Gefitinib inhibits malignant melanoma cells through the VEGF/AKT signaling pathway

XUEFENG WAN*, YALIN ZHU*, LIJUAN ZHANG and WEI HOU

Department of Dermatology and Venereology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054, P.R. China

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Abstract. Malignant melanoma (MM) is caused by melanophore cancerization in tissue pigmentation regions, leading to skin, mucous membrane, eye and central nervous system carcinogenesis. The incidence of MM has increased in previous years, and it has become the primary cause of skin cancer-associated mortality in developed countries. MM is characterized as highly malignant and readily metastasized, and has a poor prognosis. Targeting angiogenesis is an important method for MM treatment. As an important proangiogenic factor in tumor growth and metastasis, vascular endothelial growth factor (VEGF) can promote neovascularization and increase vascular permeability. Gefitinib is a novel drug targeting VEGF. The effect and mechanism of gefitinib on MM remain to be elucidated, and were investigated in the present study. The A375 MM cell line was used in the present study; it was cultured *in vitro* and divided into gefitinib groups (5 and 10 μM) and a control group. Cell proliferation was measured using an MTT assay and the activity of caspase-3 was assessed using a kit. Cell invasive ability was determined using a Transwell chamber. The mRNA and protein expression levels of VEGF and AKT were detected using reverse transcription-quantitative polymerase chain reaction and western blot analyses. Gefitinib significantly inhibited MM cell proliferation, enhanced the activity of caspase 3 and suppressed tumor cell invasion ($P<0.05$). In addition, gefitinib significantly downregulated the mRNA and protein expression levels of VEGF and AKT, and these changes were dose-dependent ($P<0.05$). Taken together, gefitinib suppressed MM cell proliferation and invasion *in vitro* by regulating the VEGF/AKT signaling pathway.

Correspondence to: Dr Yalin Zhu, Department of Dermatology and Venereology, The First Affiliated Hospital of Xinjiang Medical University, 137 Liyushannan Road, Urumqi, Xinjiang 830054, P.R. China
E-mail: yalinzhu123@sina.com

*Contributed equally

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Introduction

Malignant melanoma (MM), primarily caused by melanophore cancerization and hyperplasia, can occur in the skin, mucous membranes and central nervous system (1,2). MM is a common type of malignant tumor in dermatology with a high malignancy and incidence. The median survival rate of patients with MM is only 18 months, and it is the leading cause of skin malignant tumor-associated mortality around the world (3,4). The occurrence of MM has ethnic and regional features, being higher in the European and American countries. It is the cause of skin cancer-associated mortality in developed countries (5,6). In the Asian population, primary cutaneous melanoma accounts for 50-70% of cases, where the primary pathological type is entigo maligna melanoma, followed by superficial invasive melanoma and nodular MM (7,8). Previously, the incidence of MM was low in China; however, the incidence of MM has gradually increased following changes to lifestyle. MM is characterized as highly malignant and readily metastasized, and has a poor prognosis (9).

The pathogenetic mechanism of MM is complex and remains to be fully elucidated. Multiple factors are associated with the induction of MM, including genetics, physics, chemistry, family history and long-term sun exposure (10). Following detailed investigations of the mechanism, current treatment methods for MM include chemotherapy and molecular target therapy. The aim of molecular target therapy is to interpose MM proliferation and mutation from the molecular level (11,12). Although multiple molecular anticancer drugs for MM have been examined, their curative effect remains poor. Tumor angiogenesis is important in the occurrence and development of MM; therefore, targeting angiogenesis is important for the treatment of MM (13). As an important proangiogenic factor of tumor growth and metastasis, VEGF can promote neovascularization and increase vascular permeability (14). Gefitnib is a novel target drug against VEGF (15). However, the effect and mechanism of gefitinib in MM remain to be fully elucidated. Therefore, the present study aimed to investigate the effect of gefitinib on MM cell proliferation and invasion, and the associated mechanism.

