miR-616 promotes breast cancer migration and invasion by targeting TIMP2 and regulating MMP signaling

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Received September 10, 2018; Accepted May 31, 2019

DOI: 10.3892/ol.2019.10546

Abstract. Breast cancer is one of the most frequently diagnosed cancer types in females worldwide. The aim of the present study was to investigate the expression levels, functional role and molecular mechanism of microRNA-616 (miR-616) in the progression of breast cancer cells. The relative expression levels of miR-616 in breast cancer cell lines and tumor tissues of 30 patients with breast cancer were analyzed using reverse transcription-quantitative PCR (RT-qPCR). Cell transfection was used to upregulate and downregulate the expression of miR-616 in MCF-7 and MDA-MB-231 cells, respectively. The regulatory effect of miR-616 on tissue inhibitor of metalloproteinases 2 (TIMP2) expression was also analyzed by dual-luciferase reporter assay, western blot analysis and RT-qPCR. The results of RT-qPCR analysis demonstrated significantly higher expression levels of miR-616 in tumor tissues and cancer cell lines compared with normal tissues and a normal epithelial cell line. In addition, overexpression of miR-616 significantly promoted MCF-7 cell proliferation, migration and invasion. By contrast, miR-616 silencing was associated with the opposite effects in MDA-MB-231 cells. Furthermore, the present study demonstrated that miR-616 could positively regulate the expression of matrix metalloproteinases (MMP)2 and MMP9, both at the mRNA and protein level. TIMP2 was further confirmed as a direct target of miR-616. Finally, the current study demonstrated that TIMP2 silencing rescued the proliferation and invasion capabilities and the expression levels of MMP2 and MMP9 in cells that were treated with the miR-616 inhibitor. In conclusion, the present data suggested that the miR-616/TIMP2/MMPs axis may serve an important role in the progression of breast cancer and may be a potential therapeutic target for this disease.

Introduction

Breast cancer is one of the most prevalent types of malignant tumors in females (1). Significant advances in the treatment for breast cancer have been achieved (1,2). However, breast cancer remains one of the most common causes of cancer-associated mortality in females, accounting for ~14.3% of cancer-associated mortality worldwide in 2012 (2). The majority of mortalities due to breast cancer can be attributed to metastasis (2). Metastases occur when tumor cells disseminate from the primary tumor site to the surrounding extracellular matrix, travel through the vasculature, and finally extravasate at a distant site to form a secondary tumor (3,4). In total, ~10-15% of patients diagnosed with breast cancer present with distant metastasis within 3 years (4). Therefore, identification of new molecular targets for the effective treatment of breast cancer is important.

MicroRNAs (miRNAs) are a class of small non-coding RNAs of ~20 nucleotides in length that can regulate gene expression by directly targeting mRNAs for degradation (5,6). miRNAs regulate a wide range of physiological activities in the cell, including cell proliferation, metabolism, apoptosis, invasion and migration (7). Dysregulated miRNAs are also involved in the initiation and progression of certain types of cancer, including breast cancer (8,9). miR-616 may function as an oncogene in different types of cancer, including gastric cancer, glioma, non-small cell lung cancer, hepatocellular carcinoma and prostate cancer (10-15). However, the expression, function and molecular mechanism of miR-616 in breast cancer remain unclear.

Tissue inhibitor of metalloproteinases 2 (TIMP2) is a critical inhibitor of matrix metalloproteinases (MMPs) and has been largely studied in various types of human tumor. In addition, it was demonstrated that TIMP2 is associated with the invasive and metastatic abilities of various types of tumor cell including breast cancer cells (16). Previous studies reported that certain miRNAs, including miR-130a and miR-552, regulate TIMP2 expression (17,18); however, the association between TIMP2 and miRNAs in breast cancer remains unknown.

The results from the present study demonstrated that miR-616 was upregulated in breast cancer tissues and cell lines. In vitro functional assays indicated that miR-616 could promote breast cancer cell proliferation and metastasis. In addition, this study demonstrated that TIMP2 could be a direct
target of miR-616. These results may help understanding the underlying mechanism of miR-616 in breast cancer.

