RESEARCH ARTICLE

MiR-886-5p Inhibition Inhibits Growth and Induces Apoptosis of MCF7 Cells

Lei-Lei Zhang[&], Jiang Wu^{&*}, Qiang Liu, Yan Zhang, Zhu-Lei Sun, Hong Jing

Abstract

Background and Aims: To explore the molecular mechanisms of miR-886-5p in breast cancer., we examined roles in inhibiting growth and migration of MCF-7 cells. <u>Methods</u>: MiR-886-5p mimics and inhibitors were used to express or inhibit MiR-886-5p, respectively, and MTT and clone formation assays were used to determine the survival and proliferation. Hoechst 33342/ PI double staining was applied to detect apoptosis. The expression of caspase-3, caspase-8, caspase-9, MT1-MMP, VEGF-C and VEGF-D was detected by Western blotting, and the levels of MMP2 and MMP9 secreted from MCF-7 cells were assessed by ELISA. MCF-7 cell migration was determined by wound healing and Transwell assays. <u>Results</u>: We found that the growth of MCF-7 cells was inhibited upon decreasing miR-886-5p levels. Inhibiting miR-866-5p also significantly induced apoptosis and decreased the migratory capacity of these cells. The expression of VEGF-C, VEGF-D, MT1-MMP, MMP2, and MMP9 was also found to be decreased as compared to controls. <u>Conclusions</u>: Our data show that downregulation of miR-886-5p expression in MCF-7 cells could significantly inhibit cell growth and migration. This might imply that inhibiting miR-886-5p could be a therapeutic strategy in breast cancer.

Keywords: MCF-7 - miR-886-5p - caspase - MT1-MMP - MMP2

Asian Pac J Cancer Prev, 15 (4), 1511-1515

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs that function as tumor suppressors or oncogenes by binding to the 3'-untranslated region (3'-UTR) of target mRNAs to modulate protein expression (Bartel, 2004; Zamore, 2005; Shyu et al., 2008). MicroRNAs (miRNAs) play important roles in the development, differentiation, and function of different cell types as well as in the pathogenesis of various human diseases. MiRNAs are differentially expressed in normal and cancer cells. Bioinformatics and other in silico analyses have identified that an estimated one-third of all human genes are regulated by microRNAs (Lu, 2005; Volinia et al., 2006). These miRNA molecules participate in tumorigenesis as either oncogenes or tumor suppressors. Profiling studies have led to the identification of miR-886-5p as one of the several microRNAs found dysregulated in different cancer types, such as prostate cancer, gastric cancer, cervical carcinoma and pancreatic cancer (Liang, 2010; Gao, 2011; Sharbati, 2011; Xiao et al., 2012). However, the specific function of miR-886-5p in tumor migration and invasion of breast cancer is unknown.

MiRNAs are differentially expressed in human cancers (Calin et al., 2006). The miRNA profiles of tumors are highly distinctive and are as useful or even more useful than mRNA profiles for classifying cancers (Hagan et al., 2007). The common deregulation of certain miRNAs in different types of solid cancers may indicate their involvement in fundamental pathways and their interaction with important cancer-specific protein-coding genes (Hu et al., 2009). MiRNAs down-regulate multiple target mRNAs, thereby directly or indirectly fine tuning the expression of groups of genes and entire biological pathways (Zhang et al., 2007). Collectively, these data suggest that miRNAs provide a novel posttranscriptional mechanism for modulating the expression of specific genes during oncogenesis. MiRNAs have been recently shown to be useful tools in the silencing of cancers. miRNAs regulate a variety of cellular processes and pathways critical for breast cancer transformation and progression (Kayani, 2011; Li et al., 2013).

