miR-494 suppresses the progression of breast cancer in vitro by targeting CXCR4 through the Wnt/β-catenin signaling pathway

LINGQIN SONG1, DI LIU1, BAOFENG WANG1, JIANJUN HE2, SHUQUN ZHANG1, ZHIJUN DAI1, XIAOBIN MA1 and XIJING WANG1

1Department of Oncology, The Second Affiliated Hospital, Medical School of Xi’an Jiaotong University, Xi’an, Shaanxi 710004; 2Department of Surgical Oncology, The First Affiliated Hospital, Medical School of Xi’an Jiaotong University, Xi’an, Shaanxi 710061, P.R. China

Received January 29, 2015; Accepted April 22, 2015
DOI: 10.3892/or.2015.3965

Correspondence to: Dr Lingqin Song, Department of Oncology, The Second Affiliated Hospital, Medical School of Xi’an Jiaotong University, 157 Xiwu Road, Xi’an, Shaanx 710004, P.R. China
E-mail: qinlingsongxa@163.com

Key words: breast cancer, microRNA-494, CXCR4, Wnt/β-catenin, epithelial-to-mesenchymal transition

Abstract. Breast cancer is the most common cancer among women with a high mortality worldwide, which is mainly due to tumor invasion and metastasis. Previous studies have reported that microRNA-494 (miR-494) is downregulated in breast cancer cells. The present study investigated the role of miR-494 in the progression of breast cancer and the underlying mechanisms. The levels of miR-494 were analyzed in several breast cancer cell lines by quantitative reverse transcription PCR (qRT-PCR). The miR-494 mRNA levels were significantly lower in the malignant breast cancer cells than the level in the non-malignant normal breast epithelial cells. miR-494 mimic transfection upregulated the expression levels of E-cadherin, yet downregulated N-cadherin, vimentin and α-smooth muscle actin (α-SMA) in the breast cancer cells. As expected, the expression of these markers in breast cancer cells transfected with miR-494 inhibitors exhibited the opposite variation trend. MTT and Transwell assays showed that cell proliferation and invasion were both significantly suppressed by miR-494 mimics, and were significantly promoted by miR-494 inhibitors. The protein expression level of chemokine (C-X-C motif) receptor 4 (CXCR4) in the breast cancer cells was significantly inhibited by miR-494 mimics, and enhanced by miR-494 inhibitors. Yet, the mRNA level of CXCR4 was barely affected by miR-494 mimics or inhibitors. Dual-luciferase assay confirmed that miR-494 directly interacted with the 3'-untranslated region of CXCR4 mRNA by dual-luciferase assay. The miR-494 mimics also significantly inhibited the transcription levels of β-catenin, LEF1, CD44 and cyclin-D1, which was similar to the effect of siRNA targeted to CXCR4. In conclusion, miR-494 suppresses the progression of breast cancer through the Wnt/β-catenin signaling pathway, which is mediated by CXCR4.

Introduction

Breast cancer is the most common cancer among women, with an estimated 1.7 million new cases worldwide in 2012 [International Agency for Research on Cancer (IARC), 2012]. Tumor invasion and metastasis affect more than 90% of patients with breast cancer and are the main factors that lead to high mortality (1). To date, there has been no significant breakthrough in the control of breast cancer.

Epithelial-to-mesenchymal transition (EMT) is believed to be one of the main factors that promotes breast cancer progression. EMT has been known to impart metastasis and stemness characteristics in several types of cancers, including breast cancer, through inducing cancer cells to change from an epithelial to a mesenchymal phenotype, resulting in cancer cell invasion and metastasis (2-4). Furthermore, EMT also enhanced multidrug resistance by upregulating ATP binding cassette transporters in invasive breast cancer cells (5). The modulation of EMT includes numerous gene regulatory networks, including microRNAs (miRNAs) (6).

miRNAs are small non-coding RNA molecules that inhibit gene expression by interacting preferentially with the 3'-untranslated regions (3'-UTRs) of target mRNAs (7). Recently, a number of miRNAs, such as miR-205, miR-21, miR-30a, miR-96 and miR-107 have been shown to be involved in metastasis and cell proliferation, as well as EMT in breast cancer (8,9). In particular, miR-494 appears to play different roles in various types of tumors. miR-494 was shown to induce tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance in non-small cell lung cancer, and is considered an an oncogenic miRNA mega cluster that regulates the G1/S transition during liver tumorigenesis (10,11). In contrast, miR-494 is downregulated in human cholangiocarcinomas, prostate and breast cancer, and gastric carcinoma, and its reinforcement results in cancer growth inhibition, which indicates that miR-494 acts as an anti-oncogene (12). However, the role and the underlying mechanism of miR-494 in breast cancer are still not fully understood. In the present study, we
focused on the role of miR-494 in the progression of breast cancer and the related molecular mechanism.

