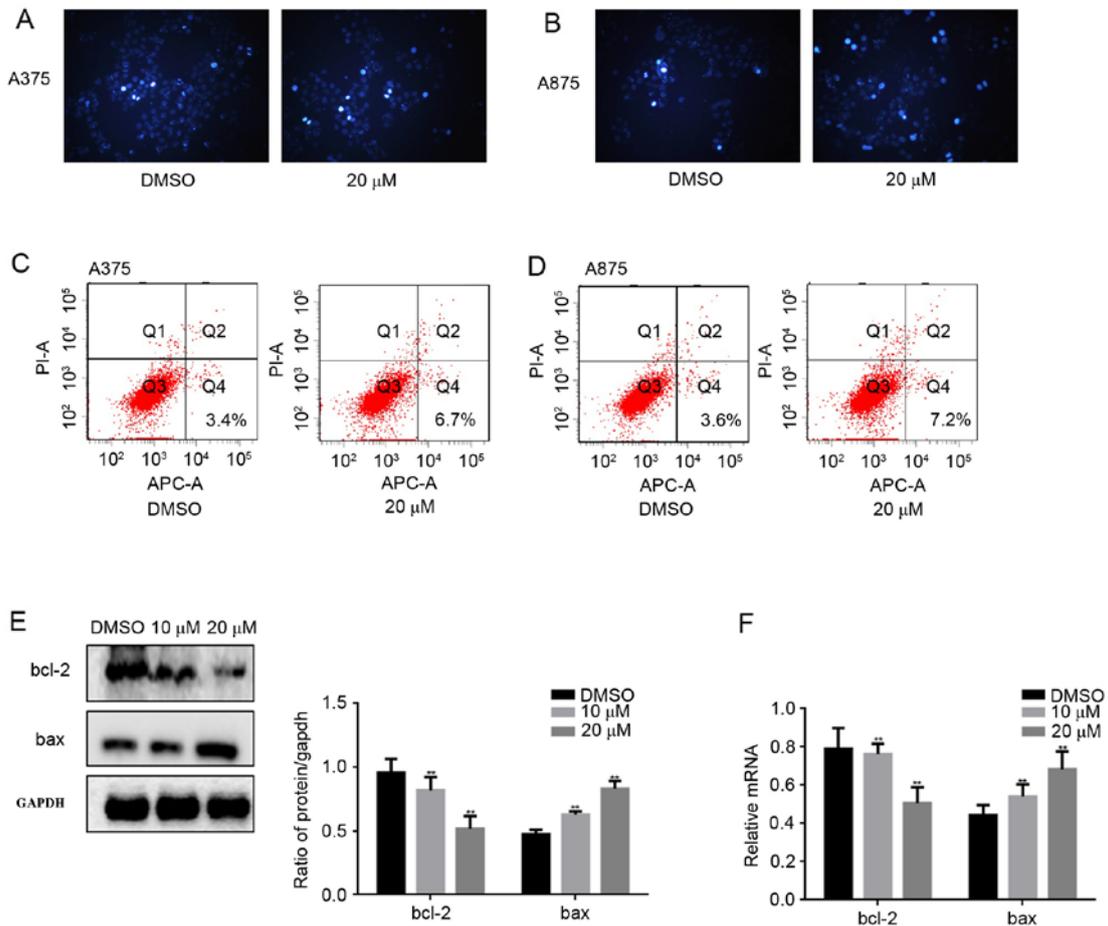


Figure 2. Fangchinoline promotes the apoptosis of melanoma cells. (A and B) A375 and A875 cells were treated with fangchinoline for 24 h and stained with Hoechst 33258. (C and D) A375 and A875 cells were treated with fangchinoline for 24 h and Annexin V-FITC/PI staining was used to detect the percentage of apoptotic cells. (E and F) A375 cells were treated with the indicated concentration of fangchinoline for 24 h, the indicated proteins and mRNA levels were detected by western blot analysis and real-time PCR. Data are shown as mean \pm SEM. ** $P < 0.01$ vs. DMSO treated group.

Statistical analysis. The data were analyzed with the SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The experiments were repeated three times and the data from three independent experiments are expressed as mean \pm SD. Statistical significance was set at $P < 0.05$.

