Function of microRNA-141 in human breast cancer through cytotoxic CD4+ T cells regulated by MAP4K4 expression

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Abstract. The present study investigated the anti-cancer effect of microRNA (miRNA)-141 on apoptosis rate of breast cancer cells and the possible underlying mechanism. In patients with breast cancer, the expression of miRNA-141 was downregulated. Overexpression of miRNA-141 reduced breast cancer cell growth, inhibited the expression of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2) and tumor necrosis factor (TNF)-α, and increased the expression levels of interleukin (IL)-10. However, downregulation of miRNA-141 resulted in upregulation of COX-2, PGE2 and TNF-α expression levels, and an inhibition of IL-10. Overexpression of miRNA-141 suppressed mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) protein expression. Downregulation of miRNA-141 markedly upregulated MAP4K4 protein expression in MCF-7 cells. Promotion of MAP4K4 protein expression reduced the effects of miRNA-141 on the toxicity of CD4+ T cells on breast cancer cells. The results of the present study indicated that miRNA-141 may cause anti-tumor effects in human breast cancer cells via cytotoxic CD4+ T cells.

Introduction

Breast cancer is the most common tumor diagnosed in women and is one of the main causes of cancer-associated mortality in women globally (1). Annually, ~1.3 million new breast cancer cases and 0.5 million breast cancer-associated mortalities occur globally (2). There were 230,000 new breast cancer cases and ~40,000 breast cancer-associated mortalities in the USA in 2013 (3). The mortality among patients with breast cancer has been decreasing in the USA since 1990 (3). The morbidity of breast cancer among Chinese women has been rising recently (3). In China 169,000 new breast cancer cases and ~45,000 mortalities are reported annually (4). Progress has been made in treatment strategies, including adjuvant chemotherapy, radiotherapy, endocrine therapy and targeted therapy (5). In addition, the detection rate of early breast cancer with good prognosis is increasing (5). Therefore, the mortality of breast cancer has been gradually decreasing (5). At present, indexes used for clinical prognostic evaluation of breast cancer include consideration of the number of metastatic lymph nodes, expression of estrogen receptor (ER), progesterone receptor and herstatin, tumor diameter and histological grade (6). These parameters and indexes have been used to guide the systemic treatment of breast cancer. Consequently, it is necessary to continue to search for more effective drugs and therapeutic strategies. Furthermore, researchers are trying to identify biological targets that may predict breast cancer prognosis and guide treatment (7).

Cyclooxygenase-2 (COX-2) is able to enhance the invasive ability of tumor cells (8). It has been demonstrated that elevated COX-2 expression may increase the activity of matrix metalloproteinase-2 (8). As a result, metalloproteinase-2 expression is upregulated, which promotes tumor invasion of the lymph nodes and metastasis (8). Furthermore, cancer cells with upregulated COX-2 expression can induce a paracrine effect (9), which may induce adjacent epithelial cells to express COX-2, which in turn may result in malignant transformation, promoting tumor proliferation. Patients with elevated COX-2 expression have low long-term disease-free survival (10). In addition, elevated COX-2 expression is associated with accelerated tumor proliferation, negative ER and lymph node metastasis. COX-2 may induce metastasis of breast cancer (10). It has been demonstrated that COX-2 expression is associated with breast cancer lymph node metastasis, tumor differentiation, blood supply and negative ER (11).

Previous studies of breast cancer have focused on its molecular mechanism. The aim of breast cancer research is to identify pathogenic and diagnostic factors similar to alpha fetoprotein in primary liver cancer (12). The arachidonic acid pathway is an important molecular pathway in tumor research; it has been studied in other tumors, especially gastrointestinal tumors (12). Prostaglandin E2 (PGE2) in this pathway is closely associated with tumors. PGE2 may affect tumorigenesis, development and transformation through multiple downstream pathways (13).

A previous study reported that the mitogen-activated protein kinase (MAPK) signaling pathway is associated with

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multiple cellular biological behaviors (14). These include apoptosis, differentiation, proliferation, cell cycle control, cell survival and malignant transformation of cells (15). MAP kinase kinase kinase kinase 4 (MAP4K4) is an upstream kinase of the MAPK signaling system (14). MAP4K4 has been demonstrated to be upregulated in multiple tumor cells and may accelerate cell transformation (16). Furthermore, it may enhance cell invasion, reduce adhesion in cultured cells and affect tumor prognosis (16).

The mechanism underlying breast cancer metastasis remains to be elucidated (17). Identification of breast cancer metastasis-associated microRNAs (miRNAs) has provided a novel approach for research into breast cancer metastasis (18). miRNAs are involved in tumorigenesis and developmental processes, including breast cancer cell growth, apoptosis, migration and invasion. miRNAs may regulate breast cancer metastasis (18). Certain miRNAs promote breast cancer metastasis, while other miRNAs serve inhibitory roles (17). Therefore, the present study aimed to investigate the anti-cancer effect of miRNA-141 on the apoptosis rate of breast cancer cells and the possible underlying mechanism.

Materials and methods

Patients and ethical approval. A total of 56 patients with breast cancer (55-64 years old, female) and 6 healthy volunteers (58-62 years old, female) included in the present study were admitted to the Department of Breast Surgery, the First Affiliated Hospital of Jinan University (Guangzhou, China) from May to October 2015. Characteristics of patients with breast cancer and healthy controls are presented in Table I. Blood samples were obtained, and serum was collected by centrifugation at 1,000 x g for 20 min at 4˚C and stored at -80˚C. All human studies were approved by the Ethics Committee of the First Affiliated Hospital of Jinan University. All patients signed written informed consent forms prior to the study. All animal experiments were approved by the Laboratory Animal Ethics Committee of Jinan University.

Isolation of CD4$^+$ T cells. C57BL/6 mice (6 weeks old, 19-20 g, n=8, male) were purchased from Animal testing center of Jinan University (Guangzhou, China) and housed at 22-23˚C, 55-60% humidity, 12 h light/dark cycle and had free access to food and water. C57BL/6 mice were anesthetized using 35 mg/kg pentobarbital sodium and sacrificed by decollation. Splenocytes were collected and homogenated using PBS. CD4$^+$ T cells were isolated from splenocytes of C57BL/6 mice using a CD4 isolation kit according to the manufacturer's protocol (Miltenyi Biotec, Inc., Cambridge, MA, USA).

Cell culture and transfection. Human breast cancer MCF7 cells were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U penicillin/ml and 10 μg streptomycin/ml at 37˚C in a humidified atmosphere containing 5% CO_2. MAP4K4 plasmid (5'-GGCGAACGACTC CCCTGCAA-3' and 5'-TGAGAGTTAGGGTTTTGCAT-3'), miRNA-141 (5'-CGGCCGGCCCTGGGTCCATC-3' and 5'-CTCCCGGGTGGGTTC-3'), anti-miRNA-141 and negative control mimics were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). MAP4K4 plasmid (500 ng), miRNA-141 (200 ng), anti-miRNA-141 (200 ng) and negative control mimics (200 ng) were transfected into MCF7 cells using 40 nM Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following transfection for 4 h, new DMEM was added into MCF7 cells and CD4$^+$ T cells (1x10^6 cells/ml).

