Int J Clin Exp Med 2017;10(6):8942-8949
www.ijcem.com /ISSN:1940-5901/IJCEM0053861

Original Article

MiR-486-5p prevents migration, invasion and EMT by regulating Smad2 in breast cancer

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Received March 27, 2017; Accepted May 10, 2017; Epub June 15, 2017; Published June 30, 2017

Abstract: Growing number of studies indicates that miRNAs play an important role in the process of tumor. Evidences have revealed the potential function of miR-486-5p in tumor development. This study aimed to investigate the role of miR-486-5p in breast cancer. First, the expression level miR-486-5p in the breast cancer cell line MCF-7 and the non-malignant breast epithelial cell (MCF-10A) was detected by qRT-PCR. And we found that miR-486-5p was relatively lower in MCF-7 cells. To investigate the role of miR-486-5p in breast cancer, miR-486-5p mimic was used, and cell proliferation, migration, invasion and EMT were determined respectively. Our results suggested that over-expression of miR-486-5p repressed MCF-7 cell proliferation, migration, invasion and EMT. In addition, the expression of EMT associated proteins was measured by western blot assay. The results indicted that miR-486-5p enhanced the level of epithelial marker (E-cadherin) and decreased the levels of mesenchymal markers (N-cadherin, Smad2, Snai1, fibronectin and vimentin) in MCF-7 cells. Moreover, we validated that miR-486-5p directly targets Smad2, and the effects of miR-486-5p on MCF-7 cells were reversed by Smad2 over-expression. In conclusion, the findings of this study highlight the critical role of miR-486-5p in the regulation of the EMT and metastasis of MCF-7 by regulating Smad2, suggesting that miR-486-5p may represent as a new treatment target and prognostic marker for breast cancer.

Keywords: miR-486-5p, breast cancer, metastasis, EMT, Smad2

Introduction

Breast cancer is the most common female malignancy worldwide, although great progress have been made in the diagnosis and treatment of this disease, breast cancer is still the main cause of tumor related death among women [1-3]. Metastasis is the primary reason that lead to breast cancer-related deaths. About 6% of patients are initially diagnosized with metastatic breast cancer (MBC), and 20-50% of the breast cancer patients finally develop into MBC [1]. Therefore, it is urgent for us to seek anti-metastatic treatment methods and target for the treatment of breast cancer.

The rapid recurrence and poor survival of malignant tumor is mainly caused by metastasis. Thus, finding molecules that can prevent metastasis is critical for cancer therapy. Epithelial-mesenchymal transition (EMT) is critical in cancer invasion and migration [4-6]. In the process of EMT, cell morphology changes from epithelium to mesenchymal, at the same time, the expression level of epithelial marker (E-cadherin) decreased and the expression level of mesenchymal markers (N-cadherin, fibronectin and vimentin) increased. The mechanism of EMT in tumor is very complex. A variety of molecules are involved in regulating EMT, including signaling pathways, transcription factors and microRNAs (miRNAs) [7-9].

miRNAs are a number of short non-coding RNAs that negatively regulate their target genes at the posttranscriptional level via mRNA inhibition or degradation [10, 11]. Since miRNA-line-4 was initially identified in 1993, more than 2042 miRNAs that regulating over a third of human genes activity have been verified [12-14]. Accumulating evidence indicates that miRNA dysfunction is implicated in proliferation, apoptosis, chemo/radio-resistance, and metastasis of tumors [15-17]. Studies have indicated that

miR-486-5p acts as a tumor inhibitor in various carcinogenesis, including glioblastoma, lung cancer, colorectal cancer, gastric cancer and breast cancer, etc [18-23]. miR-486-5p may play its tumor suppressive role via the regulation of tumor proliferation and metastasis.

To the best of our knowledge, the functional role of miR-486-5p in breast cancer remains unclear. Thus, in the present study, we determined the expression level of miR-486-5p in MCF-10A and MCF-7 cell lines, studied the role of miR-486-5p in breast cancer cells, and explored the underlying mechsnisms.