Results

Fangchinoline inhibits proliferation of melanoma cells. It has been shown that fangchinoline can inhibit the growth of tumor cells, we examined the different concentrations of fangchinoline on proliferation of HEK293, A375 and A875 cells by MTT assay. MTT assay results showed that the proliferation of cells was suppressed in a concentration-dependent manner of fangchinoline (Fig. 1A). Since the inhibition of fangchinoline on HEK293 cells was weak, the inhibition of it on melanoma cells was stronger. Thus, we infer that fangchinoline may have specific inhibitory effect on tumor cells to a certain extent. Then we learned through a large number of data analysis that fangchinoline inhibited the proliferation of A375 and A875 cells activity with IC_{50} values of 12.41 and 16.20 μ M (Fig. 1B and C). In order to clarify the inhibitory mechanism of fangchinoline on cells proliferation, cell cycle analysis was performed. A375 and A875 cells were exposed to DMSO or

indicated concentrations of fangchinoline for 24 h. The results showed that cells were arrested in G1 phase and the percentage of cells in S phase decreased (Fig. 1D and E). The phosphorylation level of FAK was upregulated in the melanoma, and it could be involved in the proliferation of melanoma through several pathways. Thus, we investigated whether fangchinoline can be influenced by the FAK signaling pathway to regulate the proliferation of melanoma cells. Since cyclin D1 is the key regulators in G1 phase of the cell cycle and is one of the downstream proteins of FAK pathway, we examined the expression of it by western blot analysis and real-time PCR (Fig. 1F and G). Results showed that the degree of FAK^{p-Tyr397} and cyclin D1 decreased significantly after exposure to fangchinoline for 24 h at the indicated concentration. These results indicated that fangchinoline could inhibit proliferation of A375 and A875 cells in a concentration-dependent manner through FAK/cyclin D1.

Fangchinoline promotes apoptosis of melanoma cells. Proliferation and apoptosis can control the growth of cells. Because fangchinoline inhibited the proliferation of melanoma cells, we further examined the effect of fangchinoline on apoptosis. Hoechst 33258 staining assay and Annexin V-FITC/PI staining showed that after treated with fangchinoline for 24 h,