Table I. Characteristics of patients with breast cancer and healthy controls.

Variable	Patients	Healthy volunteers
Number	56	6
Age (years)	55-64	58-62
Female	56	6
Male	0	0
Tumor size, ≤3.0 cm	31	n/a
Tumor size, >3.0 cm	25	n/a
Edmondson grade I-II	23	n/a
Edmondson grade III-IV	33	n/a

n/a, not applicable.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Mature mRNA was isolated using the miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany) from tissues or transfected cells and reverse-transcribed using the a miRCURY LNA™ Universal RT miRNA PCR kit (Exiqon A/S, Vedbaek, Denmark), in accordance with the manufacturer's protocol. qPCR was performed using SYBR-Green master mix (Exiqon A/S, Vedbaek, Denmark) using an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the PCR: Initial denaturation at 95˚C for 5 min; 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec; and 4˚C for 10 min. The following primers for miRNA-141 were used: Forward, 5'-CGCTAACACTGTCTGGTAAAG-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'. Primers used for U6 were: Forward, 5'-ATTGGAACGATACAGAGAAGATT-3' and reverse, 5'-GGAACGCTTCACGAATTTG-3'. The relative expression levels of target genes were calculated with the $2^{-\Delta\Delta Cq}$ method (19).

Flow cytometry. A total of 5 ml peripheral blood was collected and added into lymphocyte separation medium (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Lymphocytes were collected by centrifugation at 800 x g for 20 min at 4˚C, fixed with 4% paraformaldehyde for 15 min at room temperature, blocked with 2% BSA (Beyotime Institute of Biotechnology, Haimen, China) in PBS for 1 h at 37˚C and incubated with anti-natural killer (NK) cell (1:100, cat. no. 564537), anti-CD4-APC (1:100, cat. no. 560837), anti-CD8-PEcy5 (1:100, cat. no. 555367) at 37˚C for 15 min, all from BD Biosciences (Franklin Lakes, NJ, USA). NK cell,

and $CD4^+$ and $CD8^+$ T cell numbers were analyzed by a flow cytometer and analyzed using Flowjo 7.6.1 (FlowJo LLC, Ashland, OR, USA).

Additionally, MCF7 cells were seeded in 6-well plates at a density of $2x10^6$ cells/well, fixed with 4% paraformaldehyde for 15 min at room temperature and resuspended using a binding buffer (Nanjing KeyGen Biotech Co., Ltd.). Annexin V-enhanced green fluorescent protein and propidium iodide (both Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) were added into each well and the solution was incubated for 30 min at room temperature in the dark. Apoptosis rate was analyzed using a flow cytometer as described above.

Cell proliferation and clonogenic assay. $CD4^+$ T cells from splenocytes of WT mice and MCF7 cells following transfection were co-cultured for 24, 48 and 72 h. Cells transfected with negative control mimics served as control. Viability of MCF7 cells was determined using an MTT assay. MCF7 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well and 10 μl MTT was added into MCF7 cells and incubated for 4 h at 37˚C. Medium was subsequently removed and samples were solubilized using 150 μl dimethyl sulfoxide (DMSO), and the formazan was measured at a wavelength of 490 nm.

Determination of lactate dehydrogenase (LDH) activity and TNF-α and interleukin (IL) -10 levels. $CD4^+$ T cells and MCF7 cells following transfection were co-cultured for 48 h. MCF7 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well and cytotoxicity was detected with an LDH assay kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol.

The supernatant from $CD4^+$ T cells and MCF7 cells following transfection and co-culture for 48 h was collected following centrifugation at 1,000 x g for 10 min at 4˚C and used to measure TNF-α (cat. no. H052) and IL-10 (cat. no. H009) levels using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Optical density was measured at a wavelength of 450 nm.

Luciferase reporter assay. The 3'-untranslated region fragment of MAP4K4 containing miRNA-141 binding site was amplified by PCR and then cloned into a pGL3 luciferase reporter vector (Promega Corporation, Madison, WI, USA). The reporter vector was co-transfected with miRNA-141 or scramble control using Lipofectamine® 3000 (Invitrogen; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol provided. After 48 h, reporter activity was quantified using a Dual-Luciferase Reporter Assay kit (Promega Corporation) and normalized by comparison with *Renilla* luciferase activity.

Western blot analysis. $CD4^+$ T cells and MCF7 cells following transfection were co-cultured for 48 h. MCF7 cells were seeded in 6-well plates at $2x10^6$ cells/well, and lysed with ice-cold lysis buffer (RIPA, Beyotime Institute of Biotechnology). Protein concentration was determined using a bicinchoninic acid protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equivalent amount of proteins (50 μg/lane) were separated using 10-12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in TBS containing 0.1% Tween-20 (TBST) for 1 h at 37˚C and incubated with anti-COX-2 (cat. no. 12282; 1:1,000; Cell Signaling Technology, Inc.), anti-PGE2 (cat. no. sc-20676; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-TNF-α (cat. no. sc-8301; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-IL-10 (cat. no. sc-7888; 1:1,000; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (cat. no. sc-25778; 1:500; Santa Cruz Biotechnology, Inc.) antibodies overnight at 4˚C. The membrane was washed with TBST for 1 h and incubated with a goat anti-rabbit horseradish peroxidase conjugated IgG secondary antibody (cat. no. sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) for 2 h at 37˚C. The analysis of protein expression was visualizated using BeyoECL Moon (Beyotime Institute of Biotechnology) and resolved using Image-ProPlus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) A total of 5 μg protein was used to measure the activity of caspase-3/9 using a caspase-3 or caspase-9 assay kit (Beyotime Institute of Biotechnology).

Statistical analysis. Results are presented as means ± standard error of the mean (n=3). The data were analyzed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed by one- or two-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miRNA-141 in patients with breast cancer and the abundance of NK cells, and $CD4^+$ and $CD8^+$ T cells. In order to investigate the mRNA expression levels of miRNA-141 in patient with breast cancer, RT-qPCR was performed for the detection of miRNA-141 expression. The expression of miRNA-141 in the serum of patients with breast cancer was downregulated compared with the control group (Fig. 1A). The number of NK was not altered in patients with breast cancer (Fig. 1B). The number of $CD4^+$ T cells in patients with breast cancer was markedly reduced, compared with the control group (Fig. 1C). The number of $CD8^+$ cells was not altered in patients with breast cancer (Fig. 1D). The $CD4^+/CD8^+$ T cell ratio in patients with breast cancer was lower compared with the control group (Fig. 1F).

