Inhibition of Breast Cancer Metastasis with MicroRNA-302a by Downregulation of CXCR4 Expression

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Abstract

Metastasis remains a main cause of mortality from breast cancer and an unresolved issue. The purpose of this study is to investigate the role of miR-302a in the development of breast cancer metastasis mediated by CXCR4, a critical regulator of metastasis, and to identify miR-302a as an effective therapeutic agent for therapy and prevention of breast cancer metastasis. Our studies show that miR-302a expression levels were downregulated in metastatic breast cancer cells and tumor tissues. Additionally, the expression levels of miR-302a were inversely correlated with CXCR4 levels. More promisingly, miR-302a inhibited the invasion and metastasis of breast cancer cells \textit{in vitro} and \textit{in vivo} and reduced the expression of CXCR4. Our findings demonstrated that the repression of miR-302a levels contributes to breast cancer metastasis and restoration of miR-302a baseline expression inhibits the invasion and metastasis of breast cancer cells. These data suggest that miR-302a mimics are potential therapeutic agents for breast cancer metastasis.

Keywords

microRNA; breast cancer; metastasis; CXCR4

Introduction

Once cancer cells have spread and formed secondary masses, they are largely incurable and therefore lead to high morbidity and mortality. Metastasis remains an unresolved issue. The interaction between CXC chemokine receptor (CXCR) 4 and its ligand, stromal cell-derived factor-1 (SDF-1), which now is designated as CXCL12 [1], was recently observed to play an important role in the metastasis of various cancers [2–4]. CXCR4 expression correlated with a poor overall survival rate in patients with metastatic breast cancer [5]. Our previous studies demonstrated that CXCR4 is an excellent target to intervene in progression of metastatic tumors [6–8]. Numerous studies have shown that CXCR4 actively contributes to angiogenesis and metastasis through modulating VEGF and MMPs [9–12]. However,
regulation of CXCR4 expression and activity has been not reported. A recently discovered class of small, functional, non-coding RNA, named microRNA (miRNA), have been shown to function as regulatory molecules by inhibiting protein translation, and to play an important role in development, differentiation, cell proliferation, and apoptosis [13, 14]. More recently, miRNAs have been demonstrated to play a critical role in cancer progression [15, 16]. MicroRNA-302 has been implicated in the maintenance and establishment of pluripotency as well as regulation of differentiation and tissue formation during embryo development [17]. A study showed that miR302 cluster suppresses the proliferation and tumorigenicity of cervical carcinoma cells through the novel target AKT1 [18]. MicroRNA-302 increases reprogramming to improve drug sensitivity in hepatocellular carcinoma cells [19]. Our recent studies demonstrated that miR-302a replacement therapy restores sensitivity of breast cancer cells to ionizing radiation through directly targeting AKT and RAD52 [20]. Relatively few studies have been conducted specifically to investigate the involvement of microRNA-302a in metastasis of breast cancer so far. In the present study, we show that miR-302a expression levels were downregulated in highly metastatic breast cancer cells and restoration of miR-302a levels inhibited the invasion and metastasis of breast cancer cells by regulating CXCR4 expression.

Materials and methods

Cell lines and cell culture

The human breast cancer cell lines MDA-MB-231, MDA-MB-361, HCC1143, HCC1395, HCC1419, and HCC2218 were grown in RPMI1640 medium supplemented with 10% FBS (20% for MDA-MB-361), 100 U/ml of penicillin sodium, and 100 μg/ml of streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO₂. MCF-7, a hormone-responsive breast cancer cell line, and MCF-10A, a non-tumorigenic human breast epithelial cell lines, were cultured in DMEM medium supplemented 10% FBS, 100 U/ml of penicillin sodium, 100 μg/ml of streptomycin sulfate and 10 μg/ml of insulin 37 °C in a humidified atmosphere of 5% CO₂.

