

## Original Article

# Sunitinib treatment inhibited human breast cancer cell migration through regulation furin interaction with substrates

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**Abstract:** It has been acknowledgement that human breast cancer (MCF-7) associated with high ability of migration and angiogenesis. In our present study, we aimed to explore the mechanism of sunitinib in the inhibition of MCF-7 cell migration and angiogenesis. MCF-7 cell line was used as breast cancer cell model for investigation the regulation of cell migration upon sunitinib (0.5  $\mu$ M and 2  $\mu$ M) treatment. Western blot was used for detection the expressions of MT1-MMP and VEGF-C. The activity of MMP2 and MMP9 was monitored with gelatin zymography assay. MCF-7 cell migration and invasion were detected by wound healing assay and transwell. Immunoprecipitation was used for detection the interaction among pro-MT1-MMP, Furin and VEGF-C. Results have shown that the expressions of MT1-MMP and VEGF-C were inhibited by sunitinib. Zymography assay demonstrated that the activity of MMP2 and MMP9 decreased significantly upon sunitinib treatment. The invasion and migration of MCF-7 were inhibited. The interaction between Furin and its substrates pro-MT1-MMP, pro-VEGF-C decreased upon sunitinib treatment. These findings indicated that sunitinib may be used for inhibiting the migration and invasion of MCF-7 through down-regulated the interaction between Furin and its substrates (pro-MT1-MMP, pro-VEGF-C).

**Keywords:** MCF-7, sunitinib, furin, MT1-MMP, breast cancer, migration

## Introduction

Breast cancer is the leading cause of the cancer related death among woman in developed countries, accounting for 22% of new cases each year [1]. The occurrence of metastasis in tumorous progression is the most life-threatening aspect of breast cancer. However, the underlying mechanisms of breast cancer initiation, progression and metastasis are still not fully understood [2]. Cell migration related proteins MT1-MMP, MMP2, VEGF must be cleaved by protein convertase, followed with maturation and activation.

Furin is the best-characterized representative of the mammalian subtilisin like family of pro-protein convertase. Many protein precursors such as matrix metalloprotease, growth factors, serum proteins, receptors, and adhesion molecules have been identified as the furin substrates [3-5]. MT1-MMP proenzyme cleav-

age by furin is considered to be a principal event in the activation of this substrate and it may be play an important role in MCF-7 cell migration and invasion [6].

Furin activation plays a vital role in tumorous process [7]. Furin inhibitor  $\alpha$ 1-PDX has been used to block furin activity and to prevent cancer metastasis in cellular and animal studies [8]. Previous researches demonstrated that c-Src and furin together have been found to be upregulated in human cancers. As the most closely related members of the family of non-receptor tyrosine kinases, up-regulation of c-Src correlates with a variety of human tumors, including breast cancer [9, 10]. Whether the ubiquitous c-Src participated in the interaction between furin and its substrates still remained unknown.

Sunitinib (SU11248) is an orally bioavailable molecule, multi-targeted receptor tyrosine kina-

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se (RTK) inhibitor for the treatment of tumor angiogenesis, including vascular endothelial growth factor receptors and platelet-derived growth factor receptors [11]. Clinically, sunitinib antitumor activity was shown in renal carcinoma, neuroendocrine and breast cancers [12, 13]. Both c-Src and AKT are down-stream of various tyrosine kinase receptor signaling pathways. However, the antitumor effects of sunitinib in inhibiting tumor migration and angiogenesis on breast cancer remained unknown.

In the present study, we show that sunitinib induces tumor cell migration and invasion arrest in MCF-7 cells. The biological effects of sunitinib on tumor cell migration were associated with inhibition of Furin and its substrates interaction. Our findings suggest the potential use of sunitinib for the treatment of breast cancer cell metastasis.

### Materials and methods

#### *Cell culture and experimental reagents*

MCF-7 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 µg/mL streptomycin. All the cells were cultured in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. Sunitinib (SU11248, Sigma PZ0012) was purchased from Sigma and dissolved in DMSO. In some experiments, logarithmic growth cells were incubated with sunitinib (0.5 or 2 µM) whenever necessary as indicated in the figure legend.