Materials and methods

Main instruments and reagents. The MM A375 cell line was purchased from the American Type Culture Collection Cell Bank (ATCC; Mannasas, VA, USA). DMEM, FBS, and penicillin-streptomycin were obtained from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA). Dimethyl sulfoxide and MTT powder were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Enzyme-EDTA was from Sigma-Aldrich; Merck Miilipore (Darmstadt, Germany). The caspase-3 activity detection kit and PVDF membrane were from Pall Life Sciences (Ann Arbor, MI, USA). EDTA was purchased from Hyclone; GE Healthcare Life Sciences. The reagents associated with western blot analysis were from Beyotime Institute of Biotechnology (Haimen, China). ECL reagent was from GE Healthcare Life Sciences. Rabbit anti-human VEGF (cat. no. 2463) and AKT (cat. no. 4691) monoclonal antibodies, and mouse anti-rabbit horseradish peroxidase (HRP)-tagged IgG secondary antibody (cat. no. 5127) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The Transwell chamber was from Corning Inc. (Corning, NY, USA). The ABI 7700 Fast fluorescence quantitative PCR reaction apparatus was from Applied Biosystems; Thermo Fisher Scientific, Inc. The RNA extraction kit and reverse transcription kit were from Axygen Biosceiences (Union City, CA, USA). Other common reagents were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The Labsystem version 1.3.1 microplate reader was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

MM A375 cell culture and grouping. The A375 cell line stored in liquid nitrogen was thawed in a 37˚C water bath and centrifuged at 300 x g for 3 min at room temperature. The cells were then resuspended in 1 ml medium and cultured in a 50 ml flask at 37˚C and 5% CO_2 for 24-48 h. The cells were passaged every 2-3 days and were used for experiments in the logarithmic phase at passages 2-8. The cells were divided into three groups, including the control, 5 μM gefitinib group and 10 μM gefitinib group. The cells in the treatment two groups were treated with gefitinib for 48 h at 37˚C.

MTT assay. The A375 cells in the logarithmic phase were seeded into 96-well plate at 5xl0^3/well for 24 h. The cells were divided into control and gefitinib groups with three replicates, which were cultured for 48 h. Subsequently, the plate was treated with 20 μl 5 g/l MTT solution and incubated for 4 h at 37˚C. Following removal of the supernatant, 150 μl DMSO was added to the plate for 10 min and read at 570 nm to calculate the proliferation rate.

Transwell assay. The Transwell chamber was coated with 50 mg/l Matrigel at 1:5 for 24 h and then air dried at 4˚C. A total of 500 μl DMEM containing 10% FBS were added to the lower chamber, and 100 μl tumor cell suspension in FBS-free medium was added to the upper chamber with three replicates. The cells in the control were cultured in a Transwell chamber without Matrigel. After 48 h, the chamber was washed in PBS and fixed in ice ethanol. Following staining with crystal violet, the cells on the lower membrane were counted under a light microscope (BX43; Olympus Corporation, Tokyo, Japan). All experiments were repeated three times.

Detection of caspase-3 activity. Caspase 3 activity was detected using a kit according to the manufacturer's instructions. The cells were digested in enzyme and centrifuged at 600 g and 4˚C for 5 min. The cells were then placed on ice for 15 min and centrifuged at 20,000 g and 4˚C for 5 min. Following the addition of 2 mM Ac-DEVD-pNA, the sample was read at 405 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the A375 cells using TRIzol and reverse transcribed into cDNA. The primers used were designed by Primer 6.0 software (Premier Biosoft, Palo Alto, CA, USA) and synthetized by Invitrogen; Thermo Fisher Scientific, Inc. (Table I). qPCR was performed in a total volume of 20 μl, including 10 μl SYBR Green qPCR Super mix, 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 5 μl cDNA and 4 μl sterile water. The reaction conditions were as follows: 55˚C for 1 min, followed by 35 cycles of 92˚C for 30 sec, 58˚C for 45 sec, and 72˚C for 35 sec. GAPDH was used as internal reference. The $2^{-\Delta\Delta Cq}$ method (16) was applied to calculate relative expression levels.

Western blot analysis. The cells were lysed in RIPA buffer (150 mM NaCL, 1% NP-40, 0.1% SDS, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, 1.5 mM EDTA and 1 mM NaVanadate) on ice for 15-30 min and ultrasonicated for 5 sec four times to extract protein. Following centrifugation at 10,000 x g and 4˚C for 15 min, the protein was moved to a new Ep tube and store at -20˚C. The protein was separated by 10% SDS-PAGE and transferred onto a PVDF membrane. Following blocking in 5% skim milk for 2 h, the membrane was incubated in VEGF primary antibody at 1:1,000 and AKT primary antibody at 1:2,000 overnight at 4˚C. The membrane was then incubated with secondary antibody at 1:2,000 for 30 min at room temperature and washed with PBST. Finally, the membrane was treated with chemiluminescent agent for 1 min, and underwent X-ray imaging. The protein image processing system and Quantity One software version 4.6 (Bio-Rad Laboratories, Inc.) were used for data analysis. All experiments were repeated four times.

