Tramadol inhibits proliferation, migration and invasion via α2-adrenoceptor signaling in breast cancer cells

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Abstract. – **OBJECTIVE:** The aim of this study was to examine the function of tramadol on cell proliferation, migration and invasion in breast cancer cells *in vitro*, and to evaluate the effect of tramadol *in vivo*. Further, we explore the mechanism accounting for the role of tramadol on breast cancer cells.

MATERIALS AND METHODS: Cell proliferation was detected by the methyl thiazolyl tetrazolium (MTT) assay. Wound healing assay and transwell assay was applied to quantify the migration and invasion ability of MDA-MB-231 cells. The expression of endogenous α2-adrenoceptor and ERK was measured by Western blotting.

RESULTS: Tramadol at a clinical dose of up to 2 μM significantly inhibited the proliferation, migration and invasion in a time-dependent manner from day 0 to 28 *in vitro*. Moreover, tramadol suppressed the growth of xenotransplant tumor *in vivo* markedly. Furthermore, the protein levels of α2-adrenoceptor and phosphorylated ERK were decreased by tramadol, whereas the expression of total ERK remained unchanged. In addition, downregulation of α2-adrenoceptor by yohimbine could mimic the effect of tramadol treatment.

CONCLUSIONS: Collectively, we demonstrated that tramadol could inhibit proliferation, migration and invasion of breast cancers via inactivating α2-adrenoceptor signaling pathway. Our data provide the experimental fundamental for further investigation of the anti-cancer effect of tramadol in breast cancer cells.

Key Words:
Tramadol, α2-adrenoceptor, Breast cancer cells, Tumorgenesis.

Introduction

Breast cancer is the most prevalent and aggressive cancer identified in women with an estimated nearly 459,000 deaths worldwide[1,2]. Recent years, despite the advances and progresses in the early detection, surgery and chemotherapy, the incidence of breast cancer and the rate of five years disease-free survival are still are on the rise[3,4]. It is essential for us to reveal the potential molecular mechanism accounting for the malignancy of this disease.

Tramadol (1RS,2RS)-2-[(dimethylamino)-methyl-1-(3-methoxyphenyl)]-cyclohexanol hydrochloride) is used as an effective and well-tolerated analgesic mainly for the treatment of moderate to severe pain, including post-operative pain, chronic non-cancer-associated pain such as neuropathic pain and cancer pain[5-7]. Tramadol exhibits dual analgesic mechanisms of action; it binds with low affinity to the μ-opioid receptor, and activates central monoaminergic pathways inhibiting the reuptake of serotonin as well as noradrenaline[8,9]. In addition, tramadol has both μ-opioid receptor mediated acute analgesic and α2-adrenoceptor mediated preventive and alleviative effects on neuropathic pain[10]. Accumulative studies have demonstrated that various anesthetics, such as morphine[11], sevoflurane[12,13] and propofol[14] can affect proliferation, migration and invasion of tumor cells. However, the effect of tramadol on tumorgenesis and carcinogenic of tumor cells, including breast cancer cells remains unclear.

Adrenoceptors include α- and β-adrenoceptor that modulate diverse intracellular processes, such as DNA synthesis through activation of mitogen-activating protein kinases (MAPKs)[15]. The α-adrenoceptor, a member of the superfamily of G protein-coupled adrenoceptors, mediates reactions of endogenous catecholamines in a variety of target cells[15] and its subtypes have been classified as α1A-, α1B-, α1D-, α2A-, α2B-, and α2C-adrenoceptors[16,17]. α2-adrenoceptors in hu-

man breast cancer cells have been proved to be associated with a increased cell proliferation *in vitro* and with increased tumour growth *in vivo*[18,19]. Furthermore, α2-adrenoceptor antagonist rauwolscine has also been demonstrated to significantly suppress tumour growth in a model of mouse mammary tumours and human breast cancer cells growing in nude mice[19,20]. Similarly, another α2-adrenoceptor antagonist, yohimbine, could also inhibit pancreatic cancer cell growth by inducing apoptosis[21]. In addition, tramadol was confirmed to induce a significant decrease in the α2-adrenoceptor in the rat brain[22]. However, whether tramadol has any function on α2-adrenoceptor in breast cancer remains unknown.

