Original Paper

MicroRNA-203 Regulates Growth and Metastasis of Breast Cancer

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Key Words

Breast cancer • miRNAs • miR203 • p21 • p27 • Bcl-2 • Matrix metalloproteinase 2 (MMP2) • MMP7 • MMP9

Abstract

Backgrounds/Aims: MicroRNAs (MiRNAs) control many biological events and play critical roles in the development of tumor. Among all miRNAs, miR203 has been recently shown to have an inhibitory effect on prostate cancer. However, its involvement in the carcinogenesis of breast cancer has not been reported. **Methods:** We examined the levels of miR203 in the breast cancer from the patients compared to the paired normal breast tissue. We also examined the levels of miR203 in several commonly used breast cancer cell lines. The effects of overexpression or depletion of miR203 on breast cancer cell growth were analyzed by a MTT assay, and on breast cancer cell invasion were examined by a scratch wound healing assay and a transwell cell migration assay. MiR203-targeted genes were analyzed by Western blot. **Results:** We detected significantly lower levels of miR203 in the breast cancer from the patients compared to the paired normal breast tissue. Moreover, the levels of miR203 were significantly lower in breast cancer tissue from the patients with cancer metastasis. Decreased miR203 levels were detected in all examined breast cancer lines. Overexpression of miR203 inhibited breast cancer cell growth and invasion, while antisense-mediated inhibition of miR203 enhanced cancer cell growth and invasion. Further analyses show that miR203 may inhibit cell growth through decreasing cell-cycle activator cyclinD2 and CDK6, increasing cell-cycle suppressor p21 and p27, and increasing apoptosis-associated protein Bcl-2. MiR203 may also inhibit cell metastasis through suppressing matrix metalloproteinase 2 (MMP2), MMP7 and MMP9. **Conclusion:** Our data thus highlight miR203 as a novel therapeutic target for breast cancer.

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Introduction

Breast cancer is a malignant tumor of high incidence and lethality in women [1]. Although the molecular mechanisms that underlie the development and carcinogenesis of breast cancer have been well investigated, our current knowledge is far from complete. For example, although activation of epidermal growth factor receptor (EGFR) signaling pathway has been shown to be critical for the tumor development and progress [2-4], specific inhibition of EGFR signaling in clinic did not always achieve satisfactory therapeutic outcome in breast cancer with an activated EGFR, largely resulting from the complexity of the downstream signaling [2-4]. Thus, further elucidation of the molecular signaling cascades in the control of the carcinogenesis of breast cancer appears extremely critical.

MicroRNA (miRNA) is a class of non-coding small RNA of comprised of about 22 nucleotides. MiRNA has been found to regulate the gene expression post-transcriptionally, through its base-pairing with the 3'-untranslated region (3'-UTR) of target mRNA [5-7]. It has been shown that miRNA regulates many biological events including cancer development. Among all miRNAs, miR203 has been only recently recognized as a tumor-suppressive microRNA in various types of cancer, including prostate cancer, lung cancer, colorectal cancer and laryngeal squamous cell carcinoma [8-19]. However, a role of miR203 in the pathogenesis of breast cancer has not been reported before.

Here, we show significantly lower levels of miR203 in the breast cancer from the patients compared to the paired normal breast tissue. Moreover, the levels of miR203 were significantly lower in breast cancer tissue from the patients with cancer metastasis. Decreased miR203 levels were detected in all examined breast cancer lines. Overexpression of miR203 inhibited breast cancer cell growth and invasion, while antisense-mediated inhibition of miR203 enhanced cancer cell growth and invasion. Further analyses show that miR203 may inhibit cell growth through decreasing cell-cycle activator cyclinD2 and CDK6, increasing cell-cycle suppressor p21 and p27, and increasing apoptosis-associated protein Bcl-2. MiR203 may also inhibit cell metastasis through suppressing matrix metalloproteinase 2 (MMP2), MMP7 and MMP9.