Materials and methods

Cell lines, culture and treatment. The human breast cancer cell lines MDA-MB-231, MCF-7, MDA-MB-468, MDA-MB-435, T47D, BT-474, SK-BR-3, ZR-75-30 and human normal breast epithelial cell line MCF-10A were all obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The 293T cells were also purchased from ATCC. The breast cancer cells were cultured in Dulbecco’s modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both from Gibco, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Enpromise, China). MCF-10A cells were cultured in Mammary Epithelial Basal Medium (Lonza, Walkersville, MD, USA). Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO2.

Uppregulation and downregulation of miR-494. miR-494 (mature sequence, UGAAACAUACCGGGAAAAACCUC) mimic, inhibitor, miRNA mimic negative control and miRNA inhibitor negative control were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The MDA-MB-231 cells were cultured to ~30-40% confluency in 6-well plates, and were transfected with miR-494 mimics, miRNA mimic negative control and miRNA inhibitor negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. After 48 h of incubation, cells were harvested for further analysis. All transfections were performed in triplicate.

CXCR4 siRNA transfection. For targeted knockdown of chemokine (C-X-C motif) receptor 4 (CXCR4), two siRNAs targeted to CXCR4 were designed according to a previous study (13) and synthesized by Genetimes Technology (Shanghai, China). The sequence were: 5’-GGAAGCUGUUGGCUGAAAAdTdT-3’. The non-specific siRNA was provided by Genetimes Technology. For targeted knockdown of chemo kinase (C-X-C motif) receptor 4 (CXCR4), two siRNAs targeted to CXCR4 were designed according to a previous study (13) and synthesized by Genetimes Technology (Shanghai, China). The sequence were: 5’-UAAAAUCUUCCUGCCCCCdTdT-3' and 5’-UAAAAUCUUCCUGCCCCCdTdT-3'. The non-specific control siRNA was provided by Genetimes Technology. For transfection, 5x10^4 MDA-MB-231 cells seeded in each well of 24-well microplates were transfected with siRNAs at a final concentration of 100 nmol/l using Lipofectamine 2000.

Quantitative reverse transcription-polymerase chain reaction. Total RNA from cultured cells was isolated using TRIzol reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer’s instructions. Reverse transcription was performed on 1 µg of total RNA from each sample using oligo(dT) primers and 200 units of SuperScript II (Life Technologies, Inc.) for extension. Quantitative real-time RT-PCR (qRT-PCR) analysis of CXCR4, β-catenin, lymphoid enhancer-binding factor 1 (LEF1), cyclin-D1, CD44, E-cadherin, N-cadherin, vimentin, α-smooth muscle actin (α-SMA) and β-actin was carried out using the primers shown in Table I with the SYBR-Green Master Mix (Takara, Dalian, China) according to the manufacturer's instructions. PCR amplifications were performed using the LightCycler 2.0 (Roche, Basel, Switzerland) with the following amplification conditions: 95°C for 15 sec, 58°C for 1 min, 72°C for 1 min and 2 sec for plate reading for 35 cycles. β-actin was used as the control for normalizing the gene expression. Each measurement was performed in triplicate. Data obtained were calculated according to the 2^(-ΔΔCt) method (14).