Primary antibodies against Furin, MT1-MMP, VEGF-C and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Furin activity elisa kit (ABIN425690) was from Global Biotech (China). Gelatin zymography kit was from merck millipore (Millipore, USA), and MMP2/MMP9 elisa kits were purchased from Nanjing Jiancheng Bioengineering Institute (China).

#### *Wound healing assay*

MCF-7 cells were plated into 24-well plates and grown to confluence. The monolayer was artificially wounded using the tip of a sterile 200 µL pipette. Cell debris was removed by washing with PBS. The cells were then incubated with sunitinib for appropriate time. The cells migrated into the wounded areas were photographed.

Wound closure was photographed at the indicated times at the same spot with an inverted microscope equipped with a digital camera. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area.

#### *Transwell invasion assay*

Matrigel Invasion Chambers were hydrated for 4 h before starting the invasion assay. Log-phase cells ( $4 \times 10^4$ ) were plated in 200 µL DMEM containing 2% FBS in the upper chamber of the transwell and the lower chamber were filled with 500 µL DMEM containing 10% FBS. After incubation for 2 h, the cells were treated with sunitinib as previously description for 24 h. The cells were allowed to migrate for 10 h at 37°C and 5% CO<sub>2</sub> followed by carrying out the invasion assay. The cells were fixed for 15 min at room temperature by replacing the culture medium in the bottom with 4% formaldehyde dissolved in PBS. The cells that remained on the bottom of the chamber were stained with 0.1% crystal violet, the migrated clones were photographed under an optical microscopy. The cell number was counted at 12 different areas. Data were averaged from three parallel experiments, which were normalized to that of the control.