We speculated that tramadol maybe affect proliferation, migration and invasion of breast cancer cells via inactivation of α2-adrenoceptor. Thus, the aim of the present study is to investigate the effects of tramadol on proliferation, migration and invasion of human breast cancer cell line MDA-MB-231 in an *in vitro* model and subcutaneous xenograft tumors in mice *in vivo*. We also investigate whether the effects of tramadol on MDA-MB-231 cells are exerted through inactivation of α2-adrenoceptor.

Materials and methods

Materials

Leibovitz's L15 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Carlsbad, CA, USA). 3,3-dimethyl-2-thionotetrahydro-1,3,5-thiadiazine (MTT), tramadol and yohimbine were purchased from Sigma Chemicals (St. Louis, MO, USA).

Concentration and the Administration Time of Tramadol and Yohimbine

According to previously results, in the present study, proliferation, migration and invasion of breast cancer were tested after treatment of 2 μM tramadol for different time (0, 7, 14, or 28d)[23,24] or 50 μM yohimbine for 48h[21].

Cell Culture

Human breast cell line MDA-MB-231 was obtained from Shanghai Cell Biology Medical Research Institute, Chinese Academy of Sciences. Cells were cultured in Leibovitz's L15 medium supplemented with 10% FBS, 100U/ml penicillin, and 100μg/ml streptomycin at 37°C in a humidified and CO_2-free conditions.

Cell Proliferation Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) was applied to measure the proliferation of breast cancer cells following the manufacturer's instructions. MDA-MB-231 cells were plated in 96-well microtiter plates at a density of 3×10^3 cells/well and incubated overnight to allow for cell adherence. The cells were incubated for 48h. Then, 20 μl of 5 mg/ml solution of MTT dissolved in PBS was added to each well and the cells were further incubated at 37°C for 4 h. The supernatant was removed, the pellet was resuspended and 150 μl dimethyl sulfoxide (DMSO) were added to each well, followed by agitation at room temperature for 15 min to dissolve the precipitates. The optical density at 490 nm was determined with an enzyme linked immunosorbent assay (ELISA) reader (Molecular Devices, Silicon Valley, CA, USA). Cell proliferation was defined as the growth of treated cells compared with untreated cells.

Would Healing Assay

Cell migration was detected by using a wound healing assay. MDA-MB-231 cells were seeded in 24-well plates (2×10^5 cells per well). When the cells reached 100% confluence, cell layers were scraped with a 200 μL micropipette tip to create a clean wound area across the central period of the well. Cells were washed three times to remove the detached cells and cellular debris. Wound closure was recorded and photographed with a light microscope (Olympus, Lake Success, NY, USA). Five randomly selected points along each wound were marked, and the horizontal distance of migrating cells from the initial wound was measured (setting the gap width at 0 hour as 0 %).

Transwell Assay

After treated with 2 μM tramadol for different time, the MDA-MB-231 cells were placed in a CO_2 incubator for an additional 24 h of culture. For invasion assay, the membrane invasion culture system (transwell membranes of 6.5 mm diameter and 8 μm pore size; Millipore, Billerica, MA, USA) was used according to the standard protocol. Cells (2×10^5 cells/well) were added into the Matrigel-coated upper compartment of the chamber for 48 h. FBS medium (20%) was added to the lower wells as a chemoattractant. 48 hours later, the noninvasive cells on the upper surface of the membrane were removed with a cotton swab. The transformed cells migrated

through the Matrigel matrix and stuck to the lower surface of the membrane. The membrane was removed and fixed with 4% paraformaldehyde, stained with 1% crystal purple. The invasive cells were then counted (five high-power fields/chamber) using an inverted microscope (Olympus, Lake Success, NY). Each tests repeated in triplicate.