Statistical analysis. All statistical analyses were performed on SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA). Measurement data are presented as the mean ± standard deviation. One-way analysis of variance was used for comparison of means. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of gefitinib on melanoma cell proliferation. An MTT assay was used to examine the effect of gefitinib on A375 cell proliferation. The results showed that gefitinib treatment for 48 h significantly suppressed A375 cell proliferation, compared with the control ($P<0.05$). Following an increase in dose, the tumor cell-suppressing effect was more marked ($P<0.05$; Fig. 1). These results suggested that gefitinib inhibited abnormal proliferation of the MM cells.

Table I. Primer sequences.

Genes	Forward (5'-3')	Reverse (5'-3')
GADPH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
VEGF	ATCCTTATCTCTGTGTGGAACTTTGTG	CTCCCTCTCAGCG CTCACAGCTTGCTG
AKT	TATCTCTCTGTCTCCCACAGAAGTC	TACTTACCTCGCATGGGGTAATTTGG

VEGF, vascular endothelial growth factor.

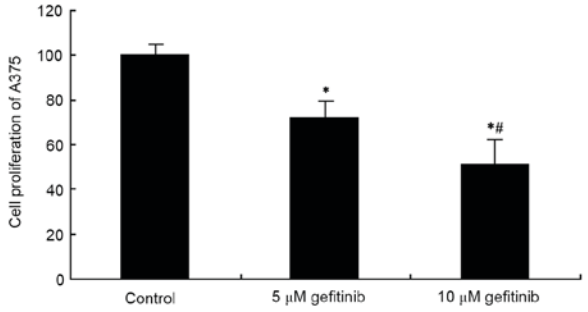

Figure 1. Effect of gefitinib on malignant melanoma cell proliferation. *P<0.05, compared with the control; #P<0.05, compared with 5 μM gefitinib.

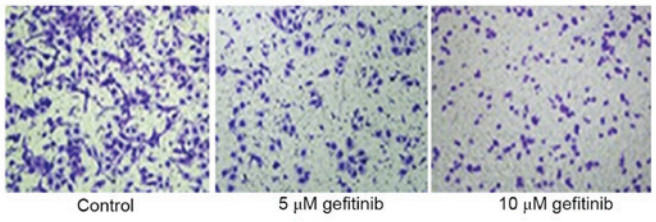

Figure 2. Effect of gefitinib on malignant melanoma cell invasion. Images of stained cells in each group (x40 magnification).

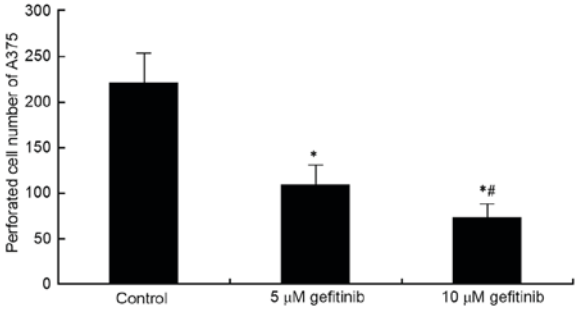

Figure 3. Effect of gefitinib on malignant melanoma cell invasion. *P<0.05, compared with the control; #P<0.05, compared with 5 μM gefitinib.

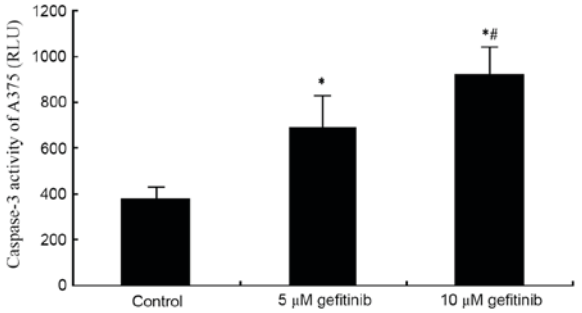

Figure 4. Effect of gefitinib on the activity of caspase-3 in malignant melanoma cells. *P<0.05, compared with the control; #P<0.05, compared with 5 μM gefitinib.

Effects of gefitinib on MM cell invasion. A Transwell assay was used to determine the effect of effect on the invasive ability of A375 cells. It was revealed that gefitinib treatment for 48 h markedly inhibited A375 cell invasion, compared with that in the control (P<0.05). Following an increase in dose, gefitinib had a more marked suppressive effect on tumor cell invasion (P<0.05; Figs. 2 and 3). These results indicated that gefitinib affected MM cell invasive ability.