Overexpression of miRNA-141 affects the toxicity of $CD4^+$ T cells to breast cancer cells. Transfection with miRNA-141 mimics and co-culture with $CD4^+$ T cells increased the expression of miRNA-141, inhibited cell proliferation, increased LDH activity and increased the apoptosis rate of MCF-7 cells, compared with the control group (Fig. 2A-D). The activity of caspase-9 and -3 in MCF-7 cells transfected with miRNA-141 and co-cultured with $CD4^+$ cells increased compared with the control group (Fig. 2E and F).

Overexpression of miRNA-141 affects COX-2, PGE2, TNF-α and IL-10 expression levels. Overexpression of miRNA-141 suppressed COX-2 and PGE2 protein expression in MCF-7 cells, compared with the control group (Fig. 3A-C). Overexpression of miRNA-141 significantly reduced TNF-α protein expression and induced IL-10 protein expression in MCF-7 cells, compared with control group (Fig. 3D-E).

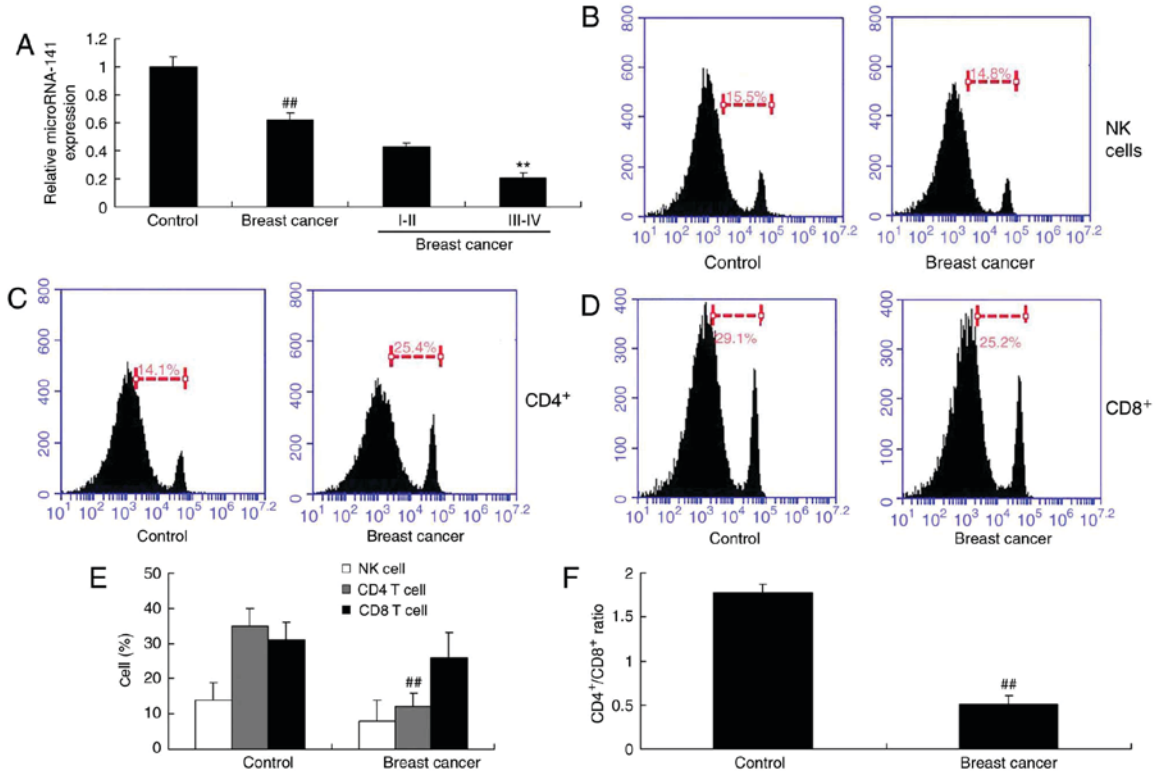

Figure 1. MicroRNA-141 expression in patients with breast cancer and NK cell, $CD4^+$ and $CD8^+$ T cell counts. (A) Expression of microRNA-141 in patients with breast cancer. Flow cytometry results for (B) NK cells, (C) CD4 T cells and (D) CD8 T cells. (E) Quantitative analysis of the results of flow cytometry. (F) $CD4^+/CD8^+$ T cell. $^{\#\#}P<0.01$ vs. the control group, $^{**}P<0.01$ vs. the breast cancer group. Control, healthy volunteers; I-II, patients with breast cancer stage I-II; III-IV, patients with breast cancer stage III-IV. NK, natural killer.

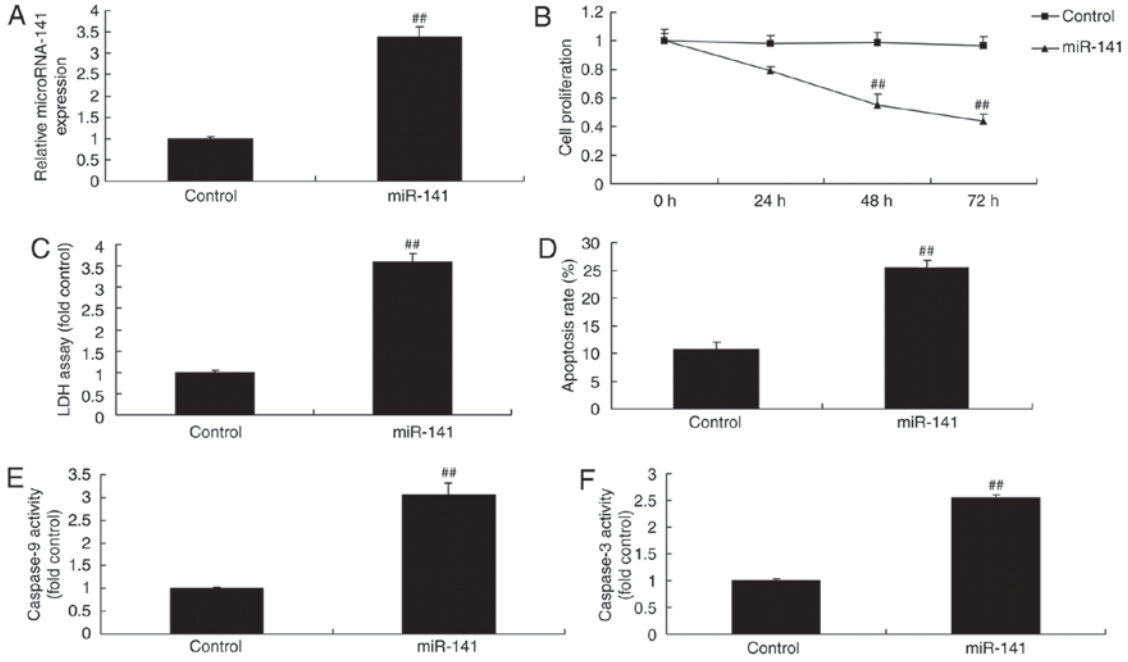

Figure 2. Overexpression of microRNA-141 affects the toxicity of $CD4^+$ T cells to breast cancer cells. (A) MicroRNA-141 expression. (B) Cell proliferation. (C) LDH activity. (D) Apoptosis rate. Activity of (E) caspase-9 and (F) caspase-3 in MCF7 cells transfected with negative control mimics and co-cultured with $CD4^+$ cells. $^{\#\#}P<0.01$ vs. the control group. Control, negative control group; miR-141, microRNA-141 over-expression group. LDH, lactate dehydrogenase.