Extraction of total RNA from formalin-fixed and paraffin-embedded tissues and quantitative real-time RT-PCR

Five 5-μm formalin-fixed paraffin-embedded breast cancer tissue sections were heated at 58°C for 30 min to dissolve the paraffin. These paraffin-dissolved specimens were washed with xylene three times for 3 min each, followed by washes with 100%, 95%, and 75% ethanol, and rinsed with PBS. Then, sections were digested overnight in a 37 °C water bath with 100 μl digestion solution containing 2% SDS and 2 mg/ml of proteinase K. Total RNA was isolated from digested tissues by using Trizol Reagent (Invitrogen) following the manufacturer’s instructions.

Quantitative and regular RT-PCR were performed following our previous descriptions [11]. Five hundred nanograms of total RNA were reverse-transcribed into cDNA in a 20 µl reaction volume at 42°C for 45 min with a GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA). Primer sequences of miR-302a, U6 snRNA and β-actin were described in previous publications [7, 20, 21]. U6 snRNA was used as internal control for
miR-302a amplification and β-actin as an internal control for CXCR4. SYBR Green quantitative PCR reaction was carried out in a 15 μl reaction volume containing 7.5 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems). The thermal profile for cDNA quantitative real-time PCR was 95°C for 10 min followed by 38 cycles of 95°C for 20s, and 60°C for 45s. In each run, a calibration sample and β-actin mRNA were run along with the unknown samples of the tissues. The relative expression levels of each sample were measured using the $2^{-\Delta\Delta C_{t}}$ method [22]. The results are presented as fold change of expression levels relative to the calibration samples.

Western blotting analyses

Twenty-five micrograms of protein were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked for 30 min in a blocking solution (5% milk in TBS-T [Tris-buffered saline containing Tween-20]) and incubated overnight at 4°C using polyclonal rabbit anti-CXCR4 antibody (Ab2; Calbiochem) at 1:500, and monoclonal antibody against β-actin (Sigma–Aldrich, St. Louis, MO) at 1:4000 in blocking solution. Enzyme-linked chemiluminescence was performed to detect hybridized protein bands.

Construction of the vector expressing miR-302a and stable transfection

MiR-302a gene double-strands were inserted into Block-iT Pol II miR RNAi Expression Vector (Invitrogen) following the manufacturer’s instructions. Then, MDA-MB-231 cells were transfected with the vector with miR-302a or control miRNA plasmids the using Lipofectamine-2000 (Invitrogen). The cells that stably expressed miR-302a were selected.

Tissue samples and immunohistochemical staining

We obtained 52 archived primary tumor tissue samples from breast cancer patients from the Avon Tissue Bank for Translational Genomics Research at Grady Memorial Hospital in Atlanta, GA. These included 30 primary breast tumor tissue samples with lymph node metastasis and 22 primary tumor samples without metastasis. The source and characteristics of tissue samples are summarized in Table 1.

For immunohistochemical staining, formalin-fixed and paraffin-embedded tissue sections were deparaffinized three times in xylene for 10 min each time after melting the paraffin for 45 min at 58°C. Gradient concentration of ethanol alcohol was used to clear deparaffinized slides. The detailed staining procedure for CXCR4 was described in previous papers [23]. The intensity of staining (brown color) was scored semi-quantitatively as follows: 1+, weak; 2+, medium; 3+, strong; and 4+, very strong staining. An immunostaining score was calculated by the multiplication of the percentage of positive tumor cells (1–100%) and the staining intensity (grade 1–4) producing a total range of 0–400 [24].

Immunofluorescence staining

Expression levels of CXCR4 protein in the miR-302a-transfected MDA-MB-231 cells and control oligonucleotide-transfected cells were detected by immunofluorescence staining as described previously [7]. Cells were cultured on chamber slides for 24 h and fixed in PBS containing 3.8% paraformaldehyde. The fixed cells were incubated with biotin-conjugated
CXCR4 antagonist TN14003 for 30 min at room temperature. After washing, slides were incubated with Rhodamine-coupled streptavidin and 1 mM Hoechst (Invitrogen) was used for nuclear counterstaining at 1:500.