Breast cancer is one of the most common malignancies in female patients (Iorio et al., 2005) Both exogenous factors and endogenous genetic agents contribute to breast cancer development (Farazi et al., 2011). Breast cancer cells are capable of co-opting normal extracellular signaling to promote their growth and survival. The role of miR-886-5p in breast tumorigenesis remains unknown. Studies have showed that levels of miR-886-5p correlate with cervical cancer progression and apoptosis (Li et al., 2011). MiR-886-5p interaction with its receptors plays an important role for the establishment of the breast cancer cell phenotype, prevention of apoptosis, and enhancing

Department of General Pathology, Huaihe Hospital, Henan University, Kai Feng, China & Equal contributors *For correspondence: llz12856@126.com

Lei-Lei Zhang et al

cell proliferation, migration, and survival.

Current therapies for breast cancer consist of excision, chemotherapy, and radiotherapy, all of which have different disadvantages including limited effects and toxic side effects (Mahamodhossen et al., 2013). Gene therapy has become a promising field of research because it provides many potential benefits for cancer treatment. For example, it might be able to overcome drug resistance, and drugs might have higher efficiency and specificity. The expression of siRNA from DNA templates offers several advantages over chemically synthesized siRNA delivery. Whether inhibiting miR-886-5p has an effect on cancer cell growth and migration, and whether it is a viable strategy for treatment, is unknown. In this study, we show for the first time that delivery of anti-miRNA-886-5p resulted in anti-tumor effects. Specific downregulation by the anti-miR-886-5p inhibited the growth of MCF-7 cells, suggesting miR-886-5p is a potential therapeutic target in human breast cancer.

Materials and Methods

Plasmids preparation and transfection MiR-886-5p:

Stem-loop prime sequence: 5'-GTCGTATCCAGTGC AGGGTCCGAGGTATTCGCACTGGATACGACCCGC CTT-3'upstream sequence: 5'-CGGGTCGGAGTTAGCT CA-3', downstream sequence: 5'-GTGCAGGGTCCGAG GT-3';

Internal control (U6snRNA): Stem-loop prime: 5'-GGAACGCTTCACGAATTTG-3', upstream prime: 5'-ATTGGAACGATACAGAGAAGATT-3', downstream prime:: 5'- GGAACGCTTCACGAATTTG-3'. MiR-886-5p mimics, negative control-microRNA mimics, MiR-886-5p inhibitor and Negative control-microRNA inhibitor were purchased from Dharmacon. To amplify the vectors, cloning was performed. The plasmid vectors were transformed into competent E. coli DH5a bacteria followed by ampicillin selection. The purified plasmid DNAs were tested for identification of the MiR-886-5p purifity by bidigestion of the clones with restriction endonuclease enzyme. The plasmids were extracted by Fastfiler Endo-free Plasmid Maxiprep kit (Omega, American).

Cell culture and reagents

MCF-7 cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μ g/mL streptomycin in a 5% CO₂ humidified atmosphere at 37°C. Transfection of MiR-886-5p mimics or inhibitor to MCF-7 cells were conducted by Fugene Transfection Kit (Roche, Germany). Cells were then selected by G418 or sort using Flow cytomertry (FACSVantage, BD). Briefly, the day before transfection, 1×10⁵ of cells were plated in antibiotic-free RPMI 1640 medium into each a 6-well plate. The cells were transfected with a mixture of 3 μ g MiR-886-5mimics or inhibitors plasmids and 9 μ l of FuGENE Transfection Reagent in 500 μ l medium per well. At 48 h post-transfection, the medium was replaced by complete medium and contained with 500 μ g/ml G418, cultured

up to 2 weeks after transfection. Stable transfection cells were harvested and RT-PCR, Western blot analysis or other experiments were performed as indicated.

Caspase3, Caspase8, Caspase9, VEGF-C, VEGF-D, MT1-MMP, MMP2 and GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Other reagents used in this study, such as Anti-mouse-IgG-HRP and Anti-Rabbit-IgG-HRP, were purchased from California Bioscience (California Bioscience, USA), Transwell Invasion Chambers were purchased from Promega, USA.