in vivo Tumor Growth Assays

Twelve to 4 weeks old BALB/c nude mice purchased from Shanghai Experimental Animal Center of the Chinese Academic of Sciences (Shanghai, China) were randomly sorted into 2 treatment groups: PBS treated (control) and tramadol treated. All mice were housed in a specific pathogen-free (SPF) circumstance and treated according to the guidelines of the Committee on Animals of Jinling Hospital (Nanjing, China). Animals were given free access to water and were monitored closely for any clinical signs of poor health throughout the study. All the animal experiments were conducted in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals. 6 mice in each group were performed under anesthesia by injecting 5×10^6 MDA-MB-231 cells in 0.2 ml volume into the right flank of the mouse. After the volume of tumor reached 100 mm^3, mice were administered with PBS or tramadol (20 mg/kg)[22] every day, for 28 days. Tumor length (a) and width (b) were measured once every 4 days for 28 days. The tumor volume (V) was calculated according to the formula $V = (ab)^2/2$[25]. Then tumor formation in mice was recorded by measuring tumor weight.

Protein Extraction and Western Blot Analysis

After treatment of tramadol, MDA-MB-231cells were cultured in 6-well plate (1×10^6 cells per well). Cells were washed with cold PBS and lysed with lysis buffer. The cell lysates were incubated on ice for 30 minutes and centrifuged at 14,000 g for 15 minutes. The supernatants were collected and measured with a protein assay reagent (Vazyme, Nanjing, China). Protein samples were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked at room temperature for 2 h in TBS-Tween 20 (TB-ST) buffer containing 5% non-fat milk and incu-

bated overnight at 4°C with the primary antibody against human ERK, α2B-adrenoceptor, GAPDH and a phosphorylated antibody against ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with an HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. Protein signals were detected using enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and quantified by densitometry using Bio-Rad Quantity One software (Bio-Rad, Hercules, CA, USA).

α2-adrenoceptor Inhibitor Treatment

MDA-MB-231 cells were treated with a α2-adrenoceptor inhibitor, yohimbine, and the protein expressions of α2-adrenoceptor and phospho-ERK were detected by Western blot. The proliferation, migration and invasion of cells were evaluated by MTT assay, wound healing assay and transwell invasion assay, respectively at 48 h post-yohimbine treatment.

Statistical Analysis

Data analysis was performed using SPSS for Windows software (ver.11.0; SPSS, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Differences in groups were analyzed using repeated measure analysis of variance (ANOVA). Differences among groups were assessed using one-way ANOVA, followed by Duncan's test for post hoc comparisons. $p < 0.05$ was considered to indicate statistical significance.

Results

Tramadol Inhibits the Proliferation of Breast Cancer Cells

To observe the proliferation inhibition effects of tramadol, MDA-MB-231 cells were treated with 2μM of tramadol at different time intervals, and the rate of proliferation inhibition was detected by MTT assay. As shown in Figure 1, treatment with tramadol could significantly inhibit the proliferation of MDA-MB-231 cells in a time-dependent manner.

Tramadol Inhibits the Migration of Breast Cancer Cells

To investigate the anti-migratory function of tramadol on MDA-MB-231 cells, *in vitro* wound healing assay was conducted. As shown in Fig-
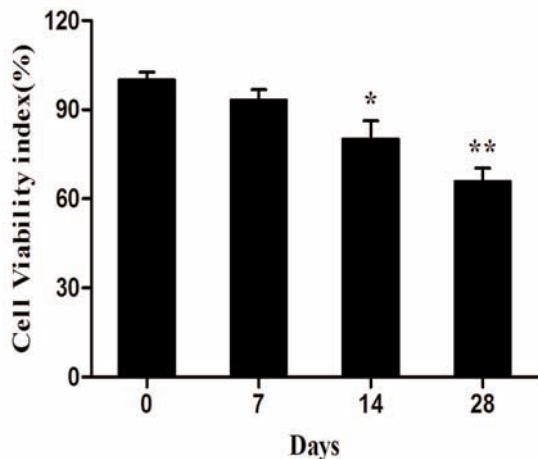
Figure 1. Tramadol inhibits breast cancer cells proliferation in vitro. MDA-MB-231 cells were treated with tramadol for different time. The rates of cell proliferation were measured using a MTT assay at various time points (0, 7, 14, and 28 days). The absorbance at 490 nm was measured. The data are presented as mean ± SD of three separate experiments. $^*p < 0.05$, $^{**}p < 0.01$.