Tumor Cell Invasion Assay

The invasion assay was performed by using a Matrigel invasion chamber from BD Biocoat Cellware (San Jose, CA) as previously described [6]. 2x10^4 of miR-302a-transfected or control oligo-transfected MDA-MB-231 cells were added into the top chamber. 200 ng/ml SDF-1 (R&D Systems, Minneapolis) was added to the bottom chamber to induce the invasion of tumor cells through the Matrigel. The Matrigel invasion chamber was then incubated for 16 hrs in a humidified tissue culture incubator. Non-invading cells were removed from the top of the Matrigel with a cotton-tipped swab. Invading cells at the bottom of the Matrigel were fixed in methanol and stained with H&E. The invasion rate was determined by counting the H&E-stained cells.

Animal experiments

Animal experiments were done on 6–8-week old athymic female nude mouse (nu/nu) divided into two groups with six mice per group. The mice were intravenously given injections of 2x10^6 MDA-MB-231 cells transfected with control vector or with the vector encoding miR-302a. The animals were sacrificed at 30 days after the tumor cell injection through tail vein. Whole lung tissues were harvested in optimum cutting temperature (OCT, Fisher Scientific, Suwanee, GA) compound and snap-frozen in liquid nitrogen. The frozen lung tissues were sectioned, fixed in ice-cold acetone, and subjected to H&E histostaining and reverse transcription for qRT-PCR human housekeep gene HPRT [7]. The tumor areas in mouse lungs were isolated with microdissection for the detection of miR-302a and CXCR4 with quantitative RT-PCr. All protocols for animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University.

Statistical analysis

Quantitative real-time RT-PCR reaction was run in triplicate for each sample and repeated at least 2 times and the data were statistically analyzed with a Student T-test.

Results

Levels of miR-302a are downregulated in highly metastatic breast cancer and inversely correlate with CXCR4

Quantitative real-time RT-PCR results showed that expression levels of miR-302a were downregulated in highly metastatic breast cancer cells compared to low metastatic breast cancer cells (Fig. 1ab). Furthermore, we analyzed expression levels of CXCR4 protein in two types of breast cancer cell lines with Western blot analysis. CXCR4 expression levels were upregulated in highly metastatic cells compared to low metastatic breast cancer cells (Fig. 1c). These results demonstrated that expression levels of miR-302a are inversely correlated with CXCR4 protein levels in breast cancer cell lines. To determine if miR-302a downregulation is clinically relevant, miR-302a expression levels were measured in 30 highly metastatic and 22 low metastatic breast cancer tissue samples with quantitative real-
Similar to breast cancer cell lines, highly metastatic breast cancer tissues expressed lower levels of miR-302a compared to low metastatic breast cancer tissues (Fig. 2a). Average expression levels of miR-302a in highly metastatic breast cancer samples are 25.4% of those in low metastatic breast tumor samples (Fig. 2a). Inversely, CXCR4 was expressed at much higher levels in highly metastatic breast cancer tissues compared to low metastatic breast cancer tissues (Fig. 2b). CXCR4 expression levels are inversely correlated with miR-302a in breast cancer tissues (Fig. 2c). These results demonstrate that decreased expression levels of miR-302a may be relevant to high metastasis of breast cancer.

MicroRNA-302a regulates expression of CXCR4

CXCR4 was identified by bioinformatic analysis with TargetScan algorithms as one of the predicted target genes of miRNA-302a (Fig. 3a). To investigate whether miR-302a participates in the regulation of CXCR4 expression, an in vitro functional assay was performed by overexpressing miR-302a in highly metastatic breast cancer cells. The miR-302a expression vector was constructed by inserting a pre-miR-302a sequence into the microRNA expression vector, and the constructed plasmids were stably transfected into MDA-MB-231 cells. MiR-302a overexpression in microRNA-transfected breast cancer cells was confirmed by qRT-PCR (Fig. 3b). Furthermore, CXCR4 expression levels were measured in these transfected cells with Western blot analysis and immunofluorescence staining. As shown in Fig. 3cd, CXCR4 expression levels were decreased by enforced expression of miR-302a in MDA-MB-231 cells. These results demonstrate that miR-302a downregulates CXCR4 expression. On the other hand, miR-302a inhibitors were transfected into MCF-7 cells to determine whether knockdown of miR-302a increases CXCR4 expression. The result shows that CXCR4 expression levels were increased in miR-302a inhibitor-transfected MCF-7 cells compared to the control oligonucleotide-transfected MCF-7 (Fig. 3e).

