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
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 enhance 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. Spleenocytes were collected by homogenization using PBS and centrifugation at 800 x g for 15 min at 4000 rpm, and added into lymphocyte separation medium (Beijing Solarbio Science & Technology Co., Ltd, China). Lymphocytes were collected by centrifugation at 1,000 x g for 20 min at 4˚C and stored at -80˚C. NK cell, anti-CD4-APC (1:100, cat. no. 560837), and anti-CD4-biotin (1:100, cat. no. 564537) were used for staining. The splenocytes were stained with anti-NK cell (1:100, cat. no. 564537) and anti-CD4 mAbs for 1 h at room temperature, blocked with 2% BSA (Beyotime Biotechnology, China) and incubated at room temperature. 

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 in PBS at 4 ˚C for 1 h at 37˚C and incubated with anti-human antibody (NK cell) (1:100, cat. no. 564537), anti-CD4-APC (1:100, cat. no. 560837), anti-CD8-PFcy5 (1:100, cat. no. 555367) at 37˚C for 15 min, all from BD Biosciences (Franklin Lakes, NJ, USA). NK cell,

<table>
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<tr>
<td>Edmondson grade I-II</td>
<td>23</td>
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<td>Edmondson grade III-IV</td>
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n/a, not applicable.

Table I. Characteristics of patients with breast cancer and healthy controls.
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; 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 visualized 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 miRNA 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).
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
activity of caspase-9 and -3 in MCF-7 cells transfected with anti-miRNA-141 was lower compared with the control group (Fig. 4F).

**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).
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.
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 F2α 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 anti-cancer 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 down-regulated 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.

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