Downregulation of miRNA-141 affects the toxicity of $CD4^+$ T cells on breast cancer cells. The present study investigated the effect of downregulation of miRNA-141 on the cytotoxicity of $CD4^+$ T cells on breast cancer cells. Anti-miRNA-141 mimics effectively reduced miRNA-141 expression, promoted cell proliferation, inhibited LDH activity and apoptosis, and increased the proliferation of MCF-7 cells, compared with the control group (Fig. 4A-D). The

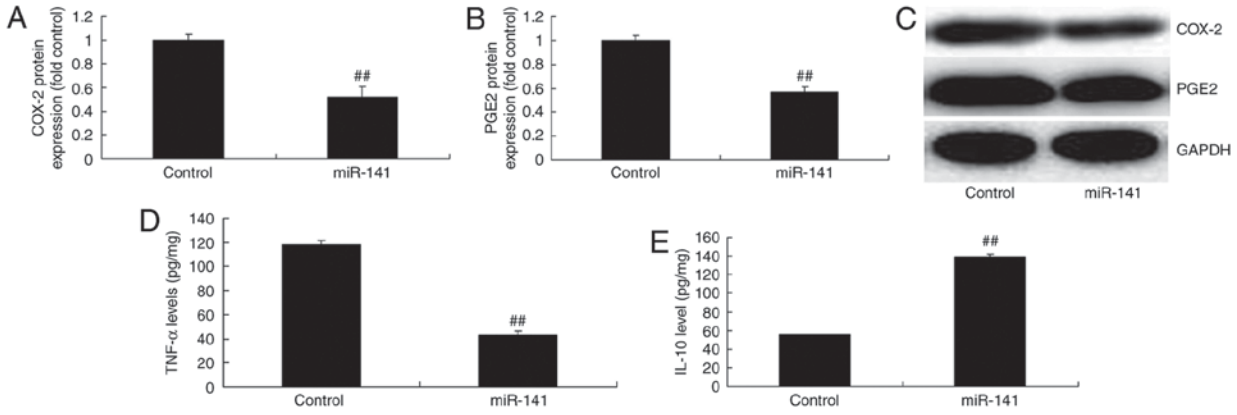

Figure 3. Overexpression of microRNA-141 affects COX-2 and PGE2 production, and TNF-α and IL-10 expression levels. Overexpression of microRNA-141 affected (A) COX-2 and (B) PGE2 protein expression, as represented by (C) western blotting. (D) TNF-α and (E) IL-10 expression levels. ##P<0.01 vs. the control group. Control, negative control group; miR-141, microRNA-141 over-expression group. COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; TNF-α, tumor necrosis factor-α; IL, interleukin.

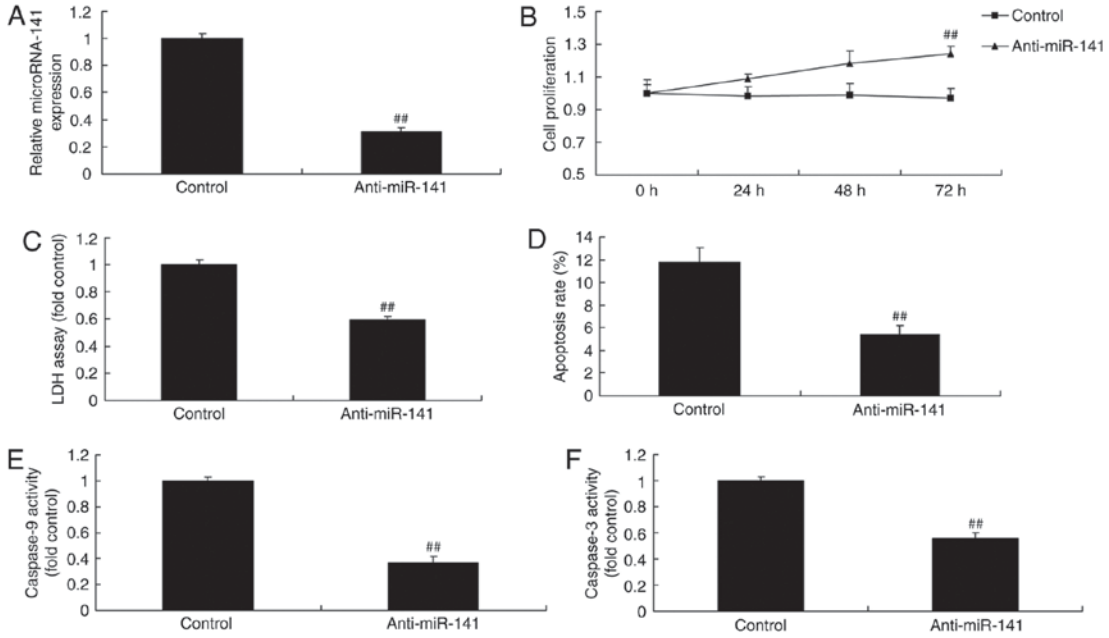

Figure 4. Downregulation of microRNA-141 affects the toxicity of $CD4^+$ T cells to breast cancer cells. (A) MicroRNA-141 expression. (B) Cell proliferation. (C) LDH activity. (D) Apoptosis rate. Activity of (E) caspase-9 and (F) caspase-3. ##P<0.01 vs. the control group. Control, negative control group; anti-miR-141, microRNA-141 downregulation group. LDH, lactate dehydrogenase.

activity of caspase-9 and -3 in MCF-7 cells transfected with anti-miRNA-141 was lower compared with the control group (Fig. 4E-F).

Downregulation of miRNA-141 affects COX-2 and PGE2 production, and TNF-α and IL-10 expression levels. Compared with the control group, overexpression of miRNA-141 significantly induced COX-2 and PGE2 protein expression in MCF-7 cells (Fig. 5A-C). Downregulation of miRNA-141 significantly induced TNF-α protein expression and suppressed IL-10 protein expression in MCF-7 cells, compared with the control group (Fig. 5D and E).

Effect of miRNA-141 on MAP4K4 protein expression. Luciferase reporter was performed to further investigate the underlying mechanism of action of miRNA-141 in breast cancer and to elucidate whether MAP4K4 is modulated in MCF-7 cells. The results of the luciferase reporter revealed that MAP4K4 may be a target gene of miRNA-141 (Fig. 6A). Overexpression of miRNA-141 significantly suppressed MAP4K4 protein expression and downregulation of miRNA-141 significantly increased MAP4K4 protein expression in MCF-7 cells, compared with the control group (Fig. 6B-E). The results suggest that MAP4K4 may be involved in the pathogenesis of breast cancer.