For miR-494 detection, the miR-494-3p was firstly obtained by reverse transcription PCR based on the total RNA using a ReverTra Ace kit (Toyobo Life Science, Shanghai, China). The primer used for reverse transcription PCR was: 5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT-3'. The reverse transcription condition was 42°C for 30 min and 95°C for 5 min. The qRT-PCR primers for miR-494 are listed in Table I. The amplification conditions were 95°C for 10 sec, 55°C for 20 sec, 72°C for 20 sec and 2 sec for plate reading for 35 cycles.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-494</td>
<td>5’-CATAGCCCCGTGAAAACATACAGC-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-GTGCAGGGTGTCGGAGGT-3’</td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-GGTGTTGCTATGTTGGCGCTTCT-3’</td>
</tr>
<tr>
<td>CXCR4</td>
<td>5’-TGGAGTGTGACAGCTTGGAG-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-TTGTGGCTGCAGAAATTGAAG-3’</td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-TGGAGTGTGACAGCTTGGAG-3’</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>CCND1</td>
<td>5’-ATGGCCATCAAGCGAATTTGGA-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-ATGGCCATCAAGCGAATTTGGA-3’</td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-ATGGCCATCAAGCGAATTTGGA-3’</td>
</tr>
<tr>
<td>Vimentin</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>α-SMA</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-ATGTGGCAATGCGTTCTCATATCCA-3’</td>
</tr>
</tbody>
</table>

CXCR4, chemokine (C-X-C motif) receptor 4 α-SMA, α-smooth muscle actin.
Western blot analysis. Total proteins were extracted and separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The bands were then electroblotted onto a nitrocellulose membrane (Amersham, Little Chalfont, UK), followed by blocking non-specific binding using 2% non-fat dry milk in Tris-buffered saline at room temperature for 2 h. Western blotting was carried out by incubating the membrane with the following antibodies: anti-CXCR4 (1:1,000; Abcam, Cambridge, UK), anti-β-actin (1:6,000; KangCheng, Beijing, China), anti-E-cadherin (1:800), anti-N-cadherin (1:800), anti-vimentin (1:2,000), anti-α-SMA (1:800) (all from Proteintech Group), anti-β-catenin (1:1,000), anti-LEF1 (1:800), anti-CD44 (1:800) and anti-cyclin-D1 (1:1,000) (all from Cell Signaling Technology, Danvers, MA, USA) antibodies diluted in the blocking buffer overnight at 4˚C. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. 4-Chloro-1-naphthol (4-CN) was used for protein visualization.

Dual-luciferase reporter gene assay. The segments of wild-type 3′-UTR of CXCR4 and mutated 3′-UTR of CXCR4 were obtained according to a previous study (15). The wild-type 3′-UTR of CXCR4 or mutated 3′-UTR of CXCR4 was cloned into the downstream of the luciferase reporter gene in the pGL3 plasmid (Promega, Madison, WI, USA) by XbaI and NolI restriction sites. Wild-type 3′-UTR of CXCR4 or mutated 3′-UTR of CXCR4 cloned in the pGL3 plasmid were co-transfected with the mimic negative control or miR-494 mimics or inhibitors into HEK293T cells (ATCC) using Lipofectamine 2000. After a 48-h incubation, the luciferase activity was measured using the Dual-Luciferase Reporter Assay kit (Promega).

MTT assay. MTT assay was performed to assess cell proliferation. MDA-MB-231 cells were transfected with miR-494 mimics or inhibitors in 96-well plates (1x10^4 cells/well). After 12, 24 and 48 h of transfection, culture medium was replaced with fresh medium containing MTT (Sangon, Shanghai, China) for 10 min, and the absorbance value at 550 nm in each well was measured with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Matrigel invasion assay. A 24-well permeable support plate (Millipore) were pre-coated with 15 µl of Matrigel (BD Biosciences, Bedford, MA, USA). Cells (2x10^4) in 200 µl growth media were placed onto the top chamber. Normal culture medium containing 100 ng/ml SDF-1 was added to the bottom chamber. Plates were incubated for 36 h at 37˚C. The top of the Matrigel coated permeable support was rubbed using a cotton swab moistened with medium to remove the non-invading cells. The cells on the lower surface of the membrane were fixed with methanol and stained with crystal violet solution. Invaded cells were counted in 10 random fields under a microscope.

Statistical analysis. Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. Results are presented as mean ± SD. Data are analyzed by the unpaired Student's t-test. Statistical comparisons between groups were performed using one-way ANOVA followed by the Student's t-test. Analyses were conducted using SPSS 13.0 software. A value of P<0.05 was considered to indicate a statistically significant result.