Material and methods

Cell culture and transfection

The breast epithelial cell line (MCF-10A) and breast cancer cell line (MCF-7) were purchased from the Cell Center of Shanghai Institute of Life Science, Chinese Academy of Science. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% FBS (Invitrogen) and 1% penicillin-streptomycin solution (Sigma-Aldrich), and incubated in an incubator at 37°C with 5% CO_2. For the restoration of miR-486-5p in MCF-7, synthesized miR-486-5p mimics, mimics control, control-plasmids or Smad2-plasmids (Genscript, Nanjing, China) was transfected into MCF-7 cells by using Lipofectamine 2000 regent (Invitrogen) following the manufacturer's instructions. 24 h after transfection, the cells were collected for following tests.

qRT-PCR assay

Total RNA from MCF-10A and MCF-7 cells was extracted by using TRIzol reagent (Invitrogen) following the manufacturer's protocol. cDNAs was established by performing reverse transcription assay, and a miScript Reverse Transcription kit (Qiagen GmbH) was used. qPCR reaction of miR-486-5p was performed by using miScript SYBR Green PCR Kit (Qiagen GmbH) following the manufacturer's protocol. Primer sequences were listed as follows: miR-486-5p-forward, 5'TCC TGT ACT GAG CTG CCC CGAG3' [the reverse primer was provided by the miScript SYBR Green PCR Kit (Qiagen GmbH)]; U6-forward, 5'CTC GCT TCG GCA GCACA3'; U6-reverse, 5'ACG CTT CAC GAA TTT GCGT3'. U6 was used as an endogenous control. This experiment was repeated 3 times, with accompanying no cDNA and no reverse transcriptase controls. The ΔΔCt method was carried out to analyze the expression level of miR-486-5p [17].

Cell proliferation assay

24 h after cell transfection, MCF-7 cell viability was detected by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To detect cell growth, ~5,000 cells were seeded into 96 well plates, then 20 μl MTT (5 mg/ml) was discarded and 150 μl dimethyl sulfoxide was replaced. After shaking for 15 min at room temperature, the optical density (OD) value at 490 nm of each sample was determined by using a microplate reader.

Cell migration assay

To investigate the effect of miR-486-5p on MCF-7 cell migration ability, cell scratch experiment was applied in the present. Briefly, 24 h after MCF-7 cells were transfected with miR-486-5p mimic or its control, a linear wound was performed by using a 200 μl pipette tip. After washing with PBS for 3 times, the cells were then incubated in DMEM medium containing 10% FBS for another 24 h. Wound healing was observed under a microscope at 0 and 24 h after wounding, respectively. All tests were performed at least three times.

Cell invasion assay

The effect of miR-486-5p on MCF-7 cell invasion ability was measured by using transwell assay. Briefly, 24 h after the MCF-7 cells were treated with miR-486-5p mimic or NC, the cells were collected, re-suspended and then seeded into the top chamber that coated with matrigel, and cell culture medium containing 20% FBS was added to the underside chamber. Then the cells were incubated for 48 h at 37°C with 5% CO_2. At the last of the test, cells on the top chambers were removed and cells on the lower chambers were subsequently fixed with 100% methanol, stained with hematoxylin-eosin solution, and then observed under a microscope (Olympus). Each experiment was repeated at least three times.

Western blotting

24 hours after cell transfection, MCF-7 cells were harvested and total proteins were extract-

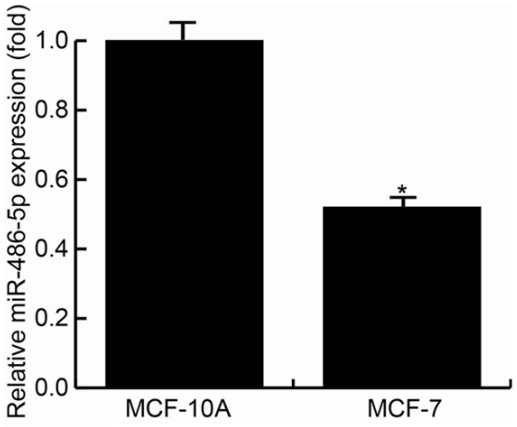

Figure 1. miR-486-5p expression determination. Relative miR-486-5p expression level in the non-metastatic MCF-10A cell line and metastatic MCF-7 cell line was detected by using qRT-PCR. Data are expressed as mean ± SD. *, P<0.05.