MAP4K4 plasmid induced the effects of miRNA-141 on protein expression. Transfection with a MAP4K4 plasmid significantly promoted MAP4K4 protein expression and induced COX-2 and PGE2 protein expression in MCF-7 cells transfected with miRNA-141, compared with the miRNA-141 only group (Fig. 7).

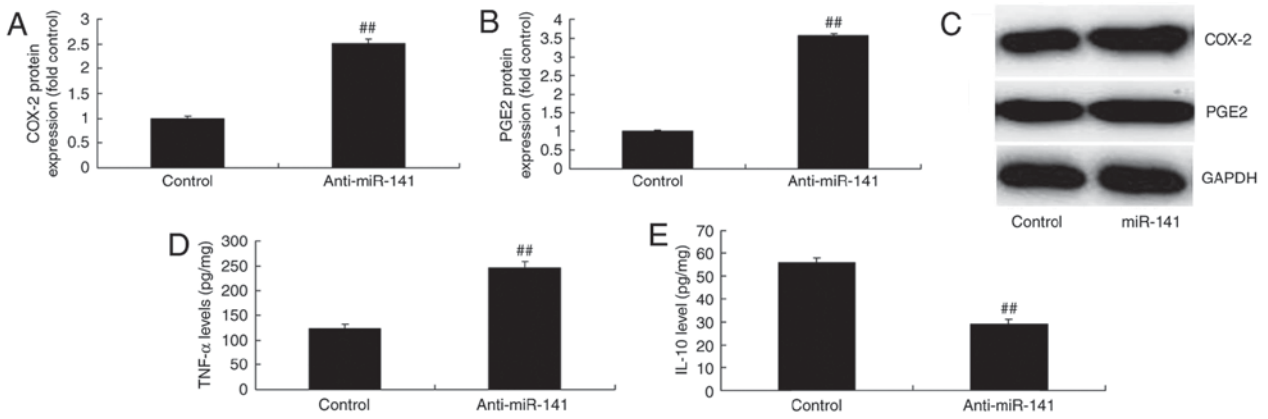

Figure 5. Downregulation of microRNA-141 affects COX-2 and PGE2 production, and TNF-α and IL-10 expression levels. The expression of (A) COX-2 and (B) PGE2 as demonstrated by (C) western blotting. (D) TNF-α and (E) IL-10 expression levels. ##P<0.01 vs. the control group. Control, negative control group; anti-miR-141, microRNA-141 downregulation group. COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; TNF-α, tumor necrosis factor-α; IL, interleukin.

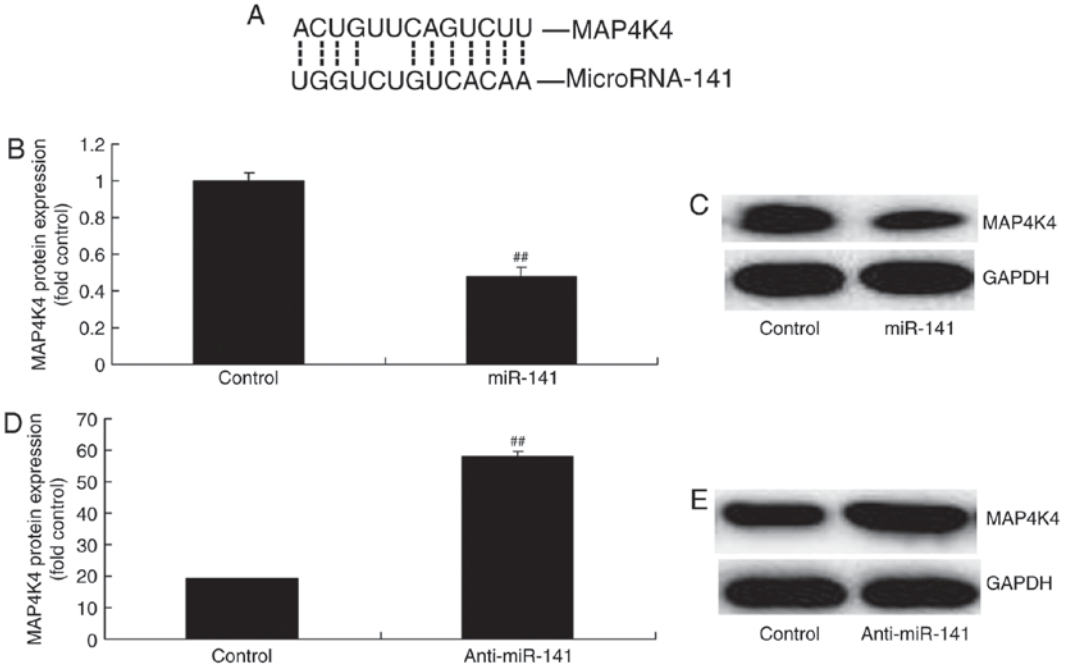

Figure 6. Effects of microRNA-141 on MAP4K4 protein expression. (A) Luciferase reporter predicted that microRNA-141 targeted MAP4K4. Protein expression of (B) MAP4K4 following transfection with miR-141 (C) demonstrated by western blotting. Expression levels of (D) MAP4K4 following transfection with anti-miR-141 (E) demonstrated by western blotting. ##P<0.01 vs. the control group. Control, negative control group; miR-141, microRNA-141 over-expression group; anti-miR-141, microRNA-141 downregulation group. MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4.

Promotion of MAP4K4 protein expression reduced the effects of miRNA-141 on the toxicity of CD⁺ T cells on breast cancer cells. The results revealed that, compared with the miRNA-141 group, the promotion of MAP4K4 protein expression increased cell proliferation, inhibited LDH activity and apoptosis rate, and reduced caspase-3 and caspase-9 activity in MCF-7 cells (Fig. 8).

Discussion

Breast cancer is one of the most common malignancies among women and its morbidity has been increasing in recent years (1). The current methods of treatment for breast cancer include surgery and chemotherapy. However, these approaches demonstrate poor efficacy for certain patients (1). Abnormal cellular apoptosis is one of the malignant manifestations of tumor cells (3). In numerous malignancies, tumor growth, invasion, metastasis and prognosis are closely associated with the level of cellular apoptosis (3). Therefore, inducing tumor cell apoptosis may inhibit tumor progression (4). Tumor metastasis is a complicated multi-step process that involves multiple factors, including oncogenes and tumor suppressor genes (4). interaction between oncogenes and tumor suppressor genes serves a role in cancer cell apoptosis. To the best of the authors' knowledge the present study was the first to demonstrate that miRNA-141 expression in the serum of patients with breast cancer was downregulated, compared with healthy controls. However, only 56 patients with breast cancer and 6 healthy volunteers were included in the present study, which is a limitation. The number of CD4⁺ T cells in patients with breast cancer was markedly reduced.