Overexpression of miR-302a inhibits invasion of highly metastatic breast cancer cells

To investigate whether overexpression of miR-302a represses the invasion of highly metastatic breast cancer cells, invasion capability change of miR-302a-transfected breast cancer cells was determined using a Matrigel invasion assay. Due to CXCR4 downregulation with miR-302a, the capability of SDF-1 in the bottom chamber to induce invasion of the cells in top chamber through the Matrigel will be decreased. Fig. 4a shows representative microphotographs for invasion cells from three different groups. The invasion of miR-302a-transfected MDA-MB-231 cells was obviously inhibited as compared to the control oligo-transfected cells (Fig. 4a). Quantitative comparison of the invasion cells between miR-302a-transfected and control oligonucleotide-transfected groups is shown in Fig. 4b. The invasion of MDA-MB-231 transfected with miR-302a was only 18% of that of the control (Fig. 4b). On the other hand, miR-302a inhibitors were transfected into MCF-7 cells to knockout the expression of miR-302a. Due to the release of miR-302a inhibition to CXCR4 expression, miR-302a inhibitor-transfected MCF-7 cells expressed higher CXCR4 and increased the ability of invasion compared to control oligonucleotide-transfected MCF-7 cells (Fig. 4cd).
MiR-302a blocks metastasis of highly metastatic breast cancer cells in a human breast cancer metastatic mouse model

The in vivo effect of miR-302a was evaluated in a metastatic human breast cancer animal model. A representative picture of whole lungs from the miR-302a-treated group in Fig. 5a shows significantly fewer visible lung metastases compared to the control group. The H&E staining of the lung tissues from the miR-302a-treated group shows the morphology of normal lung, whereas that from the control group shows invading tumor cells (Fig. 5a). These results were further confirmed by detecting the expression of human housekeeping gene hHPRT with quantitative real-time RT-PCR analysis using the specific primers that do not cross-react with its mouse counterpart. Quantitative real-time RT-PCR analyses demonstrated much higher expression levels of human HPRT mRNA in metastasis-infiltrated lungs of the mice in the control group. In contrast, there were significantly fewer metastases in the lungs of the miR-302a-treated group based on the expression levels of human HPRT in the lungs (Fig. 5b). Additionally, tumor areas in mouse lungs were isolated using microdissection for the detection of miR-302a and CXCR4 expression levels. Quantitative real-time RT-PCR analysis results show that the miR-302a vector-transfected group exhibits higher miR-302a levels (Fig. 5c) but lower CXCR4 expression levels (Fig. 5d) as compared to the control group.