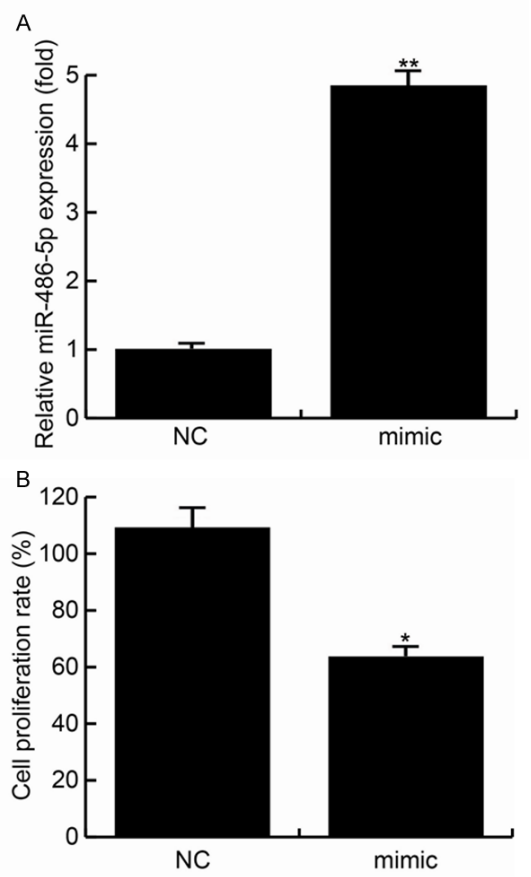

Figure 2. miR-486-5p inhibits cell proliferation in MCF-7 cells. 24 h after MCF-7 cells were transfected with miR-486-5p mimics or its negative control, miR-486-5p level was detected by qRT-PCR, and cell proliferation ability was determined by MTT assay. A: Relative miR-486-5p expression; B: MCF-7 cell proliferation ability. Data are expressed as mean ± SD. *, P<0.05; **, P<0.01.

ed by using buffer. Bicinchoninic acid protein assay was carried out to detect the protein concentration. Protein samples were resolved on 12% SDS-PAGE gels, and then transferred to PVDF membranes (Millipore, Bedford, MA). After blocking with 5% non-fat milk, the blots were incubated with antibodies against Smad2, Snai1, Vimentin, fibronectin, N-cadherin and E-cadherin (All buy from Cell Signaling Technology), and then incubated with a secondary antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system.

Luciferase reporter assay

To confirm miR-486-5p directly targets the 3'-UTR of Smad2, the vectors named Smad2-3'UTR-WT and Smad2-3'UTR-MUT with wild-type and mutated 3'UTR of Smad2 mRNA were constructed as previously described [22]. MCF-7 cells were plated in 24-well plates and then co-transfected with Smad2-3'UTR-WT or Smad2-3'UTR-MUT and miR-486-5p or its negative control (hsamiR-NC) vector by using Lipofectamine 2000 transfection reagent following the manufacturer's protocol. Dual-Luciferase Reporter Assay Kit (Promega, USA) was carried out to determine the luciferase activity.

Statistical analysis

SPSS17.0 software was used to analyze the data. Values are displayed as mean ± SD. Data were analyzed by one-way ANOVA and Student's t-test. P<0.05 was statistical significance.