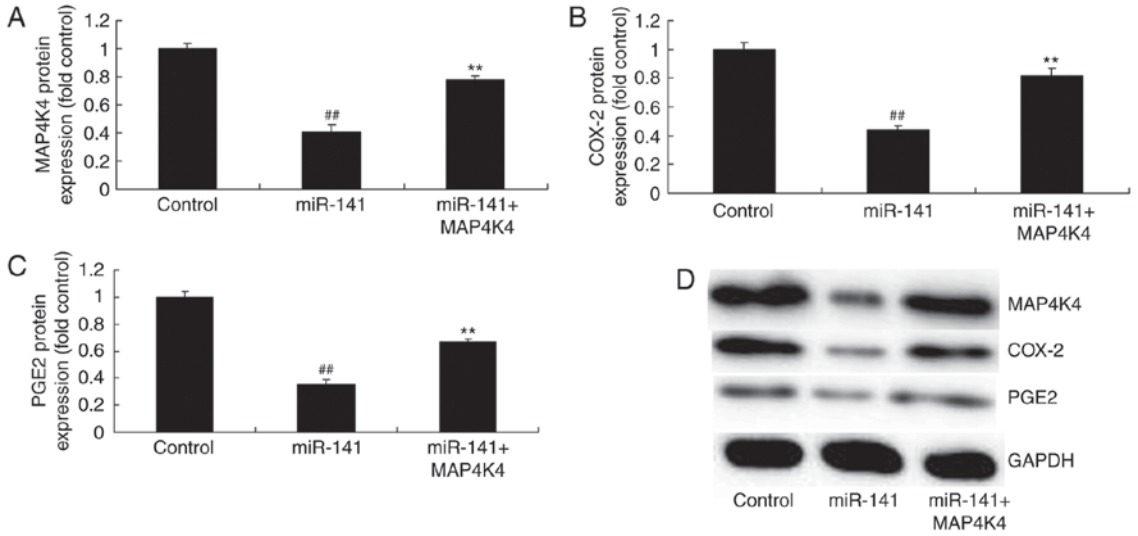

Figure 7. MAP4K4 plasmid induced the effects of microRNA-141 on protein expression. Protein expression of (A) MAP4K4, (B) COX-2 and (C) PGE2, and (D) representative western blots. ##P<0.01 vs. the control group; **P<0.01 vs. the miR-141 group. Control, negative control group; miR-141, microRNA-141 overexpression group; miR-14 + MAP4K4, microRNA-141 and MAP4K4 overexpression. MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2.

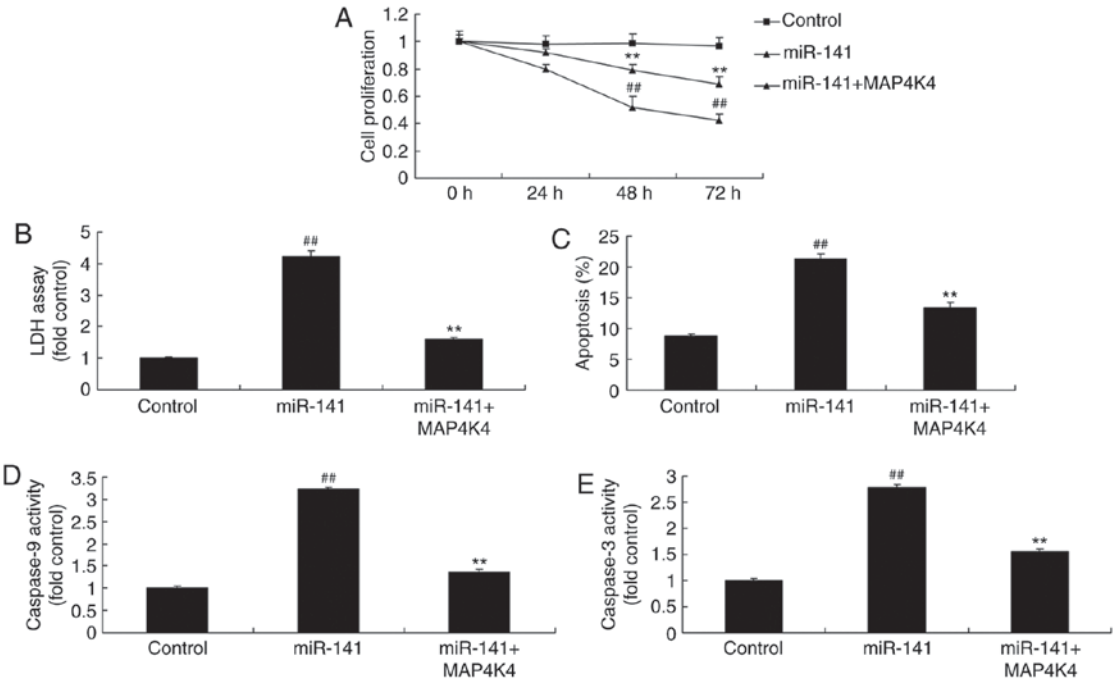

Figure 8. Promotion of MAP4K4 protein expression affects the interaction between microRNA-141 and the toxicity of $CD4^+$ T cells to breast cancer cells. (A) Cell proliferation. (B) LDH activity. (C) Apoptosis rate. Activity of (D) caspase-9 and (E) caspase-3. ##P<0.01 vs. the control group; **P<0.01 vs. the miR-141 group. Control, negative control group; miR-141, microRNA-141 overexpression group; miR-141 + MAP4K4, microRNA-141 and MAP4K4 overexpression. LDH, lactate dehydrogenase.

T cells may be classified into helper T cells (Th), cytotoxic T cells (Tc) and regulatory T cells (Treg) based on their immunological effects. Th cells serve roles in the differentiation of activated $CD4^+$ T cells (20). By contrast, Tc cells serve a role in the differentiation of activated $CD8^+$ T cells, which are cytotoxic (21). Forkhead box protein 3 (FOXP3) is a specific biomarker of $CD4^+$ Treg cells (20). FOXP3 expression reflects the number and functional activity of Treg cells. IL-10 is a cytokine expressed by a number of immune cells. It may be produced by T cells, B lymphocytes, mononuclear macrophages and keratinocytes (21). The results of the present study demonstrated that overexpression of miRNA-141 increased the toxicity of miRNA-141 on breast cancer cell growth and caspase-3/9 activity. Feng *et al* (22) demonstrated that miRNA-141 induced differentiation of $CD4^+$ T cells to induce apoptosis in colorectal cancer with lymph node metastasis.