Effect of gefitinib on the activity of caspase-3 in MM cells. A caspase 3 activity detection kit was used to measure the effect of gefitinib on the activity of caspase-3 in the A375 cells. The results demonstrated that gefitinib treatment for 48 h significantly increased the activity of caspase-3 in the A375 cells (P<0.05). Following an increase of dose, gefitinib exerted a more marked promoting effect on the activity of caspase-3 (P<0.05; Fig. 4). These results suggested that gefitinib promoted MM cell apoptosis by enhancing the activity of caspase-3.

Effects of gefitinib on the mRNA expression of VEGF and AKT in MM cells. RT-qPCR analysis was used to determine the effect of gefitinib on the mRNA expression of VEGF and AKT mRNA in A375 cells. The results showed that gefitinib treatment for 48 h markedly decreased the mRNA expression of VEGF in the A375 cells (P<0.05). Following an increase in dose, gefitinib exerted a higher suppressive effect on VEGF (P<0.05; Fig. 5). In addition, gefitinib treatment for 48 h significantly reduced the mRNA expression of AKT in the A375 cells (P<0.05). An increase in dose also resulted in an increased suppressive effect on AKT (P<0.05; Fig. 6).

Effect of gefitinib on the protein expression of VEGF in MM cells. Western blot analysis was performed to detect the effect of gefitinib on the protein expression of VEGF in A375 cells. It was found that, similar to the mRNA expression of VEGF, gefitinib treatment for 48 h weakened the protein expression of VEGF in A375 cells (P<0.05). Following dose elevation, gefitinib exerted a higher suppressive effect on VEGF (P<0.05; Figs. 7 and 8).

Effect of gefitinib on the protein expression of AKT in MM cells. Western blot analysis was used to determine the effect of gefitinib on the protein level of AKT in A375 cells. It

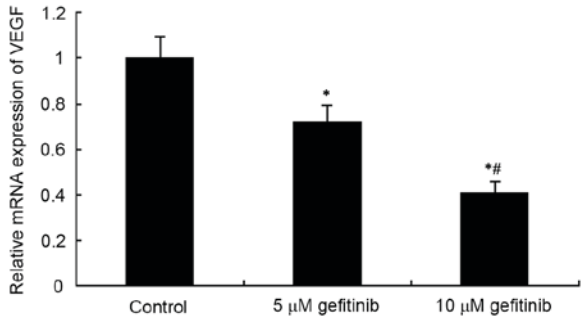

Figure 5. Effect of gefitinib on the mRNA expression of VEGF in malignant melanoma cells. *P<0.05, compared with the control; #P<0.05, compared with 5 μM gefitinib. VEGF, vascular endothelial growth factor.

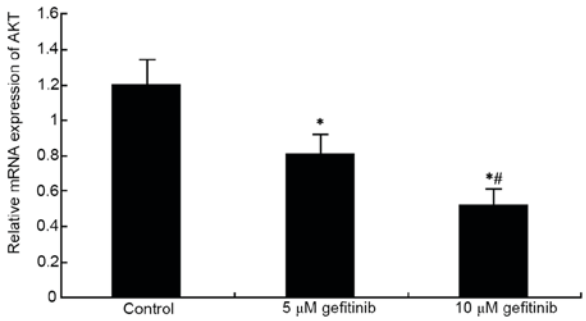

Figure 6. Effect of gefitinib on the mRNA expression of AKT in malignant melanoma cells. *P<0.05, compared with the control; #P<0.05, compared with 5 μM gefitinib.

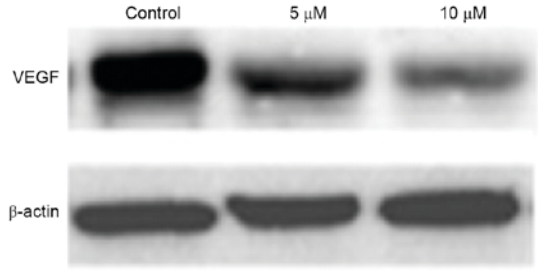

Figure 7. Effect of gefitinib on the protein expression of VEGF in malignant melanoma cells. Images show representative blots. VEGF, vascular endothelial growth factor.