Results

miR-494 is downregulated in human breast cancer cell lines. Previous studies demonstrated that miR-494 plays different roles in various types of tumors, either as an oncogenic miRNA in non-small cell lung cancer and liver tumorigenesis (10,11), or as an anti-oncogene in human cholangiocarcinomas, prostate cancer and gastric carcinoma (12,15,16). Therefore, expression of miR-494 was assessed in breast cancer cell lines in the present study. It was revealed by RT-PCR that miR-494 was significantly suppressed in all breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468, MDA-MB-435, T47D, BT-474, SK-BR-3 and ZR-75-30 are breast cancer cell lines. The human normal breast epithelial cell line MCF-10A was utilized as a control. *P<0.05 compared to the control group.

miR-494 mimics suppress EMT, proliferation and invasion of breast cancer cells. Previous studies have demonstrated that miR-494 is a potential regulatory miRNA in the EMT of cancer, which is the main cause of metastasis of breast cancer. The EMT phenotype is dictated by E-cadherin, N-cadherin, vimentin and α-SMA. To investigate the association between the expression of miR-494 and EMT of breast cancer, expression of the EMT markers was detected in the MDA-MB-231 cells transfected with miR-494 mimics or inhibitors. The mRNA level of miR-494 was increased significantly following transfection of the miR-494 mimics in the MDA-MB-231 cells. The reverse effect was shown in cells transfected with the miR-494 inhibitors (P<0.05, Fig. 2A). RT-PCR analysis demonstrated that miR-494 mimic transfection significantly increased the mRNA levels of E-cadherin and decreased that of N-cadherin, vimentin and α-SMA compared to the untreated control cells (P<0.05, Fig. 2B). As expected, in cells transfected with miR-494 inhibitors, the expression of E-cadherin was significantly decreased, and the expression of
SONG et al: miR-494 SUPPRESSES BREAST CANCER PROGRESSION

N-cadherin, vimentin and α-SMA was significantly increased compared to the untreated control cells (P<0.05, Fig. 2B). Western blot analysis showed that the expression of these proteins exhibited a similar trend after transfection with the miR-494 mimics or inhibitors, except that N-cadherin and vimentin were not significantly affected by miR-494 inhibitor transfection (P<0.05, Fig. 2C).

MTT assays showed that cells transfected with the miR-494 mimics showed a significant decrease in cell proliferation compared to the untreated control cells. Cells transfected with the miR-494 inhibitors showed a small decrease in cell proliferation (Fig. 2D). The Matrigel invasion assay was performed to evaluate the invasive ability of the MDA-MB-231 cells transfected with the miR-494 mimics or inhibitors. The relative invasive cell number was significantly decreased following transfection of the miR-494 mimics, and was markedly increased by miR-494 inhibitor transfection (P<0.05, Fig. 2E).

miR-494 mimics downregulate CXCR4 in breast cancer cells. The mechanism underlying the inhibitory effect of miR-494 on the progression of breast cancer is not fully understood. Previous studies indicated that miR-494 targets CXCR4 to suppress the proliferation, invasion and migration of prostate cancer (15). Therefore, the present study determined the effects of miR-494 on the expression of CXCR4 in the MDA-MB-231 cells. The protein level of CXCR4 was significantly decreased in the cells transfected with the miR-494 mimics compared to
the untreated control cells (P<0.05, Fig. 3A), yet the mRNA level of CXCR4 was not significantly altered (P>0.05, Fig. 3B).

To confirm that miR-494 directly interacts with CXCR4 mRNA, a dual-luciferase reporter assay was performed. Wild-type 3'-UTR of CXCR4 or mutated 3'-UTR of CXCR4 (Fig. 3C) constructed in the pGL3 plasmid were co-transfected with miR-494 mimics into HEK293T cells. Cells only transfected with wild-type or mutated 3'-UTR of CXCR4 were used as a control. The results showed that miR-494 mimics significantly inhibited luciferase activity in the wild-type 3'-UTR of CXCR4 co-transfected cells as compared with the control group (P<0.05, Fig. 3D). Yet, the reporter constructs with mutated CXCR4 3'-UTR were not affected by miR-494 mimics (P>0.05, Fig. 3D). This revealed that miR-494 binds to CXCR4 mRNA and regulated its translation.