COX-2 may be expressed in the endoplasmic reticulum or the nuclear membrane (23). Activated COX-2 is able to catalyze arachidonic acid to transport more prostaglandins

(PGs) into the nucleus, which regulates target gene transcription (23). Phospholipase A2 (PLA2) is activated by cytokines or inflammatory mediators (24). Subsequently, PLA2 is further oxidized into PGH2, the common precursor of all PGs (23,24). It can be transformed by different synthetases into bioactive end products, including prostaglandin D2 receptor 2, PGE2, SCF E3 ubiquitin ligase complex F-box protein pof2, glucose-6-phosphate isomerase 2 (24). PGE2 can prevent antigen presentation by dendritic cells. The above process allows tumors to escape immune recognition, which contributes to tumor formation. Recently, it was demonstrated that in human breast cancer specimens, aromatase cytochrome P450 is positively associated with COX-2 expression (25). T. PGE2 may increase aromatase activity, therefore elevating estrogen synthesis and directly stimulating breast cancer proliferation (26). In the present study, overexpression of miRNA-141 suppressed COX-2 and PGE2 protein expression in MCF-7 cells. Huang *et al* (27) reported that miR-141 regulates colonic leukocyte trafficking by promoting the expression of IL-10 in murine colitis and human Crohn's disease.

COX-2 is the PG synthetase, excessive expression of which may increase the levels of PG. Elevated COX-2 levels may be detected in a number of tumors (26). This may increase PG synthesis and promote tumor formation. PG is able to directly stimulate cell growth. For instance, PGE2α and the PG F2-α receptor are able to stimulate mitosis in Balb/C3T3 fibroblasts treated with pro-epidermal growth factor (EGF) (28). PGE1 and PGE2 are able to stimulate the proliferation of breast epithelial cells in the presence of EGF (28). PGE2 is able to suppress T and B cell proliferation, and cytokine synthesis, and reduce the cytotoxicity of NK cells. PGE2 may inhibit TNF-α and IL-10 production (29). IL-10 demonstrates immune inhibitory effects and is expressed in a variety of immune cells (30). T cells, B lymphocytes, mononuclear macrophages and keratinocytes secrete IL-10 (30). In addition, IL-10 exhibits a dual role of promotion and inhibition of tumors (31).

Inhibition of IL-10 increases the occurrence rate of tumors and promotes cancer cell metastasis. IL-10 demonstrates anticancer effects and suppresses breast cancer cell growth (31). In the present study, over-expression of miRNA-141 markedly reduced TNF-α protein expression and induced IL-10 protein expression in MCF-7 cells. Saito *et al* (32) demonstrated that miRNA-141 may decrease myocardial ischemia-reperfusion injury via suppression of TNF-α expression.

MAP4K4 is upregulated in multiple tumors (15). Furthermore, it has been demonstrated to serve a role in the acceleration of tumor cell transformation, the promotion of cell invasion and the reduction of cell adhesion (33). Upregulated MAP4K4 expression in pancreatic cancer is positively associated with postoperative recurrence, frequency of distant metastasis, tumor size and the number of metastatic lymph nodes (34). The present study demonstrated that overexpression of miRNA-141 markedly suppressed MAP4K4 protein expression in MCF-7 cells, while the promotion of MAP4K4 protein expression reduced the effects of miRNA-141 on the toxicity of $CD4^+$ T cells on breast cancer cells. Feng *et al* (22) demonstrated that the expression of miRNA-141 was downregulated in colorectal cancer and that MAP4K4 protein expression was increased.

In conclusion, the present study demonstrated an anti-cancer effect of microRNA-141 on breast cancer by cytotoxic $CD4^+$ T cells through MAP4K4 expression. The authors of the present study hypothesize that miRNA-141 may be a novel target for the therapy of breast cancer cells through cytotoxic $CD4^+$ T cells and COX-2, PGE2, TNF-α and IL-10 expression by MAP4K4 expression.

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Availability of data and materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QZ designed the experiments. HX and TF performed the experiments. QZ and HX analysed the data and QZ wrote the manuscript.

Ethics approval and consent to participate

All human studies were approved by the Ethics Committee of The First Affiliated Hospital of Jinan University. All patients signed written informed consent forms prior to the study. All animal experiments were approved by the Laboratory Animal Ethics Committee of Jinan University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Pu Z, Zhang X, Chen Q, Yuan X and Xie H: Establishment of an expression platform of OATP1B1 388GG and 521CC genetic polymorphism and the therapeutic effect of tamoxifen in MCF-7 cells. Oncol Rep 33: 2420-2428, 2015.
2. Børøsund E, Cvancarova M, Moore SM, Ekstedt M and Ruland CM: Comparing effects in regular practice of e-communication and Web-based self-management support among breast cancer patients: Preliminary results from a randomized controlled trial. J Med Internet Res 16: e295, 2014.
3. Maass N, Schem C, Bauerschlag DO, Tiemann K, Schaefer FW, Hanson S, Muth M, Baier M, Weigel MT, Wenners AS, *et al*: Final safety and efficacy analysis of a phase I/II trial with imatinib and vinorelbine for patients with metastatic breast cancer. Oncology 87: 300-310, 2014.
4. Walker EM, Rodriguez AI, Kohn B, Ball RM, Pegg J, Pocock JR, Nunez R, Peterson E, Jakary S and Levine RA: Acupuncture versus venlafaxine for the management of vasomotor symptoms in patients with hormone receptor-positive breast cancer: A randomized controlled trial. J Clin Oncol 28: 634-640, 2010.