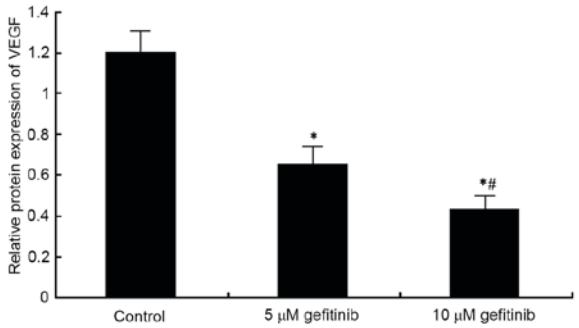

Figure 8. Effect of gefitinib on the protein expression of VEGF in malignant melanoma cells. *P<0.05, compared with the control; #P<0.05, compared with 5 μM gefitinib. VEGF, vascular endothelial growth factor.

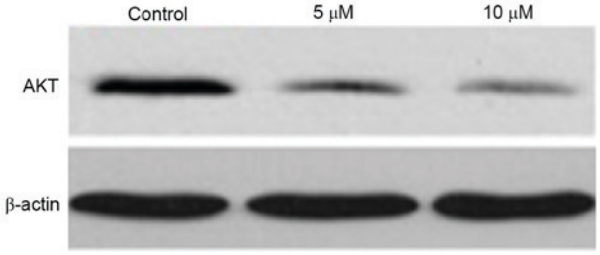

Figure 9. Effect of gefitinib on the protein expression of AKT in malignant melanoma cells. Images show representative blots.

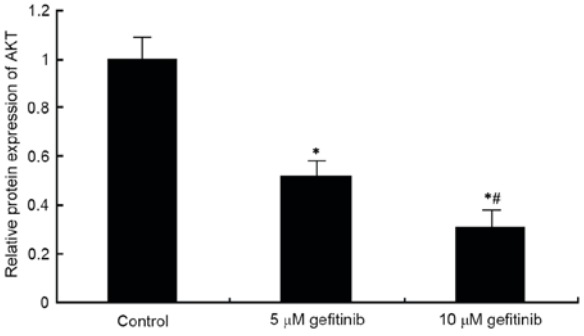

Figure 10. Effect of gefitinib on the protein expression of AKT in malignant melanoma cells. *P<0.05, compared with the control; #P<0.05, compared with 5 μM gefitinib.

was found that, similar to the mRNA expression of AKT, gefitinib treatment for 48 h decreased the protein expression of AKT in the A375 cells (P<0.05). Following dose elevation, gefitinib exerted a higher suppressive effect on AKT (P<0.05; Figs. 9 and 10).

Discussion

The incidence of MM has gradually increased over time. Due to its lack of apparent symptoms in the early stage and its ability to metastasize, the majority of patients present with metastasis at diagnosis, leading to poor surgical outcome. In addition, chemotherapy drug resistance leads to MM treatment inefficiency (17). The present study showed that molecular target drugs have certain curative effects on MM. Therefore, identifying suitable molecular target drugs to inhibit MM-associated pathways is likely to improve the survival rates and prognosis of patients with MM (18).

As one of the most important proangiogenic factors, VEGF is expressed in endothelial cells. It can promote vascular endothelial cell proliferation, differentiation, migration and movement, and form vessel structures by enhancing blood vessel permeability and degrading extracellular matrix (19). VEGF can promote neovascularization in tumorigenesis (20). The binding of VEGF to VEGF receptor, synergized with angiogenin-2, can facilitate lymphatic vessel hyperplasia surrounding the tumor to ensure that new capillaries can provide nutrition for the tumor and promote tumor metastasis (21). It has been shown that the protein kinase AKT is an important molecule involved in various biological behaviors of cells; for example, the overexpression of AKT promotes MM

metastasis (22). In the present study, MM cells were treated with gefitinib targeting VEGF, and its effect and mechanism were analyzed. The results showed that gefitinib suppressed MM cell proliferation and inhibited cell invasive ability in a dose-dependent manner. Gefitinib promoted tumor cell apoptosis by enhancing the activity of caspase-3. Analysis of the mechanism confirmed that gefitinib suppressed the mRNA and protein expression of VEGF and AKT, suggesting that gefitinib may reduce the occurrence and development of MM through the VEGF/AKT pathway.

In conclusion, the present study confirmed that gefitinib suppressed MM cell proliferation and invasion *in vitro* through regulating the VEGF/AKT signaling pathway. These results indicate a potential molecular target and theoretical basis for the treatment of MM.

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