ure 2A, compared with the group control, treatment with tramadol for 7, 14, and 28d was able to gradually slow the scratch wounds closure and consequently inhibit the migration rate of MDA-MB-231 cells.

Tramadol Suppresses the Invasion of Breast Cancer Cells

To determine the rolet of tramadol on the invasion of MDA-MB-231 cells, we determined the number of invasive cells using Transwell assay. As shown in Figure 2B, the number of MDA-MB-231 cells adherent to the outer surface of the membrane was significantly decreased in the tramadol-treated group compared to the control group, suggesting that tramadol suppressed the invasive ability of breast cancer cells. Moreover, the inhibition effect of tramadol on the invasion was in a time-dependent manner.

Administration of Tramadol Suppresses Tumor Growth in vivo.

In order to explore whether tramadol regulates breast cancer carcinogenesis, we further assessed the effects of tramadol on tumor growth *in vivo*. MDA-MB-231 cells were implanted into the right flank of the mouse in two groups. When the tumor volume reached 100 mm³, mice were administered with PBS or tramadol every day, for 28 days. During the treatment periods, tumor size

was measured starting from day 1 of post-induction of cancer and recorded once every 4 days, till the end of experiments, 28 day of post-induction. The mean volumes and weight of tumors generated from tramadol-treated were smaller than control group (Figure 3A-B). The result suggested that tramadol had proficient anticancer effects *in vivo*.

Tramadol-Mediated Anti-Tumorigenic Function is α2-adrenoceptor Dependent ERK Downregulation.

The expression of a cloned α2-adrenoceptor allows the coupling of this receptor to the p21ras-mitogen-activated protein (MAP) kinase cascade in hamster lung fibroblasts[26]. It is well known that Ras/Raf/mitogen-activated protein (MPA)-extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signaling is critical for many fundamental processes, including cell proliferation[27], survival[28], motility and migration[29]. To understand whether tramadol-mediated anti-tumorigenic function in MDA-MB-231 cells is through the inhibition of ERK signaling, we evaluated the effect of tramadol on the inhibition of ERK by western blot. Treatment with tramadol could decrease the levels of the phosphorylated ERK protein, whereas the expression level of total ERK remains unchanged (Figure 4A). When examining the potential upstream regulation of ERK, we found that the α2B-adrenoceptor protein level was also down-regulated in the same treatment of tramadol (Figure 4A). These results suggested that tramadol down-regulated the α2-adrenoceptor protein level and consistently inhibited ERK signaling.

To further explore whether tramadol-mediated inhibition of proliferation, migration and invasion is α2-adrenoceptor dependent ERK down-regulation, we treated MDA-MB-231 cells with a α2-adrenoceptor inhibwitor, yohimbine. The results of the western blot analysis revealed that the yohimbine treatment both decreased the levels of α2-adrenoceptor and reduced the ERK expression (Figure 4B). The yohimbine treatment inhibited the proliferation (Figure 5), invasion (Figure 6A) and migration (Figure 6B) of MDA-MB-231 cells relative to the group control.