5. Ohsumi S, Shimozuma K, Ohashi Y, Takeuchi A, Suemasu K, Kuranami M, Ohno S and Watanabe T: Subjective and objective assessment of edema during adjuvant chemotherapy for breast cancer using taxane-containing regimens in a randomized controlled trial: The National surgical adjuvant study of breast cancer 02. Oncology 82: 131-138, 2012.
6. Hoffman CJ, Ersser SJ, Hopkinson JB, Nicholls PG, Harrington JE and Thomas PW: Effectiveness of mindfulness-based stress reduction in mood, breast- and endocrine-related quality of life and well-being in stage 0 to III breast cancer: A randomized, controlled trial. J Clin Oncol 30: 1335-1342, 2012.
7. Khan HM, Saxena A, Gabbidon K, Rana S and Ahmed NU: Model-based survival estimates of female breast cancer data. Asian Pac J Cancer Prev 15: 2893-2900, 2014.
8. Ueno T, Chow LW and Toi M: Increases in circulating VEGF levels during COX-2 inhibitor treatment in breast cancer patients. Biomed Pharmacother 60: 277-279, 2006.
9. Generali D, Buffa FM, Deb S, Cummings M, Reid LE, Taylor M, Andreis D, Allevi G, Ferrero G, Byrne D, *et al*: COX-2 expression is predictive for early relapse and aromatase inhibitor resistance in patients with ductal carcinoma in situ of the breast and is a target for treatment. Br J Cancer 111: 46-54, 2014.
10. Brandao RD, Veeck J, Van de Vijver KK, Lindsey P, de Vries B, van Elssen CH, Blok MJ, Keymeulen K, Ayoubi T and Smeets HJ: A randomised controlled phase II trial of pre-operative celecoxib treatment reveals anti-tumour transcriptional response in primary breast cancer. Breast Cancer Res 15: R29, 2013.
11. Chuah BY, Putti T, Salto-Tellez M, Charlton A, Iau P, Buhari SA, Wong CI, Tan SH, Wong AL, Chan CW, *et al*: Serial changes in the expression of breast cancer-related proteins in response to neoadjuvant chemotherapy. Ann Oncol 22: 1748-1754, 2011.
12. Cao J, Yang X, Li WT, Zhao CL and Lv SJ: Silencing of COX-2 by RNAi modulates epithelial-mesenchymal transition in breast cancer cells partially dependent on the PGE2 cascade. Asian Pac J Cancer Prev 15: 9967-9972, 2014.
13. Tan T, Wang L and Wang B: Collagen and prostaglandin E2 regulate aromatase expression through the PI3K/AKT/IKK and the MAP kinase pathways in adipose stromal cells. Mol Med Rep 12: 4766-4772, 2015.
14. Gao X, Gao C, Liu G and Hu J: MAP4K4: An emerging therapeutic target in cancer. Cell Biosci 6: 56, 2016.
15. Liu YF, Qu GQ, Lu YM, Kong WM, Liu Y, Chen WX and Liao XH: Silencing of MAP4K4 by short hairpin RNA suppresses proliferation, induces G1 cell cycle arrest and induces apoptosis in gastric cancer cells. Mol Med Rep 13: 41-48, 2016.
16. Xiong T, Wang M, Zhao J, Liu Q, Yang C, Luo W, Li X, Yang H, Kristiansen K, Roy B and Zhou Y: An esophageal squamous cell carcinoma classification system that reveals potential targets for therapy. Oncotarget 8: 49851-49860, 2017.
17. Shen H, Li L, Yang S, Wang D, Zhong S, Zhao J and Tang J: MicroRNA-29a contributes to drug-resistance of breast cancer cells to adriamycin through PTEN/AKT/GSK3β signaling pathway. Gene 593: 84-90, 2016.
18. Tang X, Tang J, Liu X, Zeng L, Cheng C, Luo Y, Li L, Qin SL, Sang Y, Deng LM and Lv XB: Downregulation of miR-129-2 by promoter hypermethylation regulates breast cancer cell proliferation and apoptosis. Oncol Rep 35: 2963-2969, 2016.
19. Grimholt RM, Urdal P, Klingenberg O and Piehler AP: Rapid and reliable detection of alpha-globin copy number variations by quantitative real-time PCR. BMC Hematol 14: 4, 2014.
20. Bos PD, Plitas G, Rudra D, Lee SY and Rudensky AY: Transient regulatory T cell ablation deters oncogene-driven breast cancer and enhances radiotherapy. J Exp Med 210: 2435-2466, 2013.
21. Datta J, Berk E, Xu S, Fitzpatrick E, Rosemblit C, Lowenfeld L, Goodman N, Lewis DA, Zhang PJ, Fisher C, *et al*: Anti-HER2 CD4(+) T-helper type 1 response is a novel immune correlate to pathologic response following neoadjuvant therapy in HER2-positive breast cancer. Breast Cancer Res 17: 71, 2015.
22. Feng L, Ma H, Chang L, Zhou X, Wang N, Zhao L, Zuo J, Wang Y, Han J and Wang G: Role of microRNA-141 in colorectal cancer with lymph node metastasis. Exp Ther Med 12: 3405-3410, 2016.
23. Onguru O, Casey MB, Kajita S, Nakamura N and Lloyd RV: Cyclooxygenase-2 and thromboxane synthase in non-endocrine and endocrine tumors: A review. Endocr Pathol 16: 253-277, 2005.
24. Jana D, Sarkar DK, Maji A, Chikkala BR, Hassanujjaman S, Mukhopadhyay M and Ganguly S: Can cyclo-oxygenase-2 be a useful prognostic and risk stratification marker in breast cancer? J Indian Med Assoc 110: 429-433, 2012.
25. Olesch C, Sha W, Angioni C, Sha LK, Açaf E, Patrignani P, Jakobsson PJ, Radeke HH, Grösch S, Geisslinger G, *et al*: MPGES-1-derived PGE2 suppresses CD80 expression on tumor-associated phagocytes to inhibit anti-tumor immune responses in breast cancer. Oncotarget 6: 10284-10296, 2015.
26. Thakur A, Schalk D, Tomaszewski E, Kondadasula SV, Yano H, Sarkar FH and Lum LG: Microenvironment generated during EGFR targeted killing of pancreatic tumor cells by ATC inhibits myeloid-derived suppressor cells through COX2 and PGE2 dependent pathway. J Transl Med 11: 35, 2013.
27. Huang Z, Shi T, Zhou Q, *et al*: miR-141 regulates colonic leukocytic trafficking by targeting CXCL12β during murine colitis and human Crohn's disease. Gut 63: 1247-1257, 2014.
28. Yuan L, Jiang R, Yang Y, Ding S and Deng H: 1,25-Dihydroxyvitamin D3 inhibits growth of the breast cancer cell line MCF-7 and downregulates cytochrome P4501B1 through the COX-2/PGE2 pathway. Oncol Rep 28: 2131-2137, 2012.
29. Menetrier-Caux C, Bain C, Favrot MC, Duc A and Blay JY: Renal cell carcinoma induces interleukin 10 and prostaglandin E2 production by monocytes. Br J Cancer 79: 119-130, 1999.
30. Razmkhah M, Jaberipour M, Erfani N, Habibagahi M, Talei AR and Ghaderi A: Adipose derived stem cells (ASCs) isolated from breast cancer tissue express IL-4, IL-10 and TGF-β1 and upregulate expression of regulatory molecules on T cells: Do they protect breast cancer cells from the immune response? Cell Immunol 266: 116-122, 2011.
31. Yang C, He L, He P, Liu Y, Wang W, He Y, Du Y and Gao F: Increased drug resistance in breast cancer by tumor-associated macrophages through IL-10/STAT3/bcl-2 signaling pathway. Med Oncol 32: 352, 2015.
32. Saito S, Thuc LC, Teshima Y, *et al*: Glucose fluctuations aggravate cardiac susceptibility to ischemia/reperfusion injury by modulating microRNAs expression. Circ J 80: 186-195, 2016.
33. Ma M and Baumgartner M: Morphed and moving: TNFα-driven motility promotes cell dissemination through MAP4K4-induced cytoskeleton remodeling. Microb Cell 1: 154-157, 2014.
34. Chen S, Li X, Lu D, Xu Y, Mou W, Wang L, Chen Y, Liu Y, Li X and Li LY: SOX2 regulates apoptosis through MAP4K4-survivin signaling pathway in human lung cancer cells. Carcinogenesis 35: 613-623, 2014.