Cell viability studies by MTT assay

Same quantities MiR-886-5p mimics or inhibitor stable transfection MCF-7 cells and negative control MCF-7 cells (5×10^3 per well) were seeded with 100 µl medium in 96-well plate. The MTT assay was performed after 24h, 48 h, 72h, 96 h, respectively. MTT reagent (5 mg/mL) was added to each well, and incubated for 4 h at 37°C. The resulting formazan crystals were solubilized by the addition of 150µl DMSO to each well. The optical density at 570 nm was measured and cell viability was determined by the formula: cell viability (%) = (absorbance of the treated wells-absorbance of the blank control wells)/ (absorbance of the negative control wells -absorbance of the blank control wells)×100%. All MTT experiments were performed in triplicate and repeated at least three times.

Monolayer Cell migration Assay

A monolayer wound-healing model was performed. MCF7 cells stably transfected with miR-886-5p mimics or inhibitor were seeded in a 6-well-plate for 24 h in RPMI-1640 medium and treated as above. The 100% confluent monolayer MCF-7 cells were then scraped with a sterile 200 μ l pipette tip and cell debris were washed with PBS. The cells which migrated into the wounded areas were photographed at the indicated times with an inverted microscopy equipped with a digital camera. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area.

Transwell invasion Assay

Matrigel Invasion Chambers were hydrated for 4h prior to start of the assay. Stably transfected MCF-7 cells with miR-886-5p mimics or inhibitor were plated (1×10^5) in 200 µl RPMI-1640 containing 10% FBS in the upper chamber of the transwell and the lower chamber was filled with 500µl RPMI-1640 containing 10% FBS. The cells were allowed to migrate for 12 h at 37 °C and 5% CO₂. The cells were fixed for 15 min at room temperature by replacing the culture medium in the bottom and top of the chamber with 4% formaldehyde dissolved in PBS. The chambers were then rinsed in PBS and stained with 0.2% crystal violet for 10 min. After washing 5 times by dipping the chambers in a large beaker filled with dH2O, the cells that remained on the bottom of the chamber were stained with 0.1% crystal violet. The migrated clones were photographed under an optical microscope. The cell number was counted at 12 different areas. The experiments



Figure 1. Effect of micRNA886-5p on the Growth and Colony Formation of MCF7 Cells. MCF-7 cells were transfected with micRNA886-5p mimics, inhibitor or control micRNA for the indicated times. Cell growth was detection by MTT assay (A), and colony formation ability was examined by a colony assay (B)



Figure 2. Effect of miR-886-5p on the Apoptosis of MCF-7 Cells. MCF-7 cells were transfected with micRNA886-5p mimics, inhibitor, or control micRNA for indicated times, and the cellular morphology were detected by Hoechst33342/ PI staining. The ratio of MCF-7 apoptosis is quantified in (D) (**p*<0.05 compared to control micRNA)

were performed in triplicate and the data were compared with the control.

Hochest33342 / PI staining for apoptosis analysis

MCF7 cells stably transfected with miR-886-5p mimics or inhibitors were seeded at equal density on a plate pretreated with 0.01% poly-1-lysine. The cells were cultured with complete RPMI1640 medium for 48h. Staining was performed with 100 μ g/ml Hochest33342 and Propidium Iodide for 15min at RT in the dark. Subsequently, the samples were analyzed by fluorescence microscopy (Olympus, Japan). The percentage of cells undergoing apoptosis was determined.

Western Blot Analysis

MiR-886-5p mimics or inhibitor stable transfected cells were lysed in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5mM EDTA, 100 mM NaF, and 1mM Na3VO4) containing a protease inhibitor cocktail for 30 minutes on ice, followed by centrifuged for 30 min at 13200 rpm. Protein concentrations were determined by the BCA method (Pierce, USA). Equal total proteins were electrophoresed by 12% SDS-PAGE gel, followed by transferred to PVDF membranes using a wet transblot system (Bio-Rad, Hercules, CA). The membranes were



