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Dexmedetomidine regulate the malignancy of breast cancer cells by activating α2-adrenoceptor/ ERK signaling pathway

M. XIA[1], N.-N. JI[2], M.-L. DUAN[1], J.-H. TONG[1], J.-G. XU[1], Y.-M. ZHANG[2], S.-H. WANG[3]

[1]Department of Anesthesiology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China
[2]Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical University, Xuzhou, China
[3]Department of General Surgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China

Abstract. – OBJECTIVE: Breast cancer is one of the most aggressive and pervasive cancers identified in females. Dexmedetomidine (Dex) is an efficient anesthetic used in surgery. Our study aimed to explore the role of Dex in the malignancy of breast cancer cells *in vitro* and *in vivo*. Further, we investigate the molecular mechanism involved in the function of Dex on breast cancer cells.

MATERIALS AND METHODS: The methyl thiazolyl tetrazolium (MTT) assay was applied to detect cell proliferation. The migration and invasion capacity of MDA-MB-231 cells was tested by wound healing assay and transwell assay. Western blot analysis was performed to quantify the protein expression levels of α2-adrenoceptor and ERK.

RESULTS: The proliferation, migration and invasion ability of MDA-MB-231 cells was gradually increased after treatment of Dex in a dose-dependent manner *in vitro*. In addition, Dex could significantly elevate the volume and weight of xenotransplant tumor *in vivo*. Furthermore, Dex up-regulated the protein level of α2-adrenoceptor and consistently enhanced the phosphorylation of ERK without changing the total level of it. Similarity, over-expression of α2-adrenoceptor via its agonist Clonidine could mimic the function of Dex on breast cancer.

CONCLUSIONS: These data suggest that Dex could promote the proliferation, migration and invasion of breast cancer cells through the activation of α2B-adrenoceptor /ERK signaling.

Key Words
Dexmedetomidine, α2-adrenoceptor, Breast cancer cells, Tumorigenesis.

Introduction

Breast cancer, one of the most common and aggressive tumors, is the primary cause of cancer death in women worldwide, with an estimated nearly 232,670 new cases and 40,000 breast cancer deaths each year[1,2]. Despite advances in the early detection and diagnosis, the incidence of patients with breast cancer, following surgery and treatment with radiotherapy or chemotherapy, is still on the rise[3,4]. The cumulative evidence demonstrated that the poor prognosis of breast cancer patients is mainly due to the highly recurrence and metastasis of breast cancer cells[5]. Therefore, understanding the mechanisms associated with the progression and development of these cells is essential to improve survival in patients with breast cancer.

The adrenoceptors were a member of G protein-coupled receptors and played a key role in various biological processes by binding to catecholamines[6]. There are two main groups of adrenoceptors, α and β, with several subtypes[7,8]. α adrenoceptors could be further classified into different subtypes, including α1A-, α1B-, α1D-, α2A-, α2B-, and α2C-adrenoceptors[9]. Bruzzone et al[10] indicated that α2-adrenoceptors have a positive effect on the proliferation of mouse mammary tumor cell line *in vitro*[10]. In addition, it has been proved that α2-adrenoceptors were expressed in human breast cancer cell lines[11]. Furthermore, Xia et al[12] reported that tramadol could inhibit the proliferation, migration and invasion via α2-adrenoceptor signaling in human breast cancer cells, suggesting that α2-adrenoceptor may be a critical regulator in the genesis of human breast cancer.

Dexmedetomidine (Dex), a lipophilic α2 adrenergic agonist characterized with analgesic, sedative and hemodynamic effects, was used widely in reduce stress reaction and systemic inflammation, anti-anxiety, and maintain the normal function of cardiovascular system[13-16]. Re-

Corresponding Author: Shaohua Wang, MD; e-mail: shaohuawang_nj@sina.com

cently studies demonstrated that anesthetics such as morphine[17] and propofol[18] could influence the malignancy of solid tumors. However, the function of Dex in the tumorigenic processes of breast cancer cells is still unknown.

