MicroRNA-708-3p mediates metastasis and chemoresistance through inhibition of epithelial-to-mesenchymal transition in breast cancer

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Metastasis and chemoresistance remain major challenges in the clinical treatment of breast cancer. Recent studies show that dysregulated microRNAs (miRNAs) play an important role in metastasis and chemoresistance development in breast cancer. Herein, we identified downregulated expression of miR-708-3p in breast cancers. In particular, miR-708-3p expression was significantly decreased in specimens from breast cancer patients with metastasis compared to that in specimens from patients with no metastasis. Consistent with clinical data, our in vitro data show that miR-708-3p was more significantly decreased in invasive breast cancer cell lines. In addition, our data show that inhibition of miR-708-3p significantly stimulated breast cancer cell metastasis and induced chemoresistance both in vitro and in vivo. Furthermore, we identified that miR-708-3p inhibits breast cancer cell epithelial-to-mesenchymal transition (EMT) by directly targeting EMT activators, including ZEB1, CDH2 and vimentin. Taken together, our findings suggest that restoration of miR-708-3p may be a novel strategy for inhibiting breast cancer metastasis and overcoming the chemoresistance of breast cancer cells.

KEYWORDS
breast cancer, chemoresistance, EMT, metastasis, miR-708-3p

1 INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death in women worldwide.1 Chemotherapy is an important component in the treatment paradigm for breast cancers. Although chemotherapy can shrink a tumor mass rapidly following chemotherapy cycles, some cancer cells acquire resistance to chemotherapy, leading to subsequent recurrence and metastasis.2 Importantly, chemoresistance and metastasis are closely correlated with poor clinical outcome in patients with breast cancers,3 suggesting that metastasis and chemoresistance remain the main clinical challenges in treating breast cancer.

Epithelial-to-mesenchymal transition (EMT) is a cellular program that operates in the context of embryogenesis, wound healing and
carcinoma pathogenesis to drive epithelial cells towards a mesenchymal state. Hyperactivated EMT is often detected during cancer progression, and aberrantly activated EMT enables the cells forming these tumors to acquire the traits of highly malignant cells, notably, motility, invasiveness and an ability to disseminate to form distant metastases. In addition, recent studies show that cancer cells that have undergone EMT acquire cancer stem cell properties, thereby significantly contributing to the development of chemoresistance.

Thus, the EMT pathway is of great therapeutic interest in the treatment of cancers, including breast cancer. In fact, studies show that targeting EMT can abrogate chemoresistance and inhibit cancer metastasis. Studies show that many factors, including microRNAs (miRNAs), are involved in the regulation of EMT. miRNAs are small non-coding RNA molecules that inhibit target gene expression at the post-transcriptional level by directly binding to a target gene’s 3’-UTR. Studies show that miRNA expression was dysregulated in many cancers and that dysregulated miRNAs lead to aberrant expression of their target genes, thereby contributing to all stages of cancer progression. Accumulating evidence shows that dysregulated miRNAs are also involved in the activation of EMT in cancer. Studies show that dysregulated miRNAs induce EMT partly through targeting EMT stimulators, including ZEB1, CDH2, N-cadherin, and vimentin.

In the present study, we identified downregulated expression of miR-708-3p in specimens from breast cancer patients, and our data show that decreased expression of miR-708-3p was significantly associated with breast cancer metastasis. Additionally, we found that overexpression of miR-708-3p inhibited metastasis and enhanced chemosensitivity of breast cancer cells. Furthermore, we identified that miR-708-3p inhibited EMT by directly targeting EMT activators, including ZEB1, CDH2, and vimentin. Our findings clearly show that miR-708-3p acts as a cancer suppressor and that it carries out its anti-cancer role by inhibiting EMT in breast cancer. Additionally, restoration of miR-708-3p may be a novel strategy for treating breast cancer patients with metastasis or chemoresistance.

2 MATERIALS AND METHODS

2.1 Reagents

FBS, MTX, doxorubicin, anti-actin antibody, and cell culture medium were purchased from Sigma Chemical Co. (St Louis, MO, USA). QuantiTect SYBR Green PCR Kit was obtained from Qiagen (Germantown, MD, USA). Antibodies against E-cadherin, CDH2, vimentin, ZEB1, and β-catenin were obtained from Cell Signaling Technologies (Danvers, MA, USA). A Dual-Luciferase Assay Kit, caspase-3/7 activity assay kit and Lipofectamine 2000 were obtained from Promega (Madison, WI, USA). TRizol and a BLOCK-iT Pol II miR RNAi Expression Vector Kit were purchased from Invitrogen (Carlsbad, CA, USA). Opti-MEM, a High-Capacity cDNA Reverse Transcription Kit, a miRNA expression reporter vector, miR-708-3p mimics, antisense oligonucleotides of miR-708-3p (ASO miR-708-3p), negative control oligonucleotides (NC), and primer sets of RNU6 and miR-708-3p were purchased from Life Technologies (Carlsbad, CA, USA). Invasion Assay Kits were obtained from BD Biosciences (Franklin Lakes, NJ, USA). In Situ Cell Death Detection kits were purchased from Roche (Penzberg, Germany).

2.2 Cell culture and human samples

Cell lines MCF-10A, MCF-7, MDA-MB-468 and MDA-MB-231 were obtained from the ATCC (Manassas, VA, USA), and these cells were cultured in DMEM, supplemented with 10% FBS, at 37°C in an atmosphere of 95% air and 5% CO2.