Results

miR-486-5p was down-regulated in breast cancer cell line

The expression level of miR-486-5p was determined in the breast epithelial cell line MCF-10A and breast cancer cell line MCF-7 by using qRT-PCR. As shown in **Figure 1**, compared with MCF-10A cells, the relative expression level of miR-486-5p in the MCF-7 cells was significantly decreased.

miR-486-5p inhibits MCF-7 cell proliferation

To explore the function of miR-486-5p in breast cancer, synthesized miR-486-5p mimics or controls was transfected into MCF-7 cells. Our results showed that miR-486-5p have over-expressed after transfected 24 hours (**Figure**

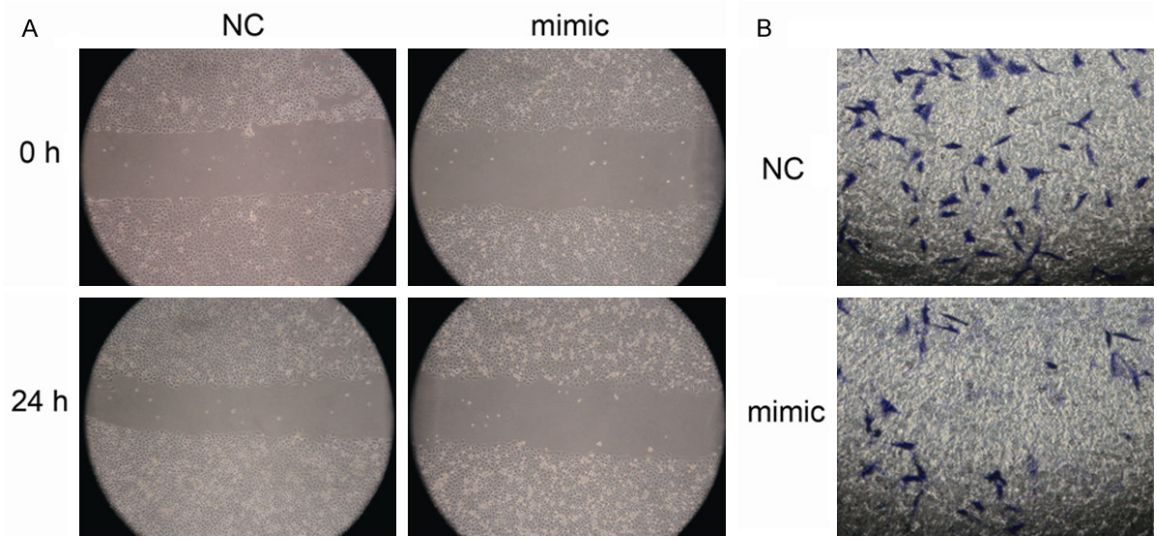

Figure 3. miR-486-5p inhibits cell migration and invasion in MCF-7 cells. 24 h after MCF-7 cells were transfected with miR-486-5p mimics or its negative control, cell migration and invasion ability in MCF-7 cells were measured. A: MCF-7 cell migration; B: MCF-7 cell invasion.

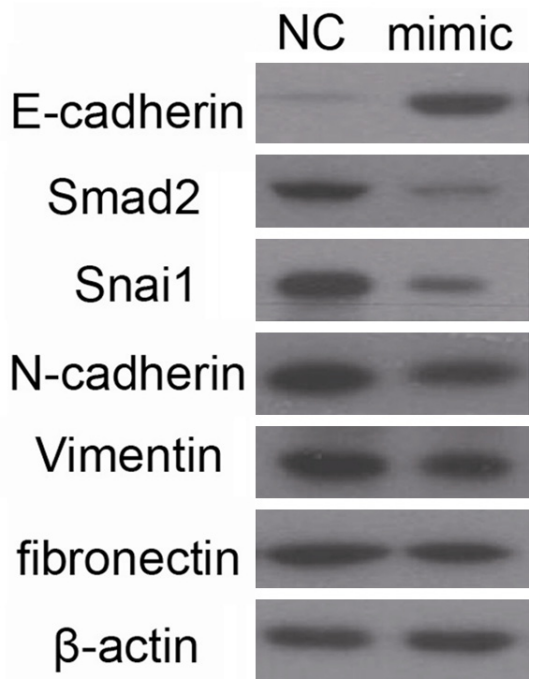

Figure 4. miR-486-5p inhibits MCF-7 cell EMT. 24 h after MCF-7 cells were transfected with miR-486-5p mimics or its negative control, EMT markers (E-cadherin, N-cadherin, Smad2, Snai1, fibronectin and vimentin) were determined by western blotting.