In the present study, we found that Dex could increase the proliferation, migration and invasion ability of MDA-MB-231 cells significantly through activation of the α2-adrenoceptor/ERK signaling pathway in a dose-dependent manner. Moreover, Dex also promoted the growth of established tumors *in vivo*. Collectively, these data suggested that Dex may not be suitable for cancer surgery in order to avoid the risk of recurrence and metastasis after cancer surgery.

Materials and Methods

Cell Culture

The human breast cancer cell line MDA-MB-231 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in Leibovitz's L15 medium (Gibco, Grand. Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified and CO_2-free conditions at 37°C.

Drug Treatments

On the basis of the previous study, to test the proliferation, migration and invasion ability of MDA-MB-231 cells, cells were treated with Dex (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at various concentrations (0.01, 0.1 or 1 μM) or 1 μM clonidine (Santa Cruz Biotechnology) for 48 hours[10].

Cell Proliferation Assay

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide (MTT) assay (Promega, Madison, WI, USA) was performed to analyze the proliferation of MDA-MB-231 cells following the manufacturer's instructions. Briefly, cells (2000 cells/well) were plated at 96-well microtiter plates and incubated overnight to allow for cell adherence. Then the cells were treated with DEX for different concentrations and incubated for 48 hours. MTT solution was added into each well and incubated at 37˚C for 4 h. The absorbance was measured at 570 nm by using a microplate reader (Molecular Devices, Silicon Valley, CA, USA).

Wound Healing Assay

Wound healing assay was used to test the migration ability of breast cancer cell. MDA-MB-231 cells were seeded in 24-well plates and cultured until they were grown to confluence. A wound was made by scraping with a 200 μL sterile plastic tip across the surface of the cell layers. After washed with PBS three times, cells were maintained in a fresh serum-free medium for 48 h. Cell migration from the initial wound was photographed with a light microscope (Olympus, Tokyo, Japan) in five randomly selected fields for each time point (0 and 48 hours, setting the width at 0 hour as 0%)

Transwell Assay

The invasion of MDA-MB-231 cells was assessed by using Transwell filters (8-μm pore size, Millipore, Billerica, MA, USA). A total of 1x 10^5 cells treated with Dex were resuspended in 400 μL serum-free medium and seeded into the filters coated with Matrigel in the upper chamber, 600 μL L15 medium containing 20% FBS was added to the lower chamber as a chemoattractant. 48 h later, we used a cotton swab to remove the non-invasive cells in the upper chamber. The invasive cells in the lower chamber were fixed with 4% paraformaldehyde, and then stained with 1% crystal purple. The numbers of invasive cells were imaged and counted in five randomly selected fields by using an inverted microscope (Olympus, Tokyo, Japan).

Western Blot Analysis

MDA-MB-231 cells were harvested and lysed in the lysis buffer (Vazyme, Nanjing, China) containing 1 mM PMSF and complete protease inhibitor cocktail (Roche, Mannheim, Germany). Then cell lysates were maintained for 30 minutes on ice and centrifuged at 12,000 g for 10 minutes. The protein concentration was analyzed using a BCA Protein Assay Kit (Vazyme). Protein samples were collected and separated on a 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gel, and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5 % non-fat milk for 2 hours at 37˚C and incubated overnight at 4˚C with the primary antibody against human GAPDH, α2B-adrenoceptor, ERK and phosphorylated antibody against ERK (1:1000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washed with TBST for 3 times, the membranes were incubated with an HRP conjugated secondary antibody (1:2000 dilutions; Santa Cruz Biotechnol-

ogy) at room temperature for 1 h. Immunoreactive bands were detected using the chemiluminescence reagent (Millipore) and measured by a densitometer (Syngene, Braintree, UK).