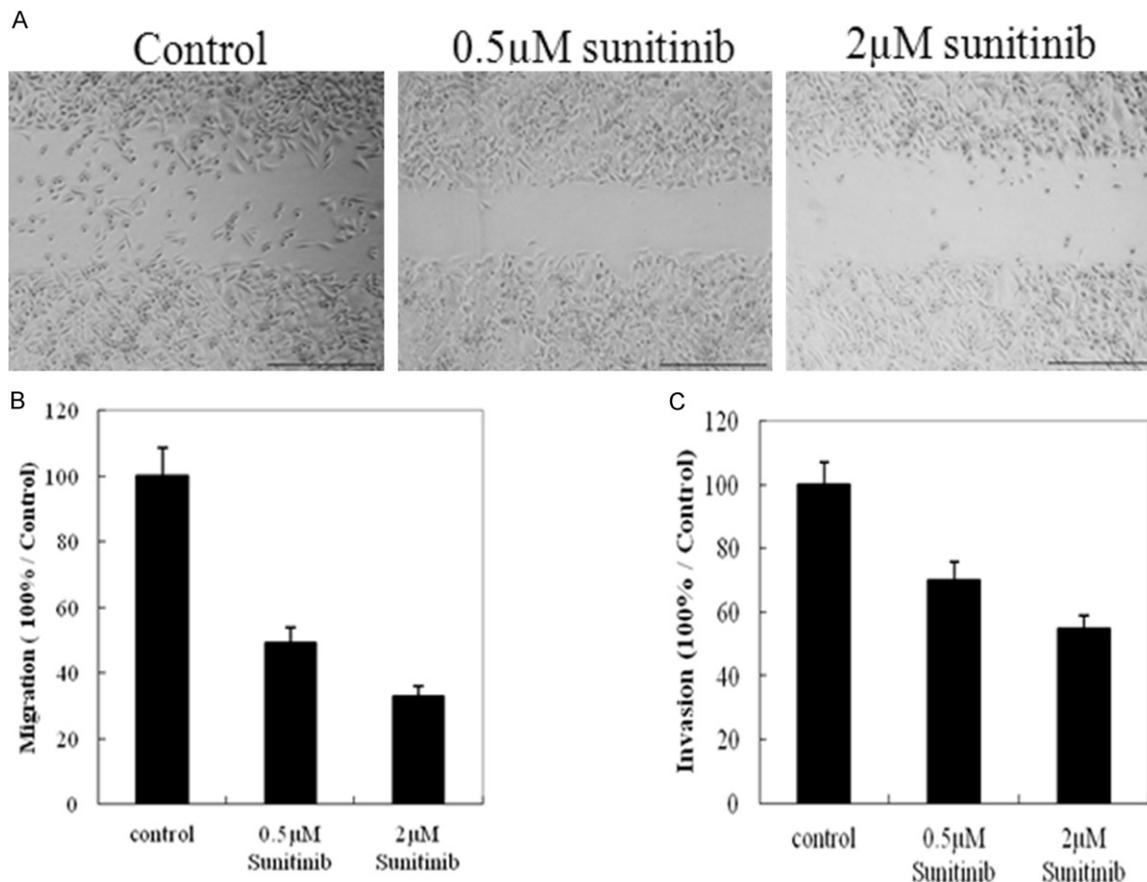
#### *Elisa detection assay*

MCF-7 cells were treated as previously description. Total cellular proteins were used for detection the activity of Furin or the concentrations of MMP2/MMP9 according with kit procedures.

#### *Gelatin zymography*

Levels of the active and latent forms of MMP-2, MMP-9 were analyzed by gelatin zymography as described in kit. MCF-7 cells were washed twice with ice-cold PBS and lysed with RIPA buffer for 30 min on ice. Mixtures were centrifuged at 12,000 g for 20 min at 4°C. The supernatant was aliquot and protein concentration was determined using BCA Protein Assay Reagent (Pierce). After electrophoresis and washing, the gel was incubated at 37°C for 24 h stained with coomassie brilliant blue R250, followed with destaining by wash buffer.

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**Figure 1.** Effects of sunitinib on the ability of MCF-7 cell migration and invasion. Confluent 90% MCF-7 cells were wounded by 200  $\mu$ l sterile pipette, then treated with varying concentrations of sunitinib for indicated time. The cells migrated into the wounded areas were photographed as A. The graph represents the mean  $\pm$  S.E. of at least three independent experiments B. The ability of MCF-7 cell invasion was monitored by transwell assay C. \* $P < 0.05$  indicated statistical significantly compared with control group.

### Furin enzyme assay

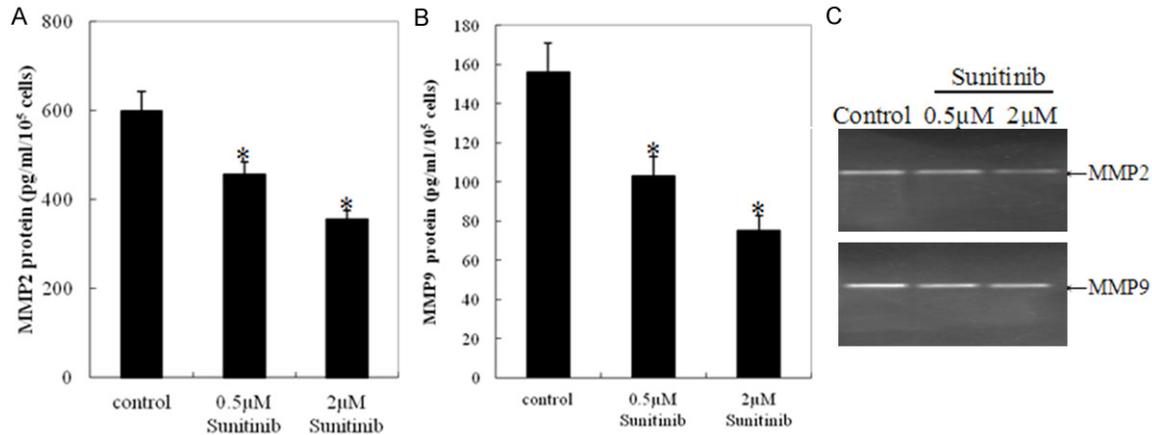
The enzyme activity of Furin in vitro was performed at 37°C in 96-well flat-bottom black plates (Corning, USA). Assay was performed using Cbz-Arg-Ser-Lys-Arg-AMC as substrates. Whole cellular lysates of MCF-7 were incubated with Furin substrate in a final volume of 100  $\mu$ L. The buffer consisted of 25 mM Mes, 25 mM Tris, and 2.5 mM CaCl<sub>2</sub> (pH 7.4). The release of highly fluorescent 7-amino 4-methyl coumarin (AMC) from Cbz-Arg-Ser-Lys-Arg-AMC was monitored by a Multi-Detection Microplate Readers (SpectraMax M5, USA) at excitation and emission wavelengths of 380 and 460 nm, respectively.