Discussion

MicroRNAs could regulate cellular migration and invasion for a primary cancer to spread and form metastases, as shown in previous studies. Ectopic expression of miR-10b dramatically increased the invasive potential of the nonmetastatic breast cancer cell line through inhibition of translation of messenger RNA encoding Homeobox D10 [25]. By targeting E-cadherin mRNA, miR-9 may initiate EMT and promote breast cancer metastasis [26]. Though some microRNAs act as pro-metastatic genes to promote the invasion and metastasis of cancer cells, few microRNAs have been reported to act as suppressors to mediate invasion and metastasis of breast cancer cells. Particularly, the role of miRNAs in breast cancer metastasis mediated by CXCR4 signaling remains largely unexplored. The present study demonstrates that MiR-302a is involved in metastasis of breast cancer via directly targeting CXCR4, a critical regulator of metastasis. Our bioinformatic studies found that CXCR4 is one of the predicted targets of miR-302a. More importantly, our data have demonstrated that miR-302a repressed invasion and metastasis of highly metastatic MDA-MB-231 cells in vitro and in vivo and reduced the expression of CXCR4, which is an important protein for metastasis of various cancer cells [2, 4, 27]. A recent luciferase reporter assay showed that a strong repression of luciferase activity in the case of miR-302a was observed when using the construct with the 3′UTR region of CXCR4 mRNA compared to the controls [28]. These findings and previous studies demonstrated that miR-302a directly mediates CXCR4 expression. Collectively, downregulation of miR-302a is involved in the metastasis of breast cancer cells, mainly through the release of suppression on CXCR4 expression. Metastasis is a complex progress that involves many genes and signaling pathways. A single miRNA can target multiple function genes to regulate a signaling network [29] through binding to the 3′-UTR of a gene in a partial complementary manner [30]. MiR-302a has been shown to reprogram skin cancer cells into a pluripotent ES-cell-
like state [18, 31, 32]. Therefore, we need to investigate further whether miR-302a directly targets breast cancer stem cells to repress metastatic progression.

Several studies suggest that miRNA replacement represents an equally viable option, although the significant focus in microRNA therapy has been directed toward antisense-mediated inhibition of oncogenic miRNAs [33, 34]. Actually, most miRNAs are downregulated in tumors [35, 36] and global miRNA repression enhances cellular transformation and tumorigenesis [37]. MiRNA mimics have the same sequence as a naturally occurring miRNA and are therefore unlikely to have “off-target” effects. MiRNA mimics simply replace the depleted endogenous miRNAs, targeting the same genes that are also affected by the naturally occurring miRNA, so they may not result in toxicity. In fact, a previous study has shown that the level of repression achieved is dependent on both the amount of mRNA and the amount of available miRNA complexes [38]. These observations suggest that re-expression of even a single miRNA in tumor cells could provide significant therapeutic benefits. Our investigation has demonstrated that miR-302a is downregulated in highly metastatic breast cancer cells compared to low metastatic breast cancer cells while expression levels of CXCR4 are upregulated. In addition, our in vivo data did not show any off-target effect. Therefore, the studies suggest that miR-302a replacement therapy may be safe.

The “seed sequence” of miRNAs, known as Nucleotides 2 to 7, is considered the most critical for selecting targets [39, 40]. On the basis of databases for miRNA target prediction, six members of the miR-302 family share the same seed sequence. MiR-302 family members (miR-302s) have been implicated in the maintenance and establishment of pluripotency as well as regulation of differentiation and tissue formation during embryo development [17, 31, 41]. It has been suggested that miR-302 family members suppress proliferation of cervical cancer cells and reprogram skin cancer cells into a pluripotent ES-cell-like state [18, 31, 32]. Recently, our studies demonstrated that miR-302a sensitized breast cancer tumor cells to radiotherapy [20]. However, the roles of miR-302a in cancer metastasis are not well understood so far. Our present study has shown that miR-302a mimics inhibit invasion and metastasis of breast cancer cells. These results suggest that miR-302a family members may interact synergistically with each other in inhibiting the metastasis of breast cancer cells in multiple steps and pathways. They may be potential therapeutic agents for preventing and treating breast cancer metastasis.

In summary, CXCR4 was identified as a functional target of miR-302a in this study. The abnormalities of the miR-302a/CXCR4 expression might contribute to the metastasis of breast cancer. Our studies demonstrate for the first time that the enforced expression of miR-302a efficiently inhibits the metastasis of breast cancer cells through downregulating the expression of CXCR4. Our results suggested that downregulation of miR-302a might play crucial role in breast cancer metastasis by regulating CXCR4. Our study on miR-302a and its target gene CXCR4 would lead to a better understanding of mechanisms mediating the development of breast cancer metastasis. MiR-302a mimic replacement therapy will open a broader path toward an efficient therapeutic strategy for breast cancer metastasis.
Acknowledgments