Tumor Formation Assay

The study protocol was approved by the National Institutes of Health, conduction and use of experimental animal in accordance with the manuals of the Committee on Animals of Jinling Hospital (Nanjing, China). Severe combined immune deficient (SCID) mice obtained from Shanghai Experimental Animal Center of the Chinese Academic of Sciences (Shanghai, China) were injected with 5×10^6 MDA-MB-231 cells into the right flank under anesthesia. When animals had developed carcinoma nodules of about 100 mm^3 in volume, the administration of DEX was initiated (day 1). The treated mice were randomly separated into 2 groups of six animals each: DEX-treated (0.05 mg/kg/day[10]) and PBS-treated. During the experiments, Tumor length (a) and width (b) were measured accurately once every 4 days by caliper measurements. At day 28, all mice were euthanized after the final drug application. Finally, tumorigenesis of mice was indicated by measuring tumor volume (volume = length x $width^2/2$) and weight.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD); Student's *t*-test was used for analysis results when only two groups were tested, and one-way analysis of variance was used to compare multiple groups. In all cases, $p < 0.05$ was regarded as statistically significant.

Results

DEX promotes the proliferation of breast cancer cells in vitro

MDA-MB-231 cells were treated with various concentrations (0.01, 0.1 or 1 μM) of Dex for 48h and the MTT assay was applied to measure the function of DEX on the proliferation of breast cancer cells. As shown in Figure 1, the rate of cell proliferation was increased markedly in a dose-dependent manner in the Dex-treated group compared with the control group, suggesting that Dex could significantly promote the proliferation of breast cancer cells.

DEX increases the migration and invasion of breast cancer cells in vitro

Wound healing assay was performed to detect the effect of Dex on cell migration. As illustrated in Figure 2A, compared with the control group, the cells treated with Dex showed considerably faster migration. The scratch wounds closure was gradually increased after treatment of Dex in a dose-dependent manner.

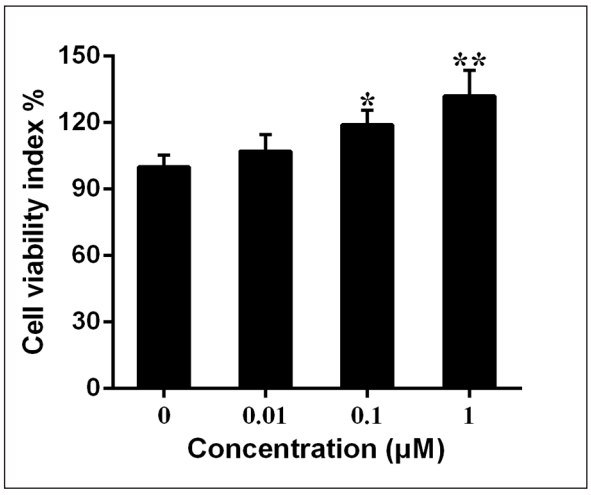

Figure 1. Dex promotes breast cancer cells proliferation in vitro. MDA-MB-231 cells were treated with different concentrations of Dex for 48 hours. The rates of cell proliferation were evaluated using a MTT assay. The absorbance at 570 nm was detected. Data are shown as the mean ± standard deviation (SD) of three independent experiments. *$p < 0.05$, **$p < 0.01$.

Having confirmed the pro-migratory role of Dex in MDA-MB-231 cells, we hypothesized that Dex might have a similar effect on the invasion of MDA-MB-231 cell. Just as we expected, treatment with Dex markedly elevated the invasion rate of breast cancer cells (Figure 2B). The total number of invaded cells was increased significantly in the Dex-treated group compared with control group. In conclusion, these above data demonstrated that DEX could enhance both the migration and invasion ability of MDA-MB-231 breast cancer cells *in vitro*.

Administration of DEX promotes the carcinogenesis of breast tumor in vivo

In order to test whether Dex regulates the carcinogenesis of breast cancer *in vivo*, we assessed the effects of Dex on tumor growth *in vivo*. The MDA-MB-231 cells (5×10^6) were hypodermic implanted into the right flank of mice. When the tumor volume reached approximately 100 mm^3, mice were treated with PBS or DEX (0.05 mg/kg/day) each day, for four weeks. During the time period, we measured and recorded the tumor size once every 4 days till the end of experiments. As shown in Figure 3A and 3B, the mean volumes and weight of