### Western blot analysis

Cells were lysed in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, 100 mM

NaF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>] containing protease inhibitor cocktail for 30 min at 4°C. All cell lysates were centrifuged at 4°C at 16,000 g for 30 min. The protein concentration was determined with the BCA method (Pierce, USA). Aliquots of cell lysates were fractionated by electrophoresis in SDS-PAGE, 8% for the analysis of Furin, 10% for the analysis of MT1-MMP and VEGF-C. Total proteins were transferred to PVDF membrane using a wet trans blot system (Bio-Rad, Hercules, CA). Blots were then blocked for 1 h at room temperature with 10% BSA or 5% nonfat dry milk. Membranes were incubated overnight at 4°C with antibodies against Furin, MT1-MMP, VEGF-C and  $\beta$ -actin (1:1000). After subsequent washing, the membranes were incubated for 1 h with horseradish peroxidase conjugated goat anti-rabbit or anti-mouse second antibody, diluted 1:5,000 in PBST. After washing, the membrane was processed using Super Signal West Pico chemilu-

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**Figure 2.** Effects of sunitinib on the expression and activity of MMP2 and MMP9 in MCF-7 cells. MCF-7 cells treated with sunitinib for indicated time, the concentrations of MMP2/MMP9 in cellular culture medium were detected by elisa assay (A and B). The results shown are representative of at least three independent experiments. At the same time, the activity of MMP2 and MMP9 were detected with Zymography Assay (C). The statistically significant cutoff  $p$  value is  $<0.05$  as shown \*.

minescent substrate (Pierce, USA), followed by exposure to Fujifilm LAS3000 Imager (Fuji, Japan). Densitometric analysis was performed with Image J densitometer.

### Co-immunoprecipitation

MCF-7 cells were washed twice with ice-cold PBS, lysed in 1 mL of RIPA buffer for 30 min on ice, clarified by centrifugation for 15 min at 10,000 g, and then the supernatant was subjected to immunoblot or immunoprecipitation. Cell lysates (500  $\mu$ g) was incubated with 2  $\mu$ g appropriate antibody (anti-Furin) overnight at 4°C. 50  $\mu$ L of protein G was added and mixed at 4°C for 2 h with gentle agitation. The pellet was washed three times with RIPA buffer, boiled with 50  $\mu$ L 2 $\times$  loading buffer (Tris pH 6.8, 0.1% SDS, 10% glycerol, and 0.025% Bromophenol blue, 20 mM DTT) for 5 min prior to gel loading, and proteins were detected by western blot with anti-Furin, MT1-MMP and VEGF-C antibody. Some experiments substituted the secondary antibody with Clean-Blot IP Detection Reagent for clear IP/Western blot results.

### Statistical analysis

Western blots were quantified by measuring the relative density of protein bands recognized by a particular antibody using Image J software (NIH, USA). The results were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was done with Student's t-test for comparison of two groups, differences with  $P<0.05$  were considered statistically significant.

## Results

### Effects of sunitinib on the invasion and migration of MCF-7 cells

In order to explore whether the invasion and migration of MCF-7 cells were regulated by sunitinib treatment, we did wound healing assay and transwell assay. As our expected that sunitinib has obvious roles in modulation the invasion and migration of MCF-7 cells. The ability of invasion and migration of the cells that treated with sunitinib decreased significantly compared with control group (**Figure 1**).