Discussion

Tramadol is used mainly for the treatment of moderate to severe pain[5-7]; however, the effect of

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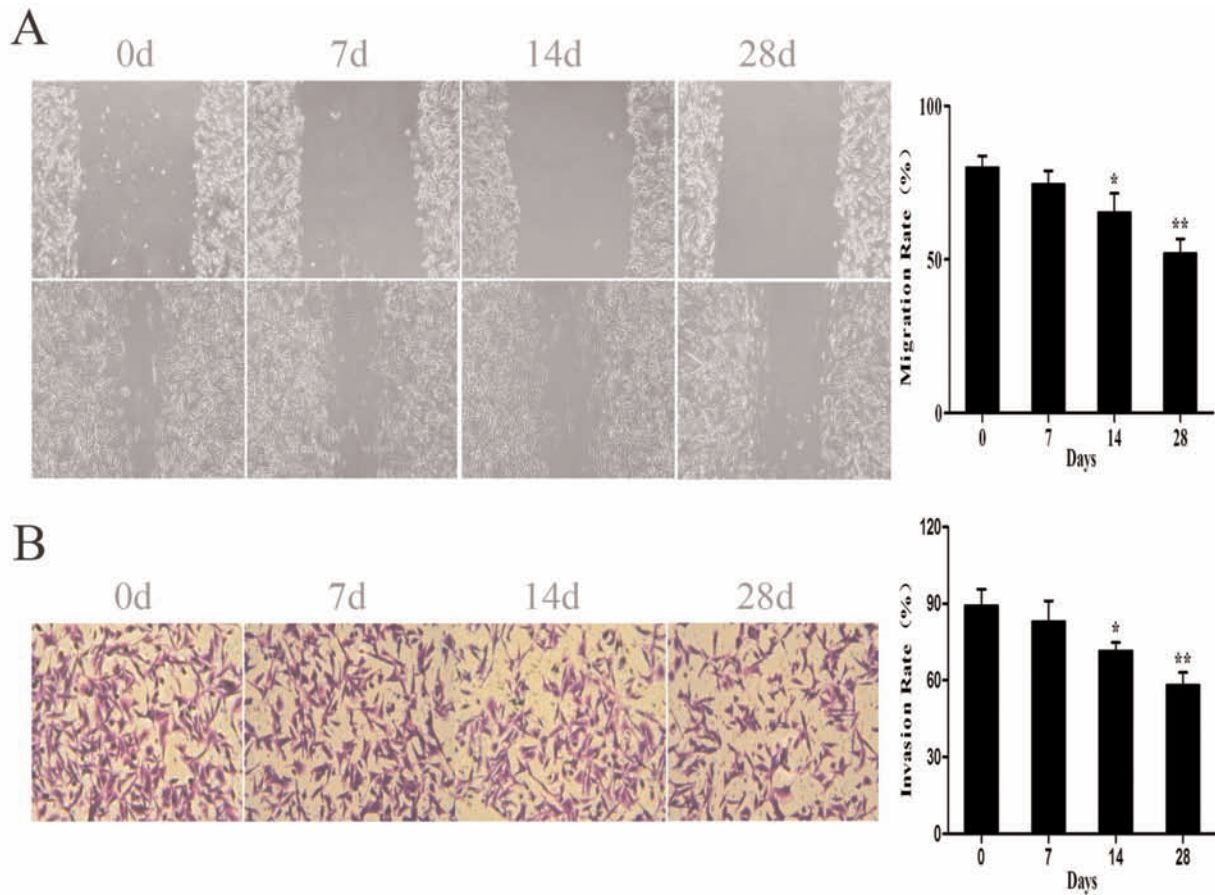

Figure 2. Tramadol decreases migration and invasion in breast cancer cells. *(A)* Representative images of wound healing assay in MDA-MB-231 cells were obtained at time point 0 and 48 h. Migration rate was quantified by measuring gap distance. *(B)* Representation of the invasive ability at different time after tramadol treatment. Data are shown as the mean ± standard deviation (SD) of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$.
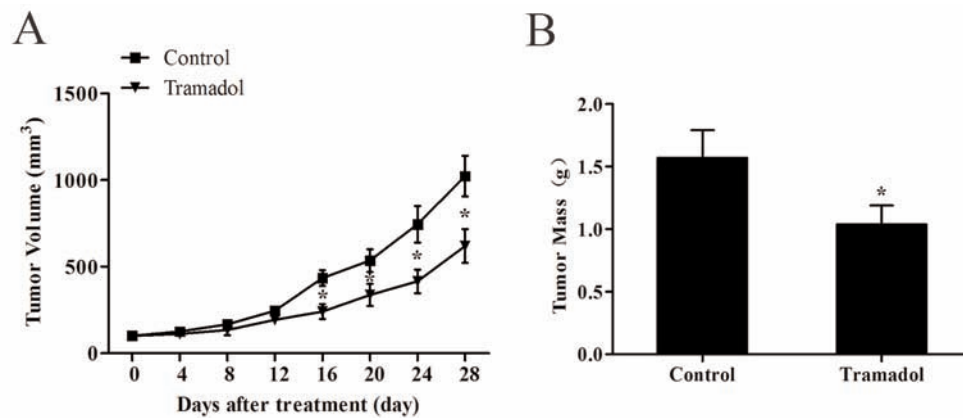