Tumor and matched adjacent normal tissues were collected from 50 patients with newly diagnosed breast cancer at the Chuncheon Sacred Heart Hospital, College of Medicine, Hallym University. Characteristics of the breast cancer patients are summarized in Table 1. This research was approved by the Research Ethics Board of the Chuncheon Sacred Heart Hospital.

2.3 Reverse transcriptase real-time quantitative polymerase chain reaction analysis (RT-qPCR)

Total RNA was isolated from cells and tissues using TRizol reagent according to the manufacturer’s protocol. Mature miR-708-3p and miR-708-3p were purchased from Life Technologies (Carlsbad, CA, USA). Invasion Assay Kits were obtained from BD Biosciences (Franklin Lakes, NJ, USA). In Situ Cell Death Detection kits were purchased from Roche (Penzberg, Germany).

### Table 1 Characteristics of patients with breast cancer in the present study

| Variables          | Low miR-708-3p | High miR-708-3p | P-value |
|--------------------|----------------|-----------------|---------|
| Age (years)        |                |                 |         |
| <50                | 11             | 13              | .60     |
| >50                | 10             | 16              |         |
| T status           |                |                 |         |
| T1-T2              | 9              | 20              | .06     |
| T3-T4              | 12             | 9               |         |
| LN metastasis      |                |                 |         |
| Yes                | 12             | 8               | .04     |
| No                 | 9              | 21              |         |
| Clinical stage     |                |                 |         |
| I-II               | 8              | 20              | .02     |
| III-IV             | 13             | 9               | .47     |
| ER status          |                |                 |         |
| Negative           | 7              | 13              | .41     |
| Positive           | 14             | 16              |         |
| PR status          |                |                 |         |
| Negative           | 8              | 14              | .47     |
| Positive           | 13             | 15              |         |
| HER2 status        |                |                 |         |
| Negative           | 11             | 18              | .49     |
| Positive           | 10             | 11              |         |

ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; LN, lymph node; miR, microRNA; PR, progesterone receptor.
the RNU6 endogenous control were analyzed using the TaqMan microRNA Assay Kit. Relative expression of miR-708-3p was normalized against RNU6 expression using the $2^{-\Delta\Delta Ct}$ method, and the miR-708-3p expression fold change in breast cancer samples matched to non-tumor control samples was evaluated using the $2^{-\Delta Ct}$ method.

On the basis of the mean fold change of miR-708-3p expression, patients were divided into high- (fold change $>\text{mean}$) and low- (fold change $<\text{mean}$) miR-708-3p expression groups. For analysis of CRYAB expression, RT and PCR were carried out with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) and a QuantiTect SYBR Green PCR Kit (Qiagen), respectively. Expression of CDH2, ZEB1 and vimentin was quantified in relation to the expression of $\beta$-actin. Primer sequences are as follows. For CDH2, forward 5’-GTCAGCAGAAGTTGAAATA-3’ and reverse 5’-AGCACTGTGTTGG-3’; for vimentin, forward 5’-GACGC CATCAACACCGAGTT-3’ and reverse 5’-CTGGT-3’; for actin, forward 5’-AGCCTAGACGTGC-3’ and reverse, 5’-AGCCTAGACGTGC-3’.

2.4 Luciferase reporter assay

Briefly, 3’-UTR segments of CDH2, vimentin and ZEB1 that were predicted to interact with miR-708-3p were amplified by PCR from human genomic DNA and inserted into the MluI and HindIII sites of the miRNA expression reporter vector. For the luciferase reporter experiments, the indicated cells were seeded into 24-well cell culture plates at a concentration of $1 \times 10^4$ per well. The next day, the cells were transfected with the indicated reporter plasmids containing firefly luciferase. Renilla luciferase plasmid was cotransfected as a transfection control. Cells were lysed 48 hours after transfection, and luciferase activity was measured by a Dual-Luciferase Assay System (Promega) according to the manufacturer’s protocol. Firefly luciferase activity was normalized by the activity of Renilla luciferase.

2.5 Western blot and immunohistochemistry assays

Western blotting and immunohistochemical assays were carried out as described by Xu et al.

2.6 MTT assay and apoptotic cell detection

For the MTT assay, cells were transfected with the indicated oligonucleotides using Lipofectamine 2000 (Promega). After 24 hours of transfection, cells were plated into 96-well plates at a density of $5 \times 10^3$ cells per well. After 12 hours of seeding, cells were incubated with or without 1 $\mu$mol/L doxorubicin for 48 hours. Cell viability was measured using MTT according to the manufacturer’s protocol. Apoptotic cells in tumor tissues were detected using an In Situ Cell Death Detection kit (Roche) according to the manufacturer’s instructions.

2.7 Invasion assay

Cells were transfected with the indicated oligonucleotides for 48 hours, and then, $1 \times 10^5$ cells in growth medium without serum were seeded in the upper wells of BD Chambers. The lower wells contained the same medium with 10% serum. After 24 hours, the cells that had invaded the lower side of the chamber were fixed with 2.5% glutaraldehyde, stained with 0.1% crystal violet, dried and counted.

2.8 Stable cell line selection

A miR-708-3p expression vector was constructed using a BLOCK-it™ Pol II miR RNAi Expression Vector Kit