Materials and methods

Tissue collection. The present study was approved by the Medical Ethics Committee of Dezhou No. 2 People's Hospital (Dezhou, China). All patients included in the current study provided written informed consent. In total, 30 paired breast tumor tissue and non-tumor breast tissue samples (>5 cm distant from tumor tissue) were obtained from 30 female patients (age range, 35-77 years; mean age, 62 years) who underwent surgical resection at Dezhou No. 2 People's Hospital (Dezhou, China) between January 2016 and July 2017. Patients who underwent chemotherapy or radiotherapy prior to surgery were excluded from the study.

Cell culture and transfection. The breast cancer cell lines MDA-MB-231, MCF-7, BT474 and MDA-MB-468, the immortal mammary epithelial cell line MCF-10A and the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in DMEM/F12 medium (GE Healthcare Life Sciences). MCF-10A cells were main-

Colony formation assay. For the assessment of colony formation, transfected breast cancer cells were seeded in 6-well plates at a density of 500 cells/well in triplicate and incubated for 1 week at 37°C. Subsequently, the plates were washed with PBS and stained with 0.5% crystal violet at room temperature for 20 min. After washing three times, colonies with >50 cells were counted and analyzed under a light microscope (magnification, x100).

Migration and invasion assays. The ability of migration and invasion was assessed using Transwell chambers (Corning Inc.). Cells were re-suspended in serum-free DMEM at a concentration of 1x10^5/ml and then 200 µl cell suspension was seeded onto the upper well of 8-µm pore Transwell inserts with or without Matrigel (Sigma Aldrich; Merck KGaA). Matrigel was only used for invasion assays. DMEM containing 10% FBS was added to the lower chamber. After 24 h incubation, cells in the upper chambers were removed with a cotton swab. The migrated and invaded cells were then stained with 0.1% crystal violet for 20 min at room temperature. Images from five different fields were captured and counted under a light microscope (magnification, x100).

Western blot analysis. Cells were lysed in cold radioimmuno-

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the breast cancer cell lines was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and 1 µg total RNA was reverse transcribed using the Reverse Transcription System kit (Promega Corporation). qPCR was performed with a Power SYBR-Green PCR Master mix (Thermo Fisher Scientific, Inc.) with human GAPDH used as an internal control. For miRNA analysis, qPCR was performed using an all-in-one miRNA RT-qPCR Detection kit (GeneCopoeia, Inc.) and U6 small nuclear RNA as an endogenous control. For the miRNA and
mRNA amplifications, the PCR thermocycling conditions were as follows: One cycle at 95˚C for 3 min, followed by 40 cycles at 95˚C for 12 sec and 62˚C for 35 sec, and a final extension step at 95˚C for 15 sec. The relative expression levels were calculated using the 2^(-ΔΔCq) method (19). The primer sequences used for amplification were as follows: miR-616, forward, 5'-ACA CTC CAG CTG GGA GTC ATT GGA GGG TTT-3', reverse, 5' -TGG TGT CGT GGA GTC G -3'; TIMP2, forward, 5' -CTC TGA TTT GGT CGT ATT GGG-3', reverse, 5' -TGG AAG ATG GTG ATG GGA TT-3'; MMP2, forward, 5' -AAG TCT GAA GAG CGT GAA GTT TGG A-3', reverse, 5' -TGA GGG TTG GTG GGA TTG GAG-3'; MMP9, forward, 5' -AGT CCA CCC TTG TGC TCT TCC TTT G-3', reverse, 5'-TCT GCC ACC CGA GTG TAA CCA T-3'; u6, forward, 5'-CTC GCT TCG GCA GCA CA-3', reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'; and GAPDH, forward, 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3', reverse, 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'.

Dual-luciferase reporter assay. The TIMP2 3'-untranslated region (3'-uTR), containing target sequences complementary to the miR-616 seed sequence, was cloned downstream of the firefly luciferase gene in the psiCHECK-2™ luciferase vector (Promega Corporation). Mutated TIMP2 3'-uTR sequences were cloned into the same luciferase vector (TIMP2-mut). The indicated reporter constructs and miR -616 mimic or inhibitor were co-transfected with the phRGTK Renilla luciferase internal control plasmid (Promega Corporation) into 293 cell line using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The luciferase activity was analyzed following 24 h transfection using a dual-luciferase reporter assay system (Promega Corporation), according to the manufacturer’s protocols.