Figure 3. Tramadol suppress the growth of established tumors in vivo. *(A)* Tumor volumes were measured and recorded every 4 days after injection. *(B)* Mice were sacrificed 28 d following tramadol injection and the tumors were weighted. Data are shown as the mean ± standard deviation (SD). $^*p < 0.05$, $^{**}p < 0.01$.
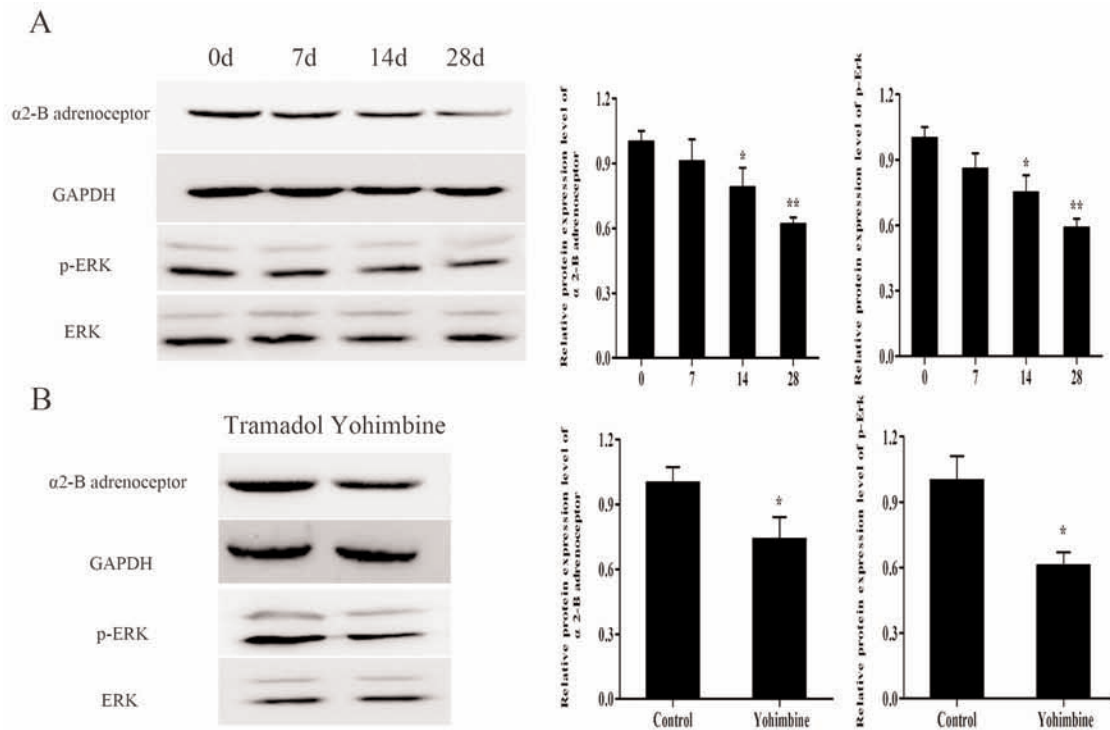
Figure 4. Tramadol and yohimbine inactivate the α2-adrenoceptor signaling. α2B-adrenoceptor, ERK and Phosphorylated ERK were evaluated by Western blot for *(A)* cells treated with tramadol at different time intervals or *(B)* treated with α2-adrenoceptor inhibitor yohimbine for 48h. $^*p < 0.05$, $^{**}p < 0.01$.