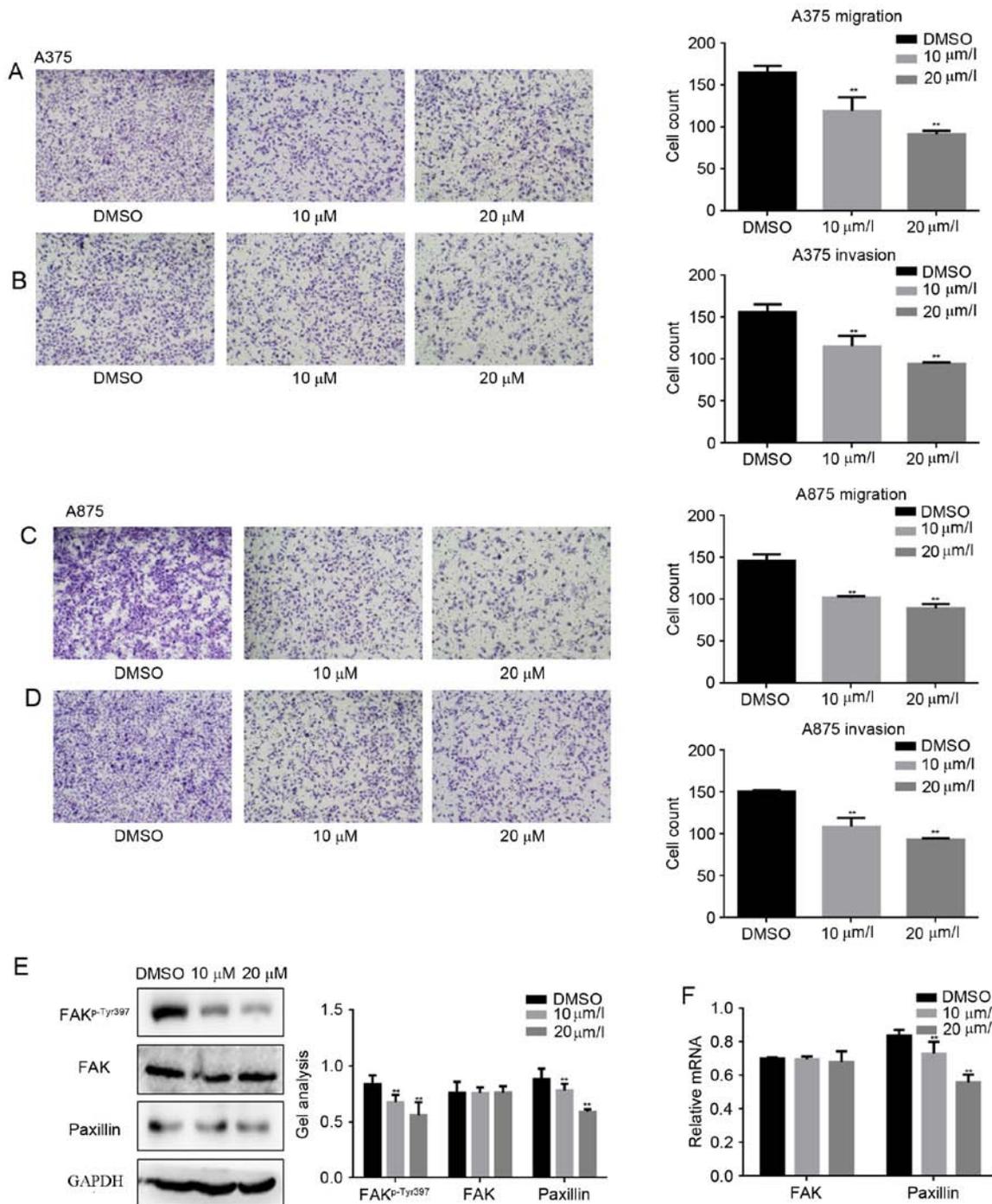


Figure 3. Fangchinoline inhibits metastasis of melanoma cells. (A and B) A375 cells were treated with fangchinoline for 24 h; Transwell assay with or without Matrigel was performed. Cells were counted and results represent the mean \pm SD of three experiments. ** $P < 0.01$ vs. DMSO treated group. (C and D) A375 cells were treated with fangchinoline for 24 h; Transwell assay with or without Matrigel was performed. Cells were counted and results represent the mean \pm SD of three experiments. ** $P < 0.01$ vs. DMSO treated group. (E and F) A375 cells were treated with the indicated concentration of fangchinoline for 24 h, the indicated proteins and mRNA levels were detected by western blot analysis and real-time PCR. Data are shown as mean \pm SEM. ** $P < 0.01$ vs. DMSO treated group.

A375 and A875 cell apoptosis was promoted (Fig. 2A-D). Then we detected bcl-2 and bax which are apoptosis-associated proteins by western blot analysis and real-time PCR (Fig. 2E and F). We found that the level of bcl-2 was downregulated by fangchinoline, the expression of bax was upregulated by fangchinoline. This result suggests that fangchinoline was able to regulate the phosphorylation level of FAK and apoptosis related pathways to promote apoptosis of melanoma cells.

Fangchinoline inhibits metastasis of melanoma cells. To study whether fangchinoline was involved in suppressing metastasis of melanoma cells, Transwell assay (with or without Matrigel) were performed (Fig. 3A-D). Results showed that fangchinoline significantly decreased the invasion and migration potential of A375 cells and A875 cells in a dose-dependent manner. It is well known that paxillin is an important protein in the regulation of cell invasion and metastasis in the FAK