2A). Then the MCF-7 cell proliferation ability was examined by using MTT assay, the results demonstrated that after miR-486-5p mimics transfected 24 hours, cell proliferation ability was notably repressed compared with negative control group (**Figure 2B**). These data indicated that loss of miR-486-5p was correlated with tumorigenesis, and restoration of miR-486-5p could suppress MCF-7 cell proliferation.

miR-486-5p inhibits MCF-7 cell migration and invasion

To investigate the metastatic effect of miR-486-5p on MCF-7 cells, cell migration and invasion assays were performed. 24 h after the miR-486-5p mimics or its negative control were transfected into MCF-7 cells, cell migration and invasion ability was determined. As shown in **Figure 3A**, miR-486-5p over-expression could repress cell migration compared with the negative control. To determine whether cell invasion ability was similarly suppressed by miR-486-5p over-expression, cell transwell assay was applied. As expected, we found that miR-486-5p overexpression expression significantly repressed the invasion of MCF-7 cells (**Figure 3B**).

miR-486-5p suppresses epithelial-mesenchymal transition

The present results indicated that miR-486-5p has potential roles in regulating breast cancer cell metastasis and invasion. As EMT is well known to play important roles in cancer metastasis regulation, we determined the effect of miR-486-5p on EMT regulation in breast can-