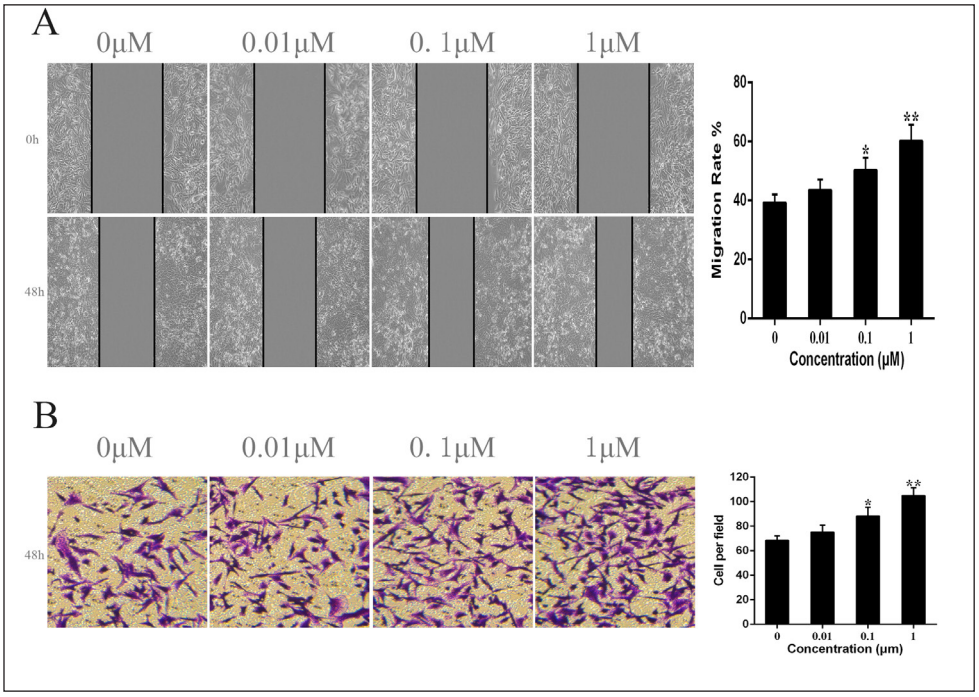

Figure 2. Dex increases the migration and invasion rate of breast cancer cells. (**A**) Images of wound healing assay in MDA-MB-231 cells obtained at time point 0 and 48 hours. Migration ability was assessed by measuring gap distance. (**B**) The invasive ability in MDA-MB-231 cells treatment with different concentrations of Dex for 48 hours. The data are presented as mean ± SD of three separate experiments. *p < 0.05, **p <0.01.

tumors in DEX-treated-mice were higher than the PBS-treated group. The result indicated that Dex might have potential carcinogenic effects *in vivo*.

Dex activates the α2-adrenoceptor/ERK signaling pathway

A previous work[11] denoted that only α2B-adrenoceptor was expressed in MDA-MB-231 cells. First, we examined the protein level of α2B-adrenoceptor post-treatment with DEX in breast cancer cells; the results revealed that DEX could up-regulate the α2B-adrenoceptor protein expression level gradually in a dose-dependent manner (Figure 4A). In addition, Xia et al[12] have shown that tramadol-mediated inhibitory effect on the malignancy of breast cancer cells is α2-adrenoceptor dependent ERK down-regulation. Therefore we hypothesized that the positive effect of Dex in MDA-MB-231 cells is through the stimulation of ERK signaling. To test this hypothesis, Western blotting analysis was applied to evaluate the expression of ERK in Dex-treated group and control group. As shown in Figure 4A, the protein level of the phosphorylated ERK was increased, while the total level of ERK remains unchanged. The result demonstrated that DEX might activate ERK signaling pathway through increasing the expression level of α2B-adrenoceptor.

