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. Methods: 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. Results: 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. Conclusions: 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

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; Sharbat, 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...
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′-GTCGTATCCAGTGCAGGGTCCAGGTACTCACTGAGACCCGCTT-3′, upstream sequence: 5′-GGGTGGTAGGTAGCT-3′, downstream sequence: 5′-GTCGTAGGGTCCGAGGTATTCGCACTGGATACCCGC

Internal control (U6snRNA): Stem-loop prime: 5′-GGAAACGGTTACAGAATTTG-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 DH5α bacteria followed by ampicillin selection. The purified plasmid DNAs were tested for identification of the MiR-886-5p purity by bidigestion of the clones with restriction 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.

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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 µg/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 cytometry (FACSVantage, BD). Briefly, the day before transfection, 1×10^5 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 µg MiR-886-5mimics or inhibitors plasmids and 9 µl of FuGENE Transfection Reagent in 500 µl medium per well. At 48 h post-transfection, the medium was replaced by complete medium and contained with 500 µg/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 (5×10^5 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 150 µl 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 µl 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 microscope 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 ddH2O, 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
system (Bio-Rad, Hercules, CA). The membranes were electrophoresed by 12% SDS-PAGE gel, followed by the BCA method (Pierce, USA). Equal total proteins were assayed by SDS-PAGE and transferred to PVDF membranes by a semi-dry transfer method. After blocking with 5% non-fat milk for 1h, 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 blot were normalized relative to the relevant GAPDH band density by Image J Analyst software (NIH). Western blot analysis was performed using three independent experiments (D) (*p<0.05 compared to control micRNA).

Statistical analysis

All experiments were performed three times and the results were expressed as mean ± 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 Hoechst 33342 and propidium iodide. The results showed that the miR-886-5p inhibitor was able to induce apoptosis in MCF7 cells (Figure 2).
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 compared 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 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 available 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.

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