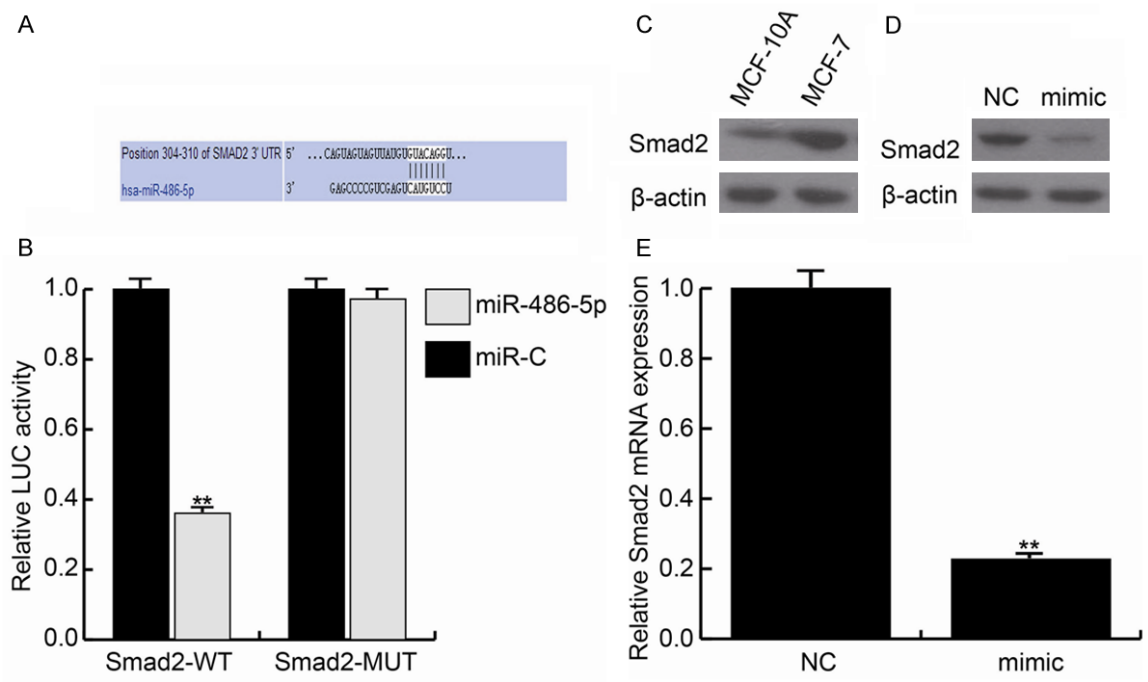

Figure 5. miR-486-5p directly targets Smad2. A: Interaction between miR-486-5p and 3'UTR of Smad2 was predicted using TargetScan; B: Luciferase activity of a reporter containing a wild-type Smad2 3'UTR or a mutant Smad2 3'UTR are shown in the bar graph (**P<0.01 *vs* control). Here, "Smad2 3'UTR-MUT" indicates the Smad2 3'UTR with a mutation in the miR-486-5p binding site. UTR, untranslated region. C: Protein expression of Smad2 in the non-metastatic MCF-10A cell line and metastatic MCF-7 cell line. D: Protein expression of Smad2 in MCF-7 cells in different groups. E: mRNA expression level Smad2 in MCF-7 cells in different groups (**P<0.01 *vs* NC).

cer cells. As shown in **Figure 4**, compared with the negative control, the protein expression level of the epithelial marker (E-cadherin) was markedly enhanced, while the the mesenchymal marker (Vimentin, Fibronectin, N-cadherin, Smad2) and the E-cadherin repressor (Snail) were notably down-regulated in MCF-7 cells that over-expressed miR-486-5p. Taken together, the data suggest that miR-486-5p regulate breast cancer invasion and metastasis through regulating EMT.

miR-486-5p directly targets Smad2

Among the EMT-promoting transcription factors, Smad2 was most markedly down-regulated at the protein level by miR-486-5p. First, we detected the level of Smad2 in MCF-7 and MCF-10A cells, the protein level was significantly high in MCF-7 cells, while the level of miR-486-5p was very low (**Figure 5C**). As expected, Smad2 was forecast as a target gene of miR-486-5p by using TargetScan (**Figure 5A**). Then luciferase reporter assay was applied to test whether miR-486-5p directly targets Smad2, and the results confirmed our prediction (**Figure 5B**). We further confirmed the negative regulation of miR-486-5p of Smad2 in MCF-7 cells by performing western blotting and qRT-PCR (**Figure 5D**, **5E**). These results indicated that the over-expression of miR-486-5p may repress EMT by suppressing Smad2 expression.

To confirm the effects of miR-486-5p on MCF-7 cells are directly though inhibiting Smad2 expression, Smad2 plasmids were used in our present study. And we observed that Smad2 plasmids could eliminate the reduction of Smad2 and the changes of EMT markers expression caused by miR-486-5p mimics (**Figure 6**).

Discussion

As a complex and multistep process, tumor metastasis and invasion is modulated by a variety of molecules, including miRNAs [24-26]. Increasing number of evidence have stated that miRNAs play important roles in the invasion and metastasis of breast cancer [27-29]. Therefore, miRNAs have been identified as potential diagnostic markers and treatment tar-