Further, we treated MDA-MB-231 cells with clonidine, a α2-adrenoceptor agonist, to examine

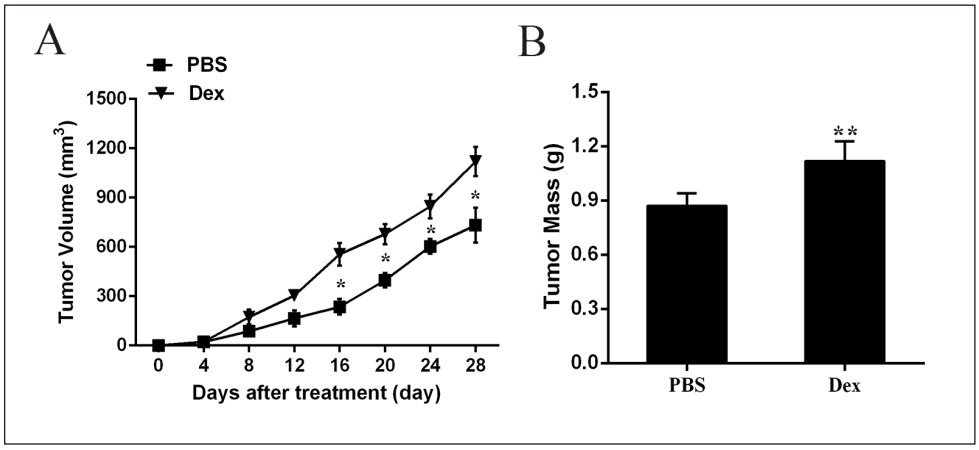

Figure 3. Dex accelerates the growth of established tumors in vivo. (**A**) Tumor volumes were detected and recorded every 4 days after injection. (**B**) Mice were sacrificed at day 28 and the tumors were weighted. *p < 0.05, **p <0.01.

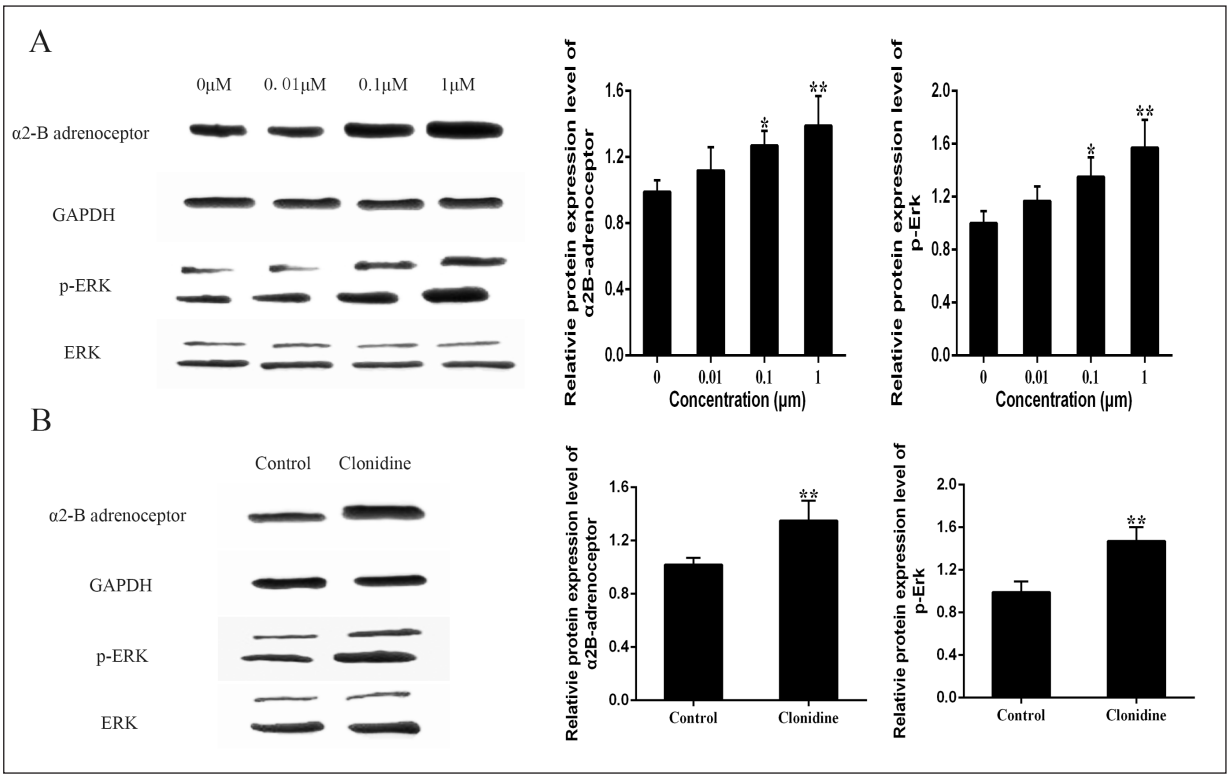

Figure 4. Dex and clonidine activate the α2-adrenoceptor signaling. α2B-adrenoceptor, phosphorylated ERK as well as total ERK were quantified by Western blot for (**A**) cells treated with Dex at different concentrations or (**B**) treated with α2-adrenoceptor agonist clonidine for 48h. Data are shown as the mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$.