tramadol on proliferation, migration and invasion of breast cancer cells remains unclear. Previous data indicated that cancer patients were treated with intramuscular or oral tramadol 300 mg/day for periods of 14 days up to 14 months[23]. Meanwhile, Lintz et al showed that after intravenous injection of 100 mg tramadol, the concentration of it in human serum is about (612.7 ± 221) ng/mL (approximately 2 μM)[24]. Taken together, we speculate MDA-MB-231 cells treated with 2 μM tramadol for various time (0, 7, 14, or 28d) may reflect concentrations that can be used in cancer patients. In our research, we reported the novel anti-tumor effect of tramadol on the human breast cancer cell line MDA-MB-231. The present study has demonstrated that tramadol inhibited cell proliferation, migration and invasion by targeting the α2-B adrenoceptor pathway.

Uncontrolled proliferation is one of the major biological features of cancer cells, and inhibiting cell proliferation could achieve the arrest of tumor growth. In this study, we found treatment with tramadol inhibited the proliferation of breast cancer cells in a time-dependent manner *in vitro* and could also inhibit tumor growth *in vivo*. A

major feature of cancer cells is their ability to migrate and settle in surrounding or distant tissues. Inhibition of the invasion and migration potential of cancer cells would exert a positive in-
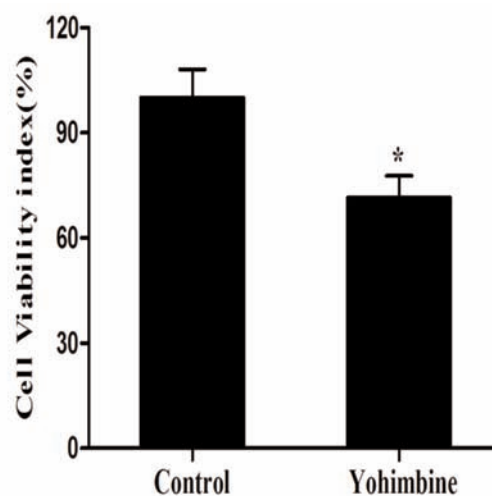

Figure 5. Yohimbine inhibits proliferation of MDA-MB-231 cells. Data are shown as the mean ± standard deviation (SD) of three independent experiments. $^*p < 0.05$.
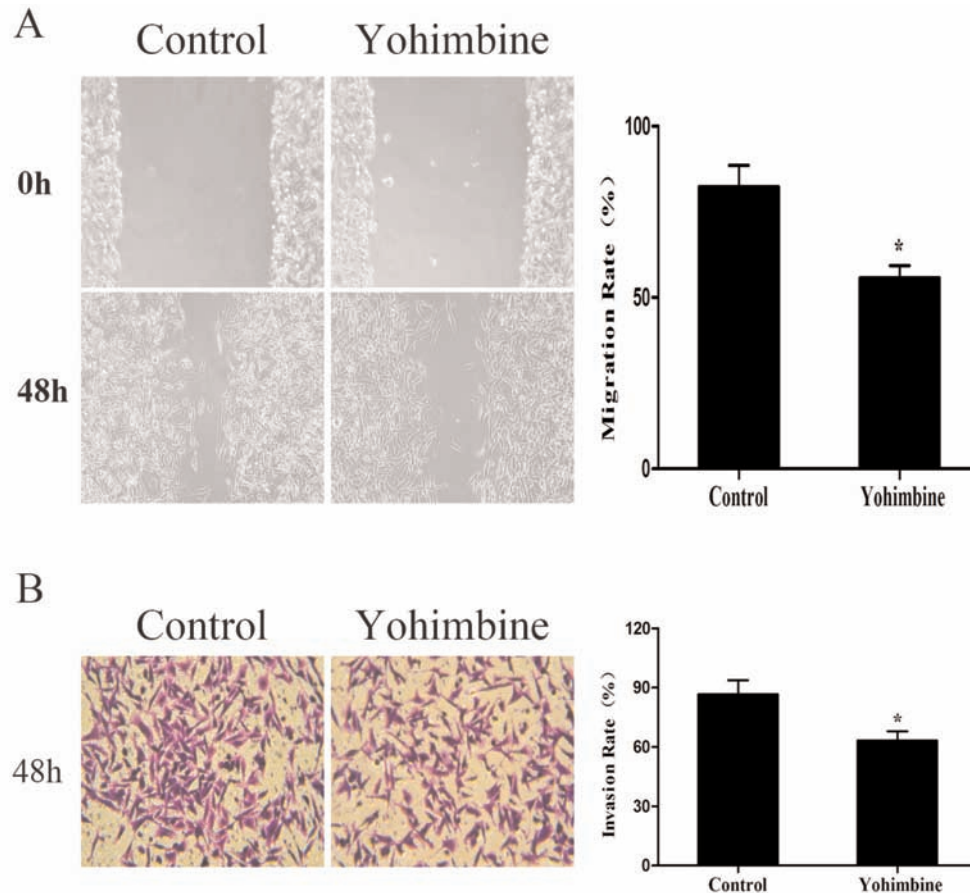
Figure 6. Yohimbine prohibits migration and invasion of MDA-MB-231 cells. (A) & (B) Influence of yohimbine on migration and invasion in MDA-MB-231 cells was detected by wound healing assay and Transwell assay. Data are shown as mean ± SEM (n=3), *p* < 0.05.