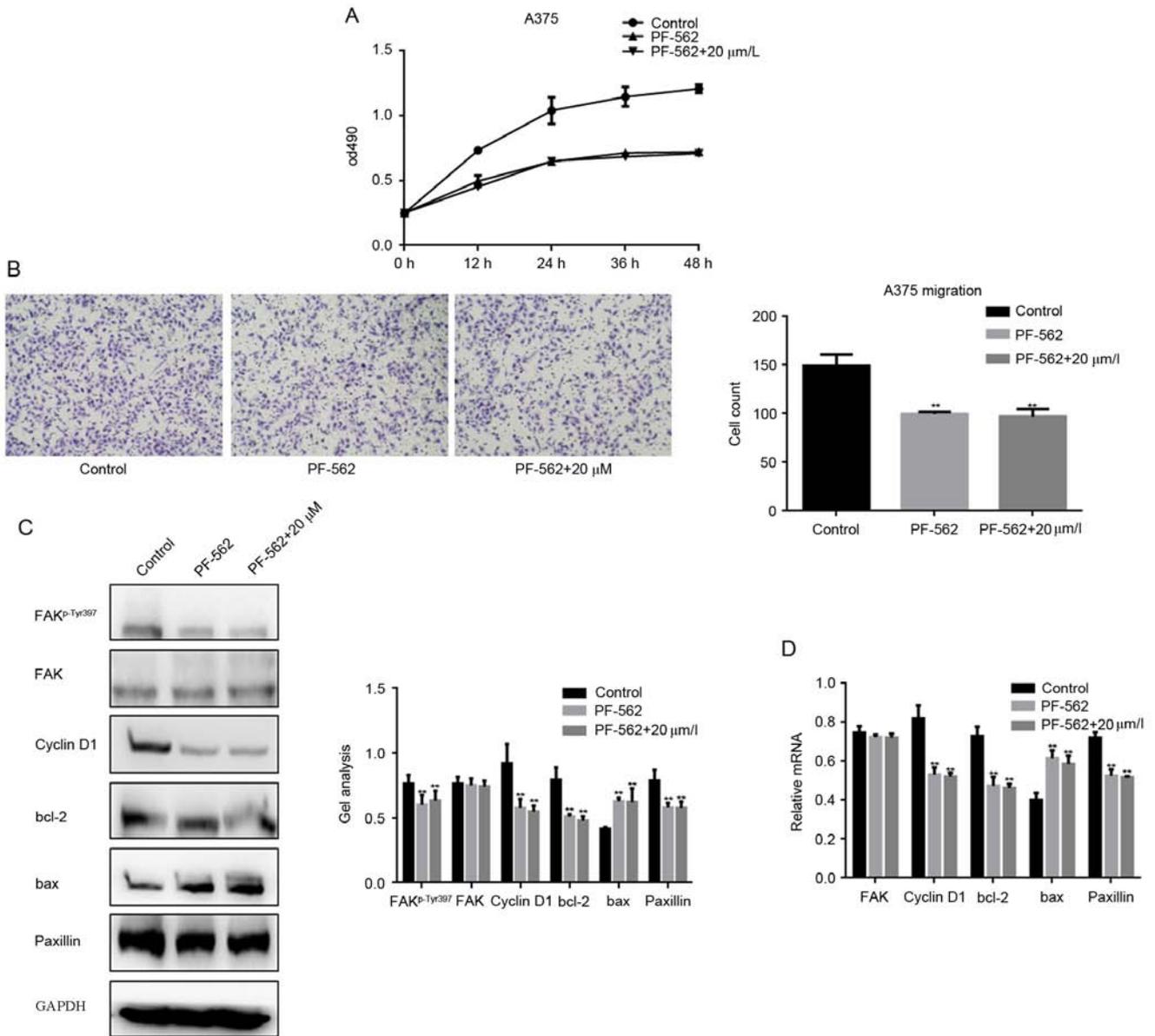


Figure 4. Fangchinoline suppresses the growth and metastasis potential of A375 cells by inhibiting the phosphorylation of FAK (A) A375 cells were treated with DMSO, PF-562271 or indicated concentrations of fangchinoline, cells growth was detected by MTT assay. Data are shown as mean ± SEM. (B) A375 cells were treated with DMSO, PF-562271 or indicated concentrations of fangchinoline for 24 h; Transwell assay without Matrigel was performed. Cells were counted and results represent the mean ± SD of three experiments. **P<0.01 vs. DMSO treated group. (C and D) A375 cells were treated with DMSO, PF-562271 or indicated concentrations of fangchinoline for 24 h; the indicated proteins and mRNA levels were detected by western blot analysis and real-time PCR. Data are shown as mean ± SEM. **P<0.01 vs. DMSO treated group.

signaling pathway. Subsequently the protein and mRNA levels of paxillin were detected by western blot analysis and real-time PCR, respectively. With the increasing concentration of fangchinoline the protein and mRNA expression of paxillin was significantly decreased, which implied fangchinoline inhibited the metastasis potential of melanoma cells by inhibiting paxillin.

Fangchinoline suppresses the growth and metastasis potential of A375 cells by inhibiting the phosphorylation of FAK. In order to explore if the inhibitory effect of fangchinoline on growth and metastasis of melanoma cells was achieved through inhibition the phosphorylation of FAK. We added the FAK inhibitor-PF-562 treatment group in our research.

MTT assay result showed that after adding the inhibitor of FAK in A375 cells, the growth effect of fangchinoline on A375 cells was not obvious (Fig. 4A). Transwell assay results showed that FAK inhibitors could inhibit the migration of A375 cells. However, when FAK inhibitors and fangchinoline were used together in A375 cells the inhibition of A375 cell migration had no significant difference with the FAK inhibitor treated alone (Fig. 4B). After that we checked the changes of FAK^{p-Tyr397}, FAK, cyclin D1, bcl-2, bax and paxillin in FAK inhibitor group, FAK inhibitor with fangchinoline group and control group (Fig. 4C and D). Western blot analysis and real-time PCR showed that when fangchinoline and FAK inhibitors were combined and applied on A375 cells the effect on these proteins was similar to FAK inhibitors alone application on

A375 cells. The data showed that the inhibitory effect of fangchinoline on A375 cells was achieved by inhibiting FAK phosphorylation.