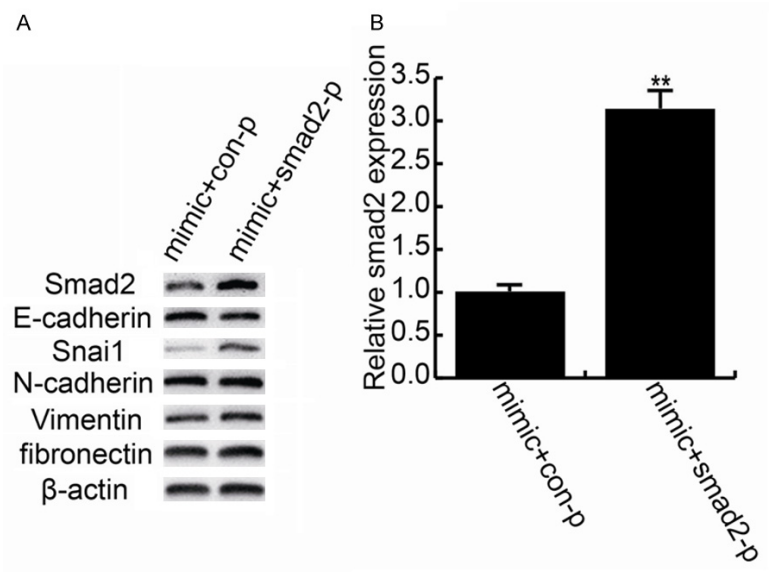

Figure 6. Smad2 plasmids reverse the reduction of Smad2 and the expression changes of EMT markers caused by miR-486-5p mimics. 24 h after MCF-7 cells were transfected with miR-486-5p mimics+control-plasmids (mimic+con-p) or miR-486-5p mimics+smad2-plasmids (mimic+smad2-p), EMT markers (E-cadherin, N-cadherin, Snai1, fibronectin and vimentin) were determined by western blotting (A), and Smad2 expression was detected by Western blotting (A) and qRT-PCR (B) respectively. ***P*<0.01 *vs* mimic+con-p.

gets for the treatment of breast cancer [28, 30]. However, studies on the roles and molecular mechanisms of miRNAs in the development of breast cancer are still needed.

The down-regulation of miR-486-5p is observed in various human cancers [20, 21]. Furthermore, miR-486-5p may function as cancer inhibitor in NSCLC through regulating metastasis via targeting ARHGAP5 [18]. In addition to the fact that miR-486-5p displays its antiproliferative role by directly down-regulating the expression of PIM-1 in breast cancer cells [21], miR-486-5p suppresses hepatocellular carcinoma cell growth by targeting PIK3R1 [20]. However, the expression level and role of miR-486-5p in MCF-7 cells has yet to be elucidated.

In the present study, we observed that compared with matched non-cancerous MCF-10A cells, miR-486-5p expressed relatively lower in the metastasis MCF-7 cells. Moreover, our results indicated that miR-486-5p over-expression significantly repressed the EMT, invasion and migration of MCF-7 cells. Furthermore, miR-486-5p exerted its functions by targeting Smad2. These data showed that miR-486-5p acts as a tumor inhibitor by directly inhibiting Smad2 expression in breast cancer process.

EMT is a key mechanism for guiding proper cell motions during embryonic development and is also critical in cancer metastasis process. EMT occurred with the reduced expressions of epithelial markers (E-cadherin) and enhanced expressions of mesenchymal markers (N-cadherin, Vimentin, etc.). In this study, we found that restoration of miR-486-5p increased the expression level of E-cadherin, and decreased the expression levels of Vimentin, Fibronectin, N-cadherin. Numerous factors, including Smad2, involve in such changes in cell phenotype and behavior. Then we detected the protein level of Smad2 in MCF-7 and MCF-10A cells, and the protein level was significantly high in MCF-7 cells, while the relative expression level of miR-486-5p was very low. The data suggested that miR-486-5p inhibit EMT through down-regulating Smad2. Further, luciferase reporter assay verified that miR-486-5p directly binding to the 3'UTR of Smad2. We also confirmed the negative regulation of miR-486-5p of Smad2 in MCF-7 cells. Moreover, we confirmed that Smad2 plasmids could eliminate the reduction of Smad2 and the expression changes of EMT markers caused by miR-486-5p over-expression. These results indicated that miR-486-5p may directly adjust the expression of Smad2 and further regulate EMT and cell metastasis in MCF-7 cells.

Taken together, the results of this study suggest that miR-486-5p is down-regulated in breast cancer MCF-7 cells and that miR-486-5p prevents breast cancer cell invasion and migration by targeting Smad2 and subsequent EMT. Indicating miR-486-5p can be used as an effective therapeutic target for breast cancer treatment.

Acknowledgements

Thanks are due to Qing Li (*Department of* pathology, *the First People's Hospital of Changzhou*) and Hongyu Zhang (*Department of* oncology, *the First People's Hospital of Chang-*

zhou) for assistance with the experiments, to Hongying Pan (*Department of Breast Surgery, Danyang People's Hospital*) for data collection and to Yuyu Jiang (Jiangnan University School of Medicine) for the paper preparation.

Disclosure of conflict of interest

None.

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