Figure 3. Effect of miR-886-5p on Cell Migration by $^{75.0}$ Wound Healing Assay. Monolayer micRNA886-5p mimics, inhibitor or control stable transfection cell grew to 100% confluent and wounded by a sterile 200μ l pipette tip. The ability $_{50.0}$ of cell migration was monitored with an inverted microscopy equipped with a digital camera (A, B, C). Statistical analysis was performed using three independent experiments (D) (*p<0.05compared to control micRNA) 25.0

blocked for 1h at room temperature with 5% nonfat dry milk and incubated overnight at 4°C with antibodies against Caspase-3, Caspase-8, Caspase-9, VEGF-C, MT1-MMP, VEGF-D and GAPDH (1:1000). After washing, the membrane was incubated for 1h with HRP-conjugated goat anti-rabbit secondary antibody diluted 1:5000 in PBST. After further washing and processed using Super Signal West Pico chemiluminescent substrate (Pierce, USA), the membrane was exposed to Fujifilm LAS3000 Imager (Fuji, Japan). The band densities of the western blots were normalized relative to the relevant GAPDH band density by Image J Analyst software (NIH).

Statistical analysis

All experiments were performed three times and the results were expressed as mean \pm SD. Statistical analysis was performed by SPSS11.0. T-tests were used to compare the average values between two different experimental groups. A *p*-value less than 0.05 was considered statistical significance.

Results

Effects of MiR-886-5p mimics and inhibitor on MCF-7 cell proliferation and apoptosis

MCF-7 cells were transfected with either mir-886-5p mimics or inhibitor. Cell survival was detected by MTT assay for the indicated times (Figure 1A). The growth of MCF-7 cells decreased upon miR-886-5p inhibitor treatment. We then tested the effects of stably transfected miR-886-5p mimics or inhibitor MCF7 cells in a colony formation assay. Decreasing miR-886-5p levels significantly inhibited the colony formation of MCF7 cells (Figures 1B, C). To determine how decreasing miR-886-5p expression in MCF7 cells decreased their growth, we performed a double staining for apoptosis using Hochest 33342 and propidium iodide. The results showed that the miR-886-5p inhibitor was able to induce apoptosis in MCF7 cells (Figure 2). 0



Figure 4. Detection of MCF-7 Cells Invasion Ability Upon miR-886-5p Mimics or Inhibitor. The invasive cells were detected by Transwell assay, and stained with crystal violet (A) and counted for statistical analysis (B) (**p*<0.05 compared to control micRNA)



Figure 5. Effect of micRNA886-5p on the Expression of MMP2, MMP9 and VEGF in MCF-7 Cell Culture Medium (*p<0.05 Compare to Control micRNA)

Effects of MiR-886-5p on the migration and invasion of MCF-7 cells

To study whether the migration and invasion of MCF-7 cells were regulated by MiR-886-5p, we performed a wound healing and Transwell assays, respectively. As expected, the miR-886-5p inhibitor was able to decrease the migratory and invasive capacities of these cells compared to the control cells (Figures 3 and 4).

Effects of MiR-886-5p on the expression of cell migration relate proteins

To fully understand the molecular mechanism which regulate migration in miR-886-5p-transfected MCF-7 cells, we detected the expression of MT1-MMP and its target molecular effectors, MMP2 and MMP9, by Western Blot and ELISA (Figures 5 and 6). The expression of MT1-MMP was significantly decreased compare to the control. This observation is consistent with the concentration of MMP2 and MMP9 in cell culture media, which also reduced upon MiR-886-5p inhibitor treatment. We also detected the expression of VEGF-C and VEGF-D by Western Blot. The results showed that VEGF-C and VEGF-D expression was also decreased in cells transfected with the miR-886-5p inhibitor (Figure 6). These results indicated that decreased miR-886-5p



Figure 6. Effects of miR-886-5p on the Expression of Caspase-3, Caspase-9, MT1-MMP, VEGF-C, VEGF-D in MCF-7 Cells. Images are represented of three independent experiments

levels inhibited the migration of MCF7 cells by regulating MT1-MMP, MMP2/9, VEGF-C/D expression.