Bioinformatics analysis. The prediction of TIMP2 3'-UTR as a miR-616 binding target was determined using TargetScan 7.1 software (www.targetscan.org).

Statistical analysis. All experiments were performed three times and data were analyzed using GraphPad Prism 5 (GraphPad Software Inc.). Differences between two groups were assessed using a two-tailed Student’s t-test. Data of >2 groups were analyzed using one-way analysis of variance with a post hoc Tukey's test. The correlation between miR-616 levels and TIMP2 mRNA expression levels in human breast cancer tissues was determined using Spearman's rank correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-616 expression is upregulated in breast cancer tissues and cell lines. The expression levels of miR-616 in breast cancer tissues and cell lines were detected by RT-qPCR. The results demonstrated that miR-616 levels were significantly upregulated in breast cancer samples compared with normal tissues (P<0.05; Fig. 1A). Furthermore, the miR-616 levels were evaluated in the breast cancer cell lines MCF-7, BT474, MDA-MB-231 and MDA-MB-468, and the immortal mammary epithelial cell line MCF-10A. The miR-616 expression levels were significantly increased in all breast cancer cell lines compared with MCF-10A cells (P<0.05; Fig. 1B), although the difference varied across the cell lines. These data indicated that miR-616 may serve an important role in the progression of breast cancer. The expression levels of miR-616 was the highest in MDA-MB-231 cells and the lowest in MCF-7 cells; therefore, these two cell lines were selected for further experiments.

miR-616 enhances breast cancer cell invasion in vitro. To investigate whether the overexpression of miR-616 affects the
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migration and invasion of breast cancer cells, Transwell assays were performed. The results revealed that overexpression of miR-616 significantly enhanced the migration and invasion potential of MCF-7 cells compared with the miR-NC group, whereas miR-616 silencing significantly inhibited the migration and invasion capacities of MDA-MB-231 cells compared with the anti-NC group (P<0.05; Fig. 3A and B).

A previous study has reported that MMP2 and MMP9 serve a critical role in the migration and invasion of breast cancer cells (20). Therefore, the current study investigated whether miR-616 affects the expression of MMP2 and MMP9. The results of RT-qPCR and western blot analysis demonstrated that overexpression of miR-616 enhanced the expression levels of MMP2 and MMP9, while miR-616 silencing significantly reduced their expression levels (P<0.05; Fig. 3C and D).

TIMP2 is a direct target of miR-616. The potential targets of miR-616 were predicted by bioinformatics analysis. Previous studies have demonstrated that TIMP2 acts as a tumor suppressor and downregulates the expression of MMP2 and MMP9 in tumor cells (20,21); therefore, out of the predicted potential targets for miR-616, TIMP2 was selected for further analysis in the current study. A schematic of the target sequence in the 3’UTR of TIMP2 is shown in Fig. 4A. Dual-luciferase reporter assays were performed to evaluate whether miR-616 directly targets TIMP2. The results revealed that miR-616 mimic significantly decreased the luciferase activity of the wild-type TIMP2 and miR-616 silencing significantly increased the luciferase activity of the wild-type TIMP2; however, no significant difference was observed in the luciferase activity of the mutant TIMP2 (P<0.05; Fig. 4B). Furthermore, RT-qPCR and western blot analysis demonstrated that the mRNA and protein expression levels of TIMP2 were negatively regulated by miR-616 expression in MCF-7 and MDA-MB-231 cells (Fig. 4C and D). When examining the tissue samples from the patient cohort, RT-qPCR results revealed that the mRNA expression levels of TIMP2 were significantly downregulated in breast cancer tissue samples compared with normal tissue samples (P<0.05; Fig. 4E). Notably, the mRNA expression levels of TIMP2 were negatively correlated with miR-616 levels in the breast cancer tissues (P<0.05; Fig. 4F).