miR-494 suppresses the Wnt/β-catenin pathway via targeting CXCR4. Aberrant activation of the canonical Wnt/β-catenin pathway plays a critical role in the development of breast cancer. Previous studies have demonstrated that SDF-1/CXCR4 promotes EMT and progression of cancer by activation of the Wnt/β-catenin signaling pathway (4). CXCR4 siRNA transfection significantly decreased the mRNA and protein levels of CXCR4 (P<0.05, Fig. 4A and B). In the MDA-MB-231 cells transfected with CXCR4 siRNA, the mRNA and protein levels of β-catenin, Lef1, CD44 and cyclin-D1 were significantly decreased compared to the untreated control (P<0.05, Fig. 4C and D). Notably, miR-494 mimics also exhibited the same effect on the Wnt/β-catenin pathway (P<0.05, Fig. 4C and D). These data suggest that miR-494 suppresses the Wnt/β-catenin pathway via targeting CXCR4.

Discussion

As the most widespread epithelial tumor among women, breast cancer has been extensively studied in the past decades. Previous studies have focused on the EMT of breast cancer cells, which is the critical factor influencing the invasion and metastasis of tumors, and leads to a poor prognosis. Previous studies have demonstrated that miR-494 is a potential regulatory microRNA in the EMT of cancer. In the present study, significant upregulation of E-cadherin and
downregulation of N-cadherin, vimentin and α-SMA were observed in the miR-494 mimic-transfected cells compared to the control cells, indicating suppression of EMT following miR-494 transfection in breast cancer cells. Notably, the expression levels of E-cadherin, N-cadherin, vimentin and α-SMA were not all significantly altered by miR-494 inhibitor transfection. This may be due to the low baseline of miR-494 in breast cancer cells.

CXCR4 is a seven-transmembrane G-protein-coupled receptor for SDF-1. It was reported that SDF-1 signaling through CXCR4 promotes primary tumor growth and metastasis in breast cancer and other malignancies (17,18). It was further found that SDF-1 signaling through CXCR4 promotes metastasis in breast cancer by increasing angiogenesis and by establishing an immunosuppressive tumor microenvironment (18). Research in prostate cancer showed that the constitutive overexpression of miR-494 downregulated the protein level of CXCR4, leading to suppression of proliferation, invasion and migration of prostate cancer (15). However, the effect of miR-494 on the SDF-1/CXCR4 signaling pathway in breast cancer has not yet been studied. In the present study, we found that miR-494 was markedly downregulated in breast cancer cell lines compared to the non-malignant breast epithelial cell line MCF-10A. Furthermore, our data showed that artificial overexpression of miR-494 suppressed the expression of CXCR4 post-transcriptionally, which is consistent with the previous findings in prostate cancer (15). We confirmed that CXCR4 is a target of miR-494. Furthermore, this effect of miR-494 was found to inhibit the invasion, EMT and metastasis of breast cancer cells, which leads to a positive prognosis.

Wingless-related proteins are important regulators of cell proliferation, differentiation and adhesion (19). The Wnt/β-catenin signaling pathway plays an important role in the development and regeneration of several tissues by stimulating the growth of stem cells and multipotential progenitors (20,21), particularly in the tumorigenesis of breast cancer by regulating expression of genes essential for mammary stem cell development. Wnt ligands, including WNT3A, WNT4, WNT6, WNT8B, WNT9A and WNT10B were found to be highly expressed in most breast cancer cell lines (22). These ligands play roles through the Wnt/β-catenin pathway. The elevated β-catenin levels in breast cells are supposed to be associated...
with a predisposition to breast cancer (22,23). In the present study, we found that the effect of miR-494 on SDF-1/CXCR4 suppressed the Wnt/β-catenin pathway, which may be a potential therapeutic target to prevent breast cancer metastasis and progression.

In conclusion, our findings demonstrated that miR-494 is downregulated in breast cancer cells, and is able to suppress cellular proliferation, migration and invasion through regulation of SDF-1/CXCR4. These results indicate that miR-494 may serve as a potential therapeutic target for breast cancer.

Acknowledgements

The present study was supported by the Science and Technology Plan Projects of Social Development Plans for Public Relations of Shaanxi Province (no. 2010k01-140).

References