Materials and Methods

Patient tissue specimens

Paired breast cancer (BC) specimen and normal breast tissue (NBT) from 40 breast cancer patients (20 with metastasis and 20 without) were collected for this study. All specimens had been histologically and clinically diagnosed at the Second Department of Oncology, the Second Hospital of Hebei Medical University from 2005 to 2013. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell culture

Human breast cancer cell lines MCF7 [20], BT20, T47D and BT474 [21] were all purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These lines represent breast cancer cells originated from adenocarcinoma (MCF and BT20) and ductal carcinoma (T47D and BT474), respectively. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA). All cell lines were incubated in a humidified chamber with 5% CO₂ at 37 °C.

Cell transfection

The miR203 construct and antisense for miR203 were both purchased from GeneCopoeia (Rockville, MD, USA). Transfection was performed using the Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Quantitative PCR

MiRNA and total RNA were extracted from resected specimen from the patients or from cultured cells with miRNeasy mini kit or RNeasy kit (Qiagen, Hilden, Germany) for cDNA synthesis. Complementary DNA (cDNA) was randomly primed from 2 µg of total RNA using the Omniscript reverse transcription kit (Qiagen). Real-time quantitative PCR (RT-qPCR) was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen) on a Rotorgene 6000 series PCR machine. Quantitative PCR (RT-qPCR) were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed with the Rotorgene software accompanying the PCR machine, using 2- $\Delta\Delta C_t$ method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α -tubulin, and then compared to controls.

MTT assay

For assay of cell growth, cells were seeded into 24 well-plate at 10^4 cells per well and subjected to a Cell Proliferation Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. Experiments were performed three times.

Scratch wound healing assay

Scratch wound healing assay was performed as has been described previously [22]. Cells were seeded in 24-well plates at a density of 10^4 cells/well in complete DMEM and cultured to confluence. The cell monolayer was serum starved overnight in DMEM prior to initiating of the experiment. Confluent cell monolayer were then scraped with a yellow pipette tip to generate scratch wounds and washed twice with media to remove cell debris. Cells were incubated at 37 °C for 24 hours with the conditioned media containing either TGF β 1, or BMP7, or both, or null control. Time lapse images were captured after 12 hours. Images were captured from five randomly selected fields in each sample, and the wound areas are calculated by NIH ImageJ software.

Transwell migration assay

Cells (10^5) were plated into the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoat™ Invasion Chambers (BD, Bedford, MA, USA) and incubated at 37°C for 22 hours. The cells inside the upper chamber with cotton swabs were then removed. Migratory and invasive cells on the lower membrane surface were fixed, stained with hematoxylin, and counted for 10 random 100x fields per well. Cell counts are expressed as the mean number of cells per field of view. Five independent experiments were performed and the data are presented as mean $\pm$ standard deviation (SD).

Western blot

Protein was extracted from the cultured cells by RIPA buffer (Sigma-Aldrich) for Western Blot. The supernatants were collected after centrifugation at 12000 $\times$ g at 4°C for 20min. Protein concentration was determined using BCA protein assay, and whole lysates were mixed with 4 $\times$ SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system (Pierce, Rockford, IL, USA) to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti-p21, anti-p27, anti-Bcl-2, anti-CDK6, anti-CyclinD2 and anti- α -tubulin (all purchased from Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson Labs, Bar Harbor, ME, USA). Images shown in the figure were representative from 5 repeats.

Statistics

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values are depicted as mean $\pm$ standard deviation and are considered significant if $p < 0.05$. Two-tailed student's t test was used for comparisons of two groups and one-way ANOVA with a Bonferoni correction was used for comparisons of three or more groups.

Fig. 1. Lower levels of miR203 were detected in breast cancer. The miR203 levels in breast cancer (BC) specimen with/without distal metastasis were compared to the normal breast tissue (NBT) in the same patient. The ratio of BC vs NBT was shown. We detected significantly lower levels of miR203 in both BC without metastasis ($48.2 \pm 5.5\%$ reduction), and BC with metastasis ($73.4 \pm 8.7\%$ reduction). Moreover, this ratio in BC with metastasis were significantly lower than those without ($p < 0.01$). Statistics: Two-tailed student's t test.