Discussion

Breast cancer is the fourth most common malignancy worldwide. Despite efforts for the development of effective diagnostic and therapeutic strategies for breast cancer, challenges still remain for those with poor prognosis. Currently, 50 % of patients with breast cancer either have metastases to bone or other sites (On et al., 2013). Patients with curatively resected breast cancer with isolated liver or lung metastases have a 5-year overall survival (OS) rate of 30–50%. More importantly, no curative chemotherapeutic treatment is currently availa ble for breast cancer with liver or lung metastases (D'Abreo et al., 2013).

MiR-886-5p levels are elevated in many types of tumors. A likely function of MiR-886-5p overexpression in tumor cells could be to reduce the cytotoxicity attributed to two TNF family members, FasL and LIGHT, leading to increased cell survival (Zheng et al., 2010). MiR-886-5p is commonly activated in human cancers and is involved in cellular survival by inhibiting apoptotic processes, and stimulating cell growth and proliferation. However, the clinical significance of miR-886-5p activation in breast cancer remains uncertain. In the present study, we found that decreasing miR-886-5p levels by an inhibitor inhibit the proliferation and induce apoptosis of MCF7 cells. In the present study, we investigated the correlation between MT1-MMP, MMP2, MMP9 and VEGF expression and miR-886-5p levels in MCF7 cells.

Tumor metastasis is a complex multistep process and is responsible for the majority of cancer deaths. A number of genes are involved during the steps of the metastatic cascade. It is well known that degradation of the extracellular matrix surrounding tumors is the most common feature for tumor cell invasion into surrounding tissues and early metastasis. Certain MMPs are found secreted by invading tumor cells which can degrade the extracellular matrix (Hsiao, 2013; Guo et al., 2013). Thus, expression levels of these MMPs effectively reflect the aggressiveness of tumor cells and are associated with poor prognosis in various cancers. Such MMPs include MMP2 and MMP9, which are pivotal in degrading the basement membrane and have been proven to facilitate tumor invasion and metastasis in many types of cancer cells (Rodrigues et al., 2013). In this study, we found that miR-886-5p inhibitor could inhibit the expression of MT1-MMP, MMP2, and MMP9. Their expression is also associated with the suppression of MCF-7 cell migration and invasion after decreasing MiR-886-5p levels. However, it is unknown how MiR-886-5p regulates MMP2 and MMP9 expression in MCF-7 cells. Further study will investigate this underlying mechanism.

VEGF is a potent angiogenic factor that promotes tumor metastasis and angiogenesis (Giuliano, 2013; Saito et al., 2013). In this study, we also detected the expression of VEGF-C and VEGF-D in MCF7 cells transfected with miR-886-5p mimics or inhibitor. The protein expression of VEGF-C and VEGF-D are also found decreased in MCF-7 cells upon inhibiting miR-886-5p. These results indicate that miR-886-5p may be important regulators of VEGF expression. Additionally, increased VEGF-C and VEGF-D expression were also significantly decreased in cell culture supernatant after inhibiting miR-886-5p expression in MCF7 cells.

In conclusion, our results demonstrated that the downregulation of miR-886-5p expression could inhibit breast cancer cell growth and migration. Further studies are needed to determine the functional role of miR-886-5p in breast cancer.

Acknowledgements

The author (s) declare that they have no competing interests.