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Abbreviations

- miRNA: microRNA
- CXCR4: CXC chemokine receptor 4
- RT-PCR: reverse transcription polymerase chain reaction
- SDF-1: stromal cell-derived factor-1
- VEGF: Vascular endothelial growth factor
- MMP: matrix metalloproteinase

References


Fig. 1.
Expression levels of miR-302a and CXCR4 in breast cancer cell lines. (a) MiR-302a expression levels determined by quantitative real-time RT-PCR are decreased in highly metastatic breast cancer cell lines. *P<0.01. (b) Agarose gel photograph of RT-PCR products. U6 snRNA was used as a loading control. (c) Western blot analysis shows that CXCR4 protein is elevated in highly metastatic breast cancer cells compared to their counterparts. β-actin was used as a loading control.
Fig. 2.
Expression levels of miR-302a are inversely correlated with CXCR4 expression levels in breast cancer tissues. (a) Quantitative comparison of miR-302a expression levels determined by quantitative real-time RT-PCR from highly and low metastatic breast cancer tissues. *P<0.01. (b) Representative microphotographs of H & E staining sections from highly and low metastatic breast cancer tissues show that CXCR4 protein is elevated in highly metastatic breast cancer tissues compared to their counterparts. Brown color represents CXCR4 and blue means nuclei counterstaining. (c) The correlation between miR-302a and CXCR4 expression levels in breast cancer patient tumor tissues. The x-axis represents CXCR4 protein expression levels determined by H & E staining and the y-axis represents miR-302a levels determined by quantitative real-time RT-PCR (n = 52). The correlation coefficient, $R=-0.8177$, indicates that there is a strong relationship between miR-302a and CXCR4 expression levels.
Fig. 3.
Overexpression of miR-302a reduced the expression of CXCR4. (a) Predicted target site of miR-302a in 3′ UTRs of CXCR4 gene. (b) Levels of miR-302a were increased in miR-302a plasmid-transfected MDA-MB-231 cells compared to control vector-transfected or wild type MDA-MB-231 cells. (c) Levels of CXCR4 protein determined by Western blot were reduced in miR-302a-transfected MDA-MB-231 cells compared to control vector-transfected or wild type MDA-MB-231 cells. β-actin was used as a loading control. (d) CXCR4 protein expression determined by immunofluorescence was reduced in miR-302a plasmid-transfected MDA-MB-231 cells. Red color represents CXCR4 staining and blue is nuclei counterstaining. (e) Levels of CXCR4 protein determined by Western blot were increased in miR-302a inhibitor-transfected MCF-7 cells compared to control oligonucleotides-transfected MCF-7 cells. β-actin was used as a loading control.
Fig. 4. MiR-302a suppressed the invasion of breast cancer cells determined by Matrigel invasion assay. (a) Representative micrographs for the invasion cells with Matrigel invasion assays using miR-302a-transfected, control oligonucleotide-transfected, or wild type MDA-MB-231 cells. (b) Comparison of invasive MDA-MB-231 cells among different groups. *P<0.01. (c) Comparison of the invasive cells from miR-302a inhibitor- or control oligonucleotide-transfected MCF-7 cells. *P<0.01. (d) Representative micrographs for the invasion cells in miR-302a inhibitor- or control oligonucleotide-transfected MCF-7 breast cancer cells.
Fig. 5. MiR-302a inhibited breast cancer metastasis in an animal experimental model. (a) Representative photographs of lungs and their H&E staining of each group from two independent experiments. (b) Expression levels of human housekeeping gene HPRT in the lungs from the control group of mice were 13.9 times as those of the miR-302a-treated group. *P<0.01. (c) Average expression levels of miR-302a from the lungs of the mice bearing miR-302a-transfected MDA-MB-231 tumor cells compared the controls. *P<0.01. (d) Average expression levels of CXCR4 from the lungs of the mice bearing miR-302a-transfected MDA-MB-231 tumor cells compared the controls. *P<0.01.
**Table 1**

Source and characteristics of tissue specimens of breast tumors

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