Fig. 2. Breast cancer cells have decreased miR203 levels. Relative miR203 expression in 4 human breast cancer cell lines MCF7, BT20, T47D and BT474 as compared to normal breast tissue (NBT). * $p < 0.05$. Statistics: one-way ANOVA with a Bonferoni correction.

Results

Lower levels of miR203 were detected in breast cancer

In order to figure out whether miR203 may be involved in the pathogenesis of breast cancer, we examined the miR203 levels in breast cancer (BC) specimen, and compared to the normal breast tissue (NBT) in the same patient. Moreover, the specimens from the patients with/without primary cancer metastasis were also analyzed separately.

We detected significantly lower levels of miR203 in both BC without metastasis (Fig. 1, $48.2 \pm 5.5\%$ reduction, $p < 0.001$), and BC with metastasis (Fig. 1, $73.4 \pm 8.7\%$ reduction, $p < 0.001$). Moreover, the levels of miR203 in BC with metastasis were significantly lower than those without (Fig. 1, $p < 0.01$). These data suggest that miR203 may play a role in the carcinogenesis of BC, and may be related to cell metastasis.

Breast cancer cells have decreased miR203 levels

Then we examined miR203 levels in four human breast cancer cell lines MCF7 [20], BT20, T47D and BT474 [21], since these lines have been commonly used and represent breast cancer cells originated from adenocarcinoma (MCF and BT20) and ductal carcinoma (T47D and BT474), respectively. We found that all these lines expressed lower miR203 compared with normal breast tissue (Fig. 2). Then we selected BT474 cells that had a median expression of miR203 for our following study.

Preparation of miR203-modified breast cancer cells

We then transfected the BT474 cells with either a miR203-overexpressing plasmid, or a plasmid carrying antisense for miR203 (as-miR203), to increase or decrease miR-2-3 levels in BT474 cells, respectively. BT474 cells were also transfected with a plasmid carrying a scrambled sequence (scr) as controls. We confirmed the modification of miR203 levels in these cells by RT-qPCR (Fig. 3).

Fig. 3. Preparation of miR203-modified breast cancer cells. BT474 cells were transfected with either a miR203-overexpressing plasmid, or a plasmid carrying antisense for miR203 (as-miR203), or a plasmid carrying a scrambled sequence (scr) as controls. The levels of these miR203-modified cells were analyzed by RT-qPCR. * $p < 0.05$. Statistics: one-way ANOVA with a Bonferoni correction.

Fig. 4. MiR203 inhibited breast cancer cell growth. The effects of miR203 modifications on cell growth were assayed in a MTT assay. * $p < 0.05$. Statistics: one-way ANOVA with a Bonferoni correction.

Fig. 5. MiR203 inhibited migration and invasion of breast cancer cells. (A-B) The invasiveness of the miR203-modified BT474 cells was examined in a scratch wound healing assay (A), and a transwell cell migration assay (B). * $p < 0.05$. Statistics: one-way ANOVA with a Bonferoni correction.

MiR203 inhibited breast cancer cell growth

The effects of miR203 modifications on cell growth were then assayed in a MTT assay. We found that miR203 overexpression significantly inhibited the growth of BT474 cells, while antisense-mediated suppression of miR203 significantly increased the growth of BT474 (Fig. 4). These data suggest that miR203 inhibit breast cancer cell growth.

MiR203 inhibited migration and invasion of breast cancer cells

Next, we examined the invasiveness of the miR203-modified BT474 cells in both a scratch wound healing assay (Fig. 5A) and a transwell matrix penetration assay (Fig. 5B). We found that miR203 overexpression significantly inhibited the migration potential and invasiveness of BT474 cells, while antisense-mediated suppression of miR203 significantly increased the migration potential and invasiveness (Fig. 5A-B). These data suggest that miR203 inhibit migration and invasion of breast cancer cells.

Molecular mechanisms of regulation of breast cancer growth and invasion by miR203

In order to understand the mechanisms that underlie the regulation of breast cancer growth by miR203, we examined protein levels of cell-cycle associated genes in miR203-

Fig. 6. Molecular mechanisms of regulation of breast cancer growth and invasion by miR203. (A) Protein levels of cell-cycle associated genes in miR203-modified breast cancer cells were examined by Western blot, and shown by representative images. (B) The mRNA levels of MMPs in miR203-modified breast cancer cells were examined by RT-qPCR. * $p < 0.05$. NS: non-significant. Statistics: one-way ANOVA with a Bonferoni correction.