References

- Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281-97.
- Calin GA, Croce CM (2006). MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res*, **66**, 7390-4.
- D'Abreo N, Hindenburg AA (2013). Sex hormone receptors in breast cancer. *Vitam Horm*, **93**, 99-133.
- Farazi TA, Horlings HM, Ten Hoeve JJ, et al (2011). MicroRNA sequence and expression analysis in breast tumors by deep sequencing. *Cancer Res*, **71**, 4443-53.
- Gao W, Shen H, Liu L, et al (2013). MiR-21 overexpression in human primary squamous cell lung carcinoma is associated with poor patient prognosis. *J Cancer Res Clin Oncol*, **137**, 557-66.
- Guo Y, Zeng Q, Yan Y, et al (2013). Proliferative effect and osteoinductive potential of extracellular matrix coated on cell culture plates. *Springerplus*, **5**, 303-8.
- Giuliano S, Pagès G (2013). Mechanisms of resistance to antiangiogenesis therapies. *Biochimi*, 95, 1110-9.
- Hagan JP, Croce CM (2007). MicroRNAs in carcinogenesis. *Cytogenet Genome Res*, **118**, 252-9.
- Hu Z (2009). Insight into microRNA regulation by analyzing the characteristics of their targets in humans. *BMC Genomics*, **10**, 594-9.
- Hsiao KC, Shih NY, Fang HL, et al (2013). Surface α-enolase promotes extracellular matrix degradation and tumor

metastasis and represents a new therapeutic target. *PLoS One*, **19**, e69354.

- Iorio MV, Ferracin M, Liu CG, et al (2005). MicroRNA gene expression deregulation in human breast cancer. *Cancer Res*, 65, 7065-70.
- Kayani MU, Kayani MA, Malik FA (2011). Role of miRNAs in breast cancer. Asian Pac J Cancer Prev, 12, 3175-80.
- Lu J, Getz G, Miska EA (2005). MicroRNA expression profiles classify human cancers. *Nature*, 435, 834-8.
- Liang SH, Li J, Al-beit M, et al (2010). Screening and identification of potential miRNA involved in ovarian cancer invasion and metastasis. *Zhonghua Zhong Liu Za Zhi*, **32**, 650-4.
- Li JY, Jia S, Zhang WH (2013). Differential distribution of microRNAs in breast cancer grouped by clinicopathological subtypes. *Asian Pac J Cancer Prev*, 14, 3197-203.
- Li JH, Xiao X, Zhang YN, et al (2011). MicroRNA miR-886-5p inhibits apoptosis by down-regulating Bax expression in human cervical carcinoma cells. *Gynecol Oncol*, **120**, 145-51.
- Mahamodhossen YA, Liu W, Rong-Rong Z (2013). Triplenegative breast cancer: new perspectives for novel therapies. *Med Oncol*, **30**, 653-60.
- On NH, Miller DW (2013). Transporter-based delivery of anticancer drugs to the brain: improving brain penetration by minimizing drug efflux at the blood-brain barrier. *Curr Pharm Des*, **714**, 56-64.
- Rodrigues M, Xin X, Jee K, et al (2013). VEGF Secreted by Hypoxic Muller Cells Induces MMP-2 Expression and Activity in Endothelial Cells to Promote Retinal Neovascularization in Proliferative Diabetic Retinopathy. *Diabetes*, 62, 3863-73.
- Shyu AB, Wilkinson MF, Hoof AV (2008). Messenger RNA regulation: to translate or to degrade. *EMBO J*, 27, 471-81.
- Sharbati J, Lewin A, Kutz-Lohroff B, et al (2011). Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon *Mycobacterium avium* subsp. Hominissuis infection. *PLoS One*, 6, e20258.
- Saito T, Takeda N, Amiya E, et al (2013). VEGF-A induces its negative regulator, soluble form of VEGFR-1, by modulating its alternative splicing. *FEBS Lett*, **587**, 2179-85.
- Volinia S, Calin GA, Liu CG, et al (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA*, **103**, 2257-61.
- Xiao W, Bao ZX, Zhang CY, et al (2010). Upregulation of miR-31* is negatively associated with recurrent/newly formed oral leukoplakia. *PLoS One*, **7**, e38648.
- Zamore PD, Haley B (2005). Ribo-gnome: the big world of small RNAs. *Science*, **309**, 1519-24.
- Zhang B, Pan X, Cobb GP (2007). Anderson TA. microRNAs as oncogenes and tumor suppressors. *Developmental Biol*, 302, 1-12.
- Zheng TS, Wang JB, Chen X, Liu LX (2010). Role of microRNA in anticancer drug resistance. *Int J Cancer*, **126**, 2-10.