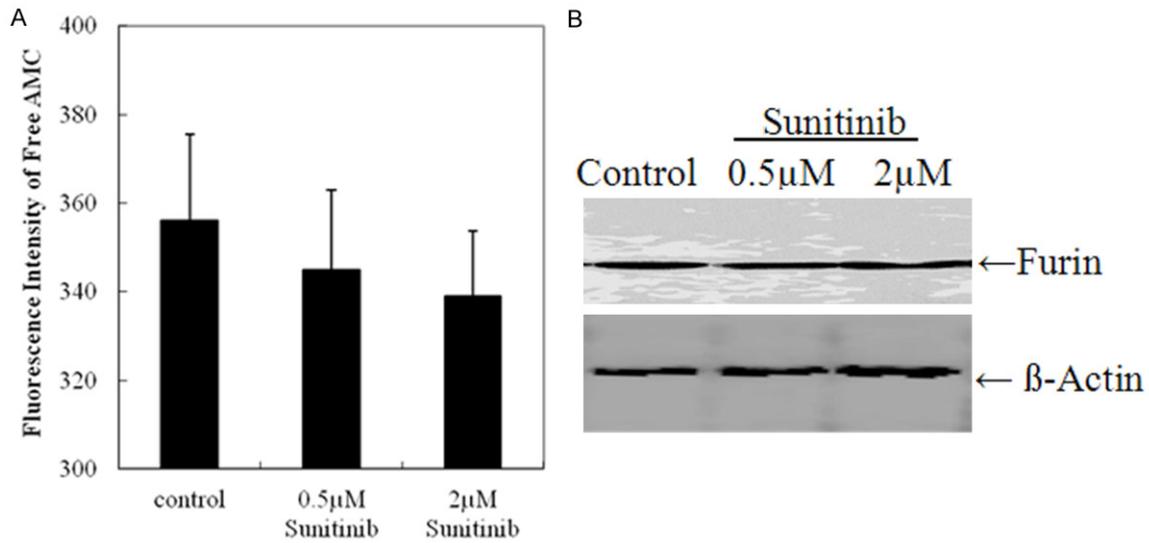
### Effects of sunitinib on the expressions of MMP2/9 in MCF-7 cell culture medium

MMP2 and MMP9 elisa kits were used for monitored the concentrations of MMP2/9 in the cell culture medium. The results have shown that the concentrations of MMP2/9 decreased significantly compare to the control group (**Figure 2A** and **2B**). The results were expressed as mean  $\pm$  SD from five independent experiments. We also detected the activity of MMP2/9 by gelatin zymography assay. As our expected, both the activity of MMP2 and MMP9 were all decreased (**Figure 2C**).

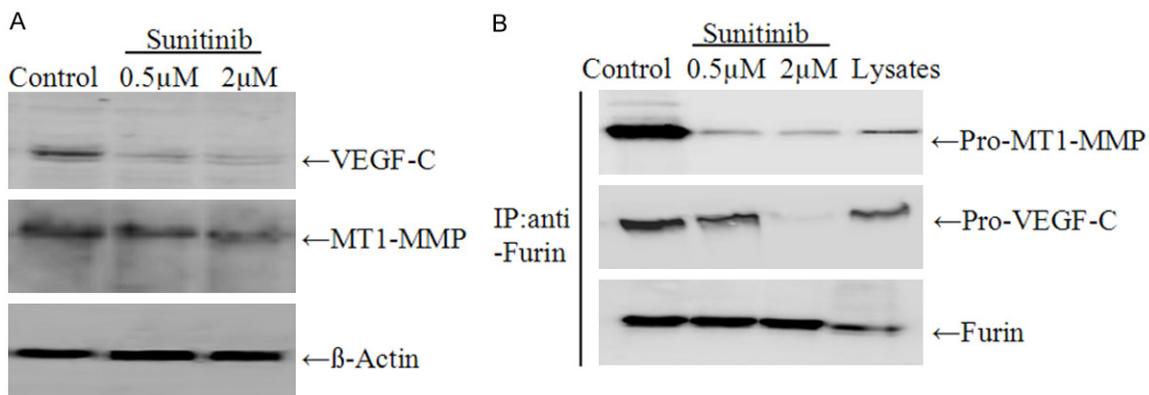
### Effects of sunitinib on the expressions of furin and its substrates in MCF-7 cells