fluence on breast cancer mortality rates[30]. Our data presented the migration and invasion ability of breast cancer cells decreased markedly after usage of tramadol in a time-dependent manner.

Growing evidences showed α2-adrenoceptors is accounting for the aggressive proliferation of human breast cancer cells *in vitro* and *in vivo*[18,19]. Shen et al reported that α2-adrenoceptor antagonist, yohimbine, plays an opposite role on cell growth by decreasing the level of α2-adrenoceptor in pancreatic cancer[21]. More important, Faron-Górecka et al suggested that the expression of α2-adrenoceptors could be notably compromising by tramadol in the rat brain[22]. To further examine the molecular mechanism of tramadol action, we studied the regulation of α2-adrenoceptor signaling pathway. Similar to Vazquez et al work[18], we found only α2B-adrenoceptor was expression in MDA-MB-231 cells. As we hypothesized, long-term effects of

tramadol significantly reduced the protein level of α2B-adrenoceptor.

The expression of a cloned α2-adrenoceptors allows the coupling of this receptor to the p21ras-mitogen-activated protein (MAP) kinase cascade in hamster lung fibroblasts[26]. ERK is one of the key signaling proteins in MAPK pathway[31,32]. We, therefore, explored whether tramadol has an impact on ERK signals in the MDA-MB-231 cells. At the molecular level, the data showed tramadol treatment significantly decreased α2B-adrenoceptor protein level and consistently decreased phosphorylation of ERK 1/2 without changing the total level of it.

Further we examined the function of α2-adrenoceptor on MDA-MB-231 cells. Treatment with α2-adrenoceptor inhibitor, yohimbine for 48h, was able to inhibit cells proliferation, migration and invasion of breast cancer cells, which is consistent with the finding that long-term use of

tramadol can suppress the malignancy of breast cancer cell *in vitro*. These results suggested that the tramadol-mediated anti-tumorigenic function in the MDA-MB-231 cell lines is through regulating the $\alpha2$-adrenoceptor expression.

Conclusions

The results from our study demonstrate that the clinical dosage of tramadol could exert an anti-cancer effect on proliferation, migration and invasion of human breast cancer MDA-MB-231 cells through inactivation of the $\alpha2$-adrenoceptor pathway in a time-dependent manner. Nevertheless, the function and mechanism of tramadol on other cancers still unknown, which merits further investigation.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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