Fig. 7. Schematic of the model.

modified breast cancer cells. We found that miR203 overexpression increased the levels of cell-cycle inhibitor p21 and p27, decreased cell-cycle activator cyclinD2 and CDK6, and induced apoptosis-associated protein Bcl-2 (Fig. 6A). In line with these findings, miR203 suppression decreased the levels of cell-cycle inhibitor p21 and p27, increased cell-cycle activator cyclinD2 and CDK6, and inhibited apoptosis-associated protein Bcl-2 (Fig. 6A). These data highlight the molecular basis of miR203-mediated breast cancer cell growth arrest.

In order to understand the mechanisms that underlie the regulation of breast cancer invasion by miR203, we examined mRNA levels of MMPs in miR203-modified breast cancer cells. We found that miR203 seemed to inhibit matrix metalloproteinase 2 (MMP2), MMP7 and MMP9, but not other examined MMPs in breast cancer cells (Fig. 6B). These data highlight the molecular basis of miR203-mediated inhibition of breast cancer cell invasion. This model is thus summarized in a schematic (Fig. 7).

Discussion

It has well-established that miRNA regulates the carcinogenesis of some types of cancer. Among all miRNAs, miR203 has been relatively less studied. However, recent evidence

pointed out a specific tumor-suppressive effect of miR203 on prostate cancer, lung cancer, colorectal cancer and laryngeal squamous cell carcinoma [8-19]. Nevertheless, so far the role of miR203 in the pathogenesis of breast cancer has not been studied, which prompted us to perform the analyses on the levels of miR203 in breast cancer specimen from the patients. Strikingly, not only did we find significantly lower levels of miR203 in breast cancer tissue, but the miR203 levels in breast cancer with distal metastasis were even lower as well. These data suggest that miR203 may play a role in the carcinogenesis of breast cancer, and it may be involved in cell metastasis.

From 4 different human breast cancer cell lines of diverse cell origin, we found that all had significantly decreased levels in miR203, consistent with our clinical findings. Using a cell line that had a median expression of miR203, we modified miR203 levels in these breast cancer cells. We found that miR203 overexpression significantly inhibited the growth and invasion potential of these breast cancer cells, while antisense-mediated suppression of miR203 significantly increased the growth and invasion potential of these breast cancer cells.

In order to understand the mechanisms that underlie the regulation of breast cancer growth and invasion potential by miR203, we examined cell-cycle associated genes and MMPs that were generally regarded as direct regulator of cell invasiveness. From a series of factors involved in cell-cycle control, we found that miR203 increased the levels of cell-cycle inhibitor p21 and p27, decreased cell-cycle activator cyclinD2 and CDK6, and induced apoptosis-associated protein Bcl-2. These data suggest that miR203 may induce G1/S phase arrest in breast cancer cells, and induced cell apoptosis. Moreover, we found that MMP2, MMP3 and MMP9 were MMPs that were affected by miR203. However, MMP7, MMP11, MMP13 and MMP26 seemed not to be regulated by miR203 in breast cancer cells. Moreover, we found similar expression patterns of these factors in specimen from breast cancer patients. Of note, miR203 may affect these factors indirectly, since we did not find any of these factors that contain miR203-targeting sequence on their 3'-UTR. In summary, these data demonstrate the molecular basis of miR203-mediated inhibition of breast cancer cell invasion.

Although our study highly suggests a molecular regulatory model in which miR203 controls the growth and invasion potential of breast cancer cells as illustrated in Fig. 7, this model needs further confirmation and extensive clarification. The UTR of genes for miR203 to bind should be examined. Moreover, a set of gain-of-function and loss-of-function experiments should be performed in future to elucidate the key factors among all the affected ones that regulate the growth and metastasis of breast cancer.

Taken together, our study sheds light on miR203 as a novel therapeutic target for breast cancer.

Disclosure Statement

The authors have declared that no competing interests exist.

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