TIMP2 silencing reverses the effects of miR-616 in breast cancer cells. To confirm that TIMP2 is a functional target of miR-616, MDA-MB-231 cells were transfected with sicontrol or siTIMP2. Western blot analysis revealed that the expression of TIMP2 was decreased in siTIMP2-transfected MDA-MB-231 cells compared with sicontrol-transfected MDA-MB-231 cells (Fig. 5A). MDA-MB-231 cells were then transfected with miR-616 inhibitor and/or siTIMP2 and their corresponding controls. CCK-8 and colony formation assays demonstrated that TIMP2 knockdown significantly promoted cell proliferation and colony formation, which were initially reduced by miR-616 silencing (P<0.05; Fig. 5B and C).
Furthermore, a Transwell assay demonstrated that TIMP2 silencing significantly increased the migration and invasion capabilities of MDA-MB-231 cells, which were initially reduced by miR-616 inhibitor (P<0.05; Fig. 5D). Finally, it was demonstrated that TIMP2 knockdown enhanced the protein and mRNA expression levels of MMP2 and MMP9, which were reduced by miR-616 inhibitor alone (P<0.05; Fig. 5E and F).

**Discussion**

Investigation of the molecular mechanisms involved in tumor progression facilitates the identification of molecules that may act as new targets for the treatment of breast cancer (22,23). miRNAs serve important roles in tumorigenic processes, including cell viability, proliferation, apoptosis, migration and invasion (22,23). miRNAs may serve as oncogenes or tumor suppressors, depending on the type of tissue and the context of expression (5). However, the underlying molecular mechanisms of miRNAs in cancer progression remain unclear.

Certain types of miRNAs, including miR-155 and miR-21, serve critical roles in the progression of breast cancer (8,24). miR-616 is a novel miRNA that serves as an oncogene in glioma, gastric cancer, prostate cancer, non-small cell lung cancer and hepatocellular carcinoma (10-15). In breast cancer, to the best of our knowledge, the expression levels, biological function and molecular mechanism of miR-616 have not been fully elucidated. In the present study, the expression levels of miR-616 were significantly upregulated in breast cancer cell lines and tissues. Functional *in vitro* experiments demonstrated that miR-616 promoted the proliferation, migration and invasion of breast cancer cells. According to these data, miR-616 may act as an oncogene and may be a potential biomarker for breast cancer.

miRNAs exert their biological roles in cancer by regulating their target genes (5). Therefore, identifying the association of a miRNA and its target gene is important for elucidating the molecular mechanism underlying its action in cancer. miR-616 can regulate numerous genes, including phosphatase and tensin homolog, SRY-box 7, tissue factor pathway inhibitor 2 and...
glycogen synthase kinase 3β (10-14). In the present study, it was
demonstrated that miR-616 could positively regulate the expres-
sion levels of MMP2 and MMP9. Using bioinformatics analysis,
it was identified that the 3'-UTR of TIMP2 contained a miR-616
response element. TIMP2 inhibits cell proliferation and migra-
tion in vitro by inhibiting the function of MMPs (21,25). In
addition, TIMP2 serves as a tumor suppressor in the progression
of breast cancer (26). However, to the best of our knowledge,
the mechanism of TIMP2 in the promotion of breast cancer
remains unclear and the association of TIMP2 with miRNAs
has not been fully investigated in breast cancer.

For the first time, the present study demonstrated the
expression and function of miR-616 in breast cancer. A nega-
tive correlation was identified between TIMP2 and miR-616
in breast cancer, which was consistent with a previous study
regarding ovarian cancer (15). Notably, the present study
identified that miR-616 promoted the expression of MMP2 and
MMP9 via TIMP2 in breast cancer. However, certain limita-
tions were present in the current study. Firstly, the experiments
were performed in vitro. Therefore, in vivo experiments are
required to further support the conclusions of the current
study. In addition, associations between the expression of
miR-616 and clinical features were not investigated; therefore,
this is required in future studies. Finally, other molecular
mechanisms of miR-616 in the progression of breast cancer
should be examined in the future.

In summary, the present data demonstrated that miR-616
enhanced the proliferation, migration and invasion of breast
cancer cells by directly targeting TIMP2. Although clinical
applications should be further investigated, the present study
revealed that the miR-616/TIMP2/MMP axis may serve as role
in the regulation of breast cancer progression and identified
miR-616 as a potential novel therapeutic target for the treatment of breast cancer.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data analyzed during this study are included in the published article.

Authors’ contributions

CY conceived, designed and conducted all experiments, and wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Dezhou No. 2 People’s Hospital (Dezhou, China). All patients included in the current study provided written informed consent.

Patients consent to participate

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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