The above results indicated that fangchinoline could suppress the growth and metastasis of melanoma cells by specially targeting FAK^{p-Tyr397}.

Discussion

FAK can promote the proliferation of tumor cells by regulating the cell cycle and apoptosis. At present, it is believed that FAK could promote the proliferation of tumor cells by increasing the cellular DNA synthesis and accelerating the transformation of G1/S phase. The studies found that the activity of FAK increased significantly in a variety of highly invasive cells (12-14). It was demonstrated that FAK could bind to the death domain kinase receptor interacting protein RIP and inhibit the tumor-suppressing apoptotic function of RIP (15). In addition, FAK was reported to interact with p53 and to inhibit p53 apoptotic activity (16). It was demonstrated that FAK could activate the PI3K/Akt pathway and induce function of NF- κ B to achieve the anti-apoptotic function in HL-60 leukemia cells (17). FAK was shown to mediate cell invasion and metastasis through promotion of epithelial-to-mesenchymal transition (18). Studies showed the importance of FAK activation to enable proliferation of micrometastatic cancer cells disseminated in the lungs (19). In melanoma, when the activity of FAK was inhibited, the growth and metastasis of melanoma were inhibited (3-5,12,20-22).

Fangchinoline is a bisbenzylisoquinoline alkaloid isolated from *Radix Stephaniae tetrandrae* S. Previous reports showed that, fangchinoline could induce cell cycle arrest, apoptosis and metastasis in cultured cancer cells. Fangchinoline may be a potential drug candidate for the prevention of lung cancer by the downregulation of cellular CDK4, CDK6 and cyclin D1 levels then blocking cell cycle progression (6). Fangchinoline could target PI3K in tumor cells that express PI3K abundantly and inhibit the growth and metastasis ability of SGC7901 cells (23). Studies showed that fangchinoline induced G1/S arrest by modulating expression of p27, PCNA and cyclin D in human prostate carcinoma cancer PC3 cells and tumor xenografts (7,9). Fangchinoline also induced autophagic cell death via p53/sestrin2/AMPK signaling in human hepatocellular carcinoma cells (24). Reports also indicated that fangchinoline inhibited cell proliferation via Akt/GSK-3 β /cyclin D1 signaling and induce apoptosis in MDA-MB-231 breast cancer cells (10,11,25). Researchers pointed out that fangchinoline could achieve the inhibition of lung cancer and glioma role by regulating multiple signaling pathways (26,27).

Many reports pointed out that fangchinoline could inhibit cell proliferation, promote cell apoptosis, inhibit cell metastasis and inhibit tumor angiogenesis to achieve the purpose of inhibiting the occurrence and development of tumors. However, there was no report on whether fangchinoline could play the same role in melanoma cells as in other cancer cells. In the present study, we observed the effect of fangchinoline on different cell line proliferation through the MTT assay. The results showed that fangchinoline inhibited melanoma cell proliferation dose-dependently. The flow cytometry experiment pointed out that fangchinoline could

repress the cell cycle from G1 to S phase transformation. The effect of this was through the inhibition of cyclin D1. At the same time we found that apoptosis of fangchinoline could promote the apoptosis of melanoma. In addition, through Transwell experiments we found that fangchinoline regulated the proliferation of melanoma cells, and it could inhibit melanoma cell metastasis through inhibiting paxillin. By detecting the protein level and mRNA level of FAK and FAK^{p-Tyr397} we found that these functions were likely to be due to the inhibition of fangchinoline by phosphorylation of FAK. We found that when FAK inhibitor was added the inhibition of fangchinoline to melanoma cell growth and metastasis almost disappeared, which confirmed that the inhibitory effect of fangchinoline on growth and metastasis of melanoma cells may be achieved by inhibiting the phosphorylation of FAK in the opposite direction.

In summary, our data clarified that fangchinoline could inhibit the growth and metastasis of melanoma cells by inhibiting FAK^{p-Tyr397} and suppressing the FAK pathway. Although further *in vivo* tests are needed to confirm our results, our data can still provide a certain experimental basis for the treatment of melanoma.

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