We first found that the expression and activity of Furin have no obvious variation upon sunitinib treatment (**Figure 3A** and **3B**). However,

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**Figure 3.** Effects of sunitinib on the expression and activity of Furin in MCF-7 cells. The expression of Furin in MCF-7 cells upon sunitinib treatment was detected by western blot (A) and the activity of Furin was monitored by enzyme assay (B).



**Figure 4.** Effects of sunitinib on the interaction between Furin and pro-MT1-MMP, pro-VEGF-C in MCF-7 cells. MCF-7 cells were treated as previous description. Whole cell proteins were collected and 2 μg anti-Furin antibody was used for immunoprecipitation for overnight. The immunoprecipitates were used for detection the expression of pre-MT1-MMP or pre-VEGF-C by specific antibody.

the immunoblot indicated that a quantitative decrease in the intensities of MT1-MMP and VEGF-C bands were observed upon treatment with sunitinib (**Figure 4A**). These results indicated that some mechanisms maybe exit in regulation the maturation of MT1-MMP and VEGF-C.

*The interaction between furin and its substrates was inhibited by sunitinib in MCF-7 cells*

It is unclear whether the binding between Furin and its substrates was affected upon sunitinib

treatment in MCF-7 cells. We then did co-immunoprecipitation assay. Whole cell lysates were immunoprecipitated with anti-Furin antibody, and then detected the expressions of pro-MT1-MMP and pro-VEGF-C. As shown in **Figure 4B**, there was almost no band detection in sunitinib treated group. Similar results have been got for VEGF-C detection.

### Discussion

Clinical data has shown that sunitinib has been used in anticancer therapy through inhibiting cell proliferation or angiogenesis. However, its

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role in cancer cell metastasis remained unknown. The precursor proteins of cell metastasis association, such as MT1-MMP, MMP2, must be shorn by Furin in secretory pathway compartments [14]. Inhibition of Furin activity or interaction with its substrates may decrease substrate activation, proliferation rate and invasive potential of cancer cells. So, it is a potentially useful target for cancer therapeutics [15].

Our present study first found sunitib treatment may directly regulate the Capan-1 cell invasion and migration through modulation the maturation of MT1-MMP/VEGF-C. In our study, we first found that the ability of Capan-1 cells invasion and migration decreased upon sunitib treatment. To explore the mechanism, we then detected the effects of sunitib on the expressions of MT1-MMP or VEGF-C in Capan-1 cells. MT1-MMP and VEGF-C have played a vital role in regulation of cancer cell invasion and migration. Upregulation of MT1-MMP can effectively cause elevated invasiveness in human cancer cell [16-18]. Results have shown that MT1-MMP or VEGF-C decreased significantly in accordance with c-Src activity, but the activity of furin had no obviously variation. The results indicated that regulation of MT1-MMP or VEGF-C not dependent on the down-regulation of furin, another mechanism may exist.

To be active, the zymogen of MT1-MMP or VEGF must be cleaved the pro-peptide by protein convertase. We then hypothesized that sunitinib may directly inhibited the interaction between Furin and pro-MT1-MMP/pro-VEGF in vivo. So it is necessary to detect the effects of sunitinib on the interaction between Furin and its substrates. Our results showed that sunitib treatment decreased the formation of the complex between Furin and pro-MT1-MMP/pro-VEGF. Similar results got in interaction between Furin and VEGF-C.

In conclusion, we examined the role of sunitib in the process of Furin proteolysis its substrates. The potential inhibitor to block Furin and subsequent processing activity are more attractive therapeutic agents for MCF-7 cancer.

### Disclosure of conflict of interest

None.

### Abbreviations

CHIP, Chromatin immunoprecipitation; MMPs, Matrix metalloproteinases.

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