whether Dex indeed exerts its function through up-regulation of α2B-adrenoceptor. Western blotting analysis revealed that the expression level of α2B-adrenoceptor and phosphorylated ERK was up-regulated in clonidine-treated group (Figure 4B). Moreover, treatment with clonidine could significantly accelerate the proliferation (Figure 5), migration (Figure 6A) and invasion (Figure 6B) of breast cancer cells, which further supports that the Dex-mediated pro-tumorigenic effect on MDA-MB-231 cells is through the activation of α2B-adrenoceptor /ERK signaling.

Discussion

Various common anesthetics were put into use in tumor patients' surgery, such as morphine, pentobarbital, tramadol, sufentanil and Dex. A recent study[19] demonstrated that intermittent hypodermic injections of morphine are nontoxic and highly effective for the management of terminal cancer patients who were undergoing dyspnea. It has also been revealed that pentobarbital had the protective effect of radiation[20,21]. Moreover, Skolnick at al[22] found that pentobarbital could improve the radio-therapeutic function in brain tumors.

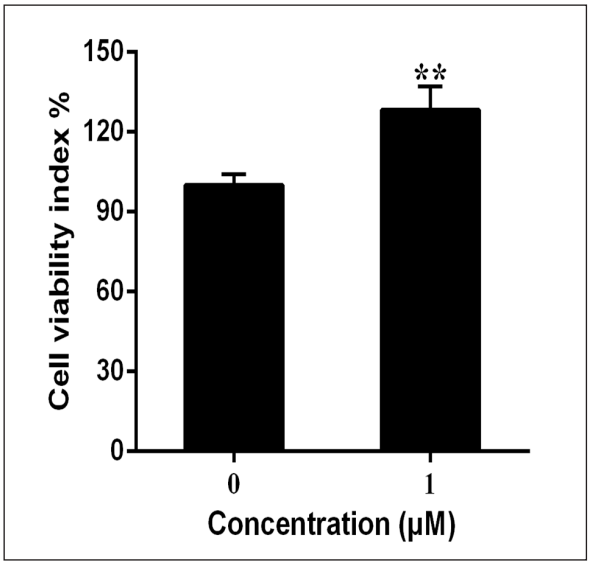

Figure 5. Clonidine promotes proliferation of MDA-MB-231 cells. Data are shown as mean ± SD (n=3), **$p < 0.01$.

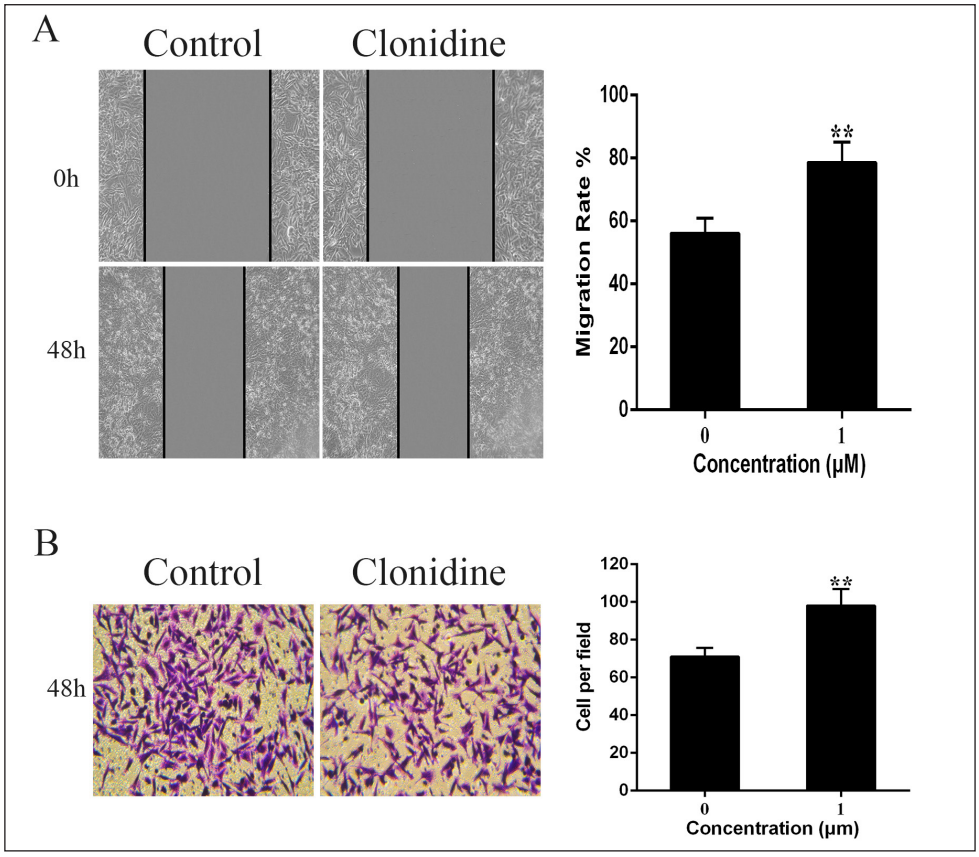

Figure 6. Clonidine increases migration and invasion of MDA-MB-231 cells. (**A**) & (**B**) Influence of clonidine on the migration and invasion ability of MDA-MB-231 cells was measured by wound healing assay and transwell assay. Data are shown as the mean ± standard deviation (SD) of three independent experiments. ** <0.01.

Dex, a α2-adrenoceptor agonist, is an efficient analgesic used to reduce the systemic inflammation and intense pain of patients in intensive care units and to improve diaphragmatic function in perioperative patients under mechanical ventilation[23]. Recently, Dex was found to have a protective effect of cell apoptosis in lung alveolar epithelial cells by promoting the cell proliferation[14]. Furthermore, α2-adrenoceptors was reported to contribute to the aggressive proliferation of mouse mammary tumor cells[10]. Therefore, we hypothesized that Dex might have a positive effect in human breast cancer tumors. As a consequence, we demonstrated that Dex could promote the malignancy of MDA-MB-231 cells *in vitro* via increasing the protein level of α2B-adrenoceptor, which is inconsistent with the role of Dex found in lung alveolar epithelial cells. More important, we also found that DEX could promote the carcinogenesis of breast tumor *in vivo*. Collectively, these results suggested that Dex may not be suitable for surgery in breast cancer patients.

Extracellular signal-regulated kinase (ERK1/2) is one of the key signaling proteins of the Ras/Raf/MAPK signaling pathway[24,25], which is involved in various functions including the regulation of carcinogenesis and progression of tumor cells[26-28]. We wonder whether Dex has any impact on the expression of ERKs. In our present study, the protein expression of phosphorylated ERK was up-regulated significantly post-treated with Dex for 48 hours in a dose-dependent manner.

To further determine whether α2-adrenoceptor is the key mechanism for Dex to regulate the development and progression of breast cancer, MDA-MB-231 cells were treated with α2-adrenoceptor agonist, Clonidine for 48h. Similarly, cell proliferation, migration as well as invasion were promoted after treatment with Clonidine. These data indicated that the Dex-mediated pro-tumorigenic function in breast cancer cells is dependent on the positive regulating of α2B-adrenoceptor.

Conclusions

Our data presented that Dex could significantly increase the proliferation, migration and invasion of MDA-MB-231 cells via activation of the α2B-adrenoceptor/ERK signaling pathway in a dose-dependent manner, which means Dex may not be suitable for the surgery of breast cancer patients.

Conflict of Interests

The Authors declare that there are no conflicts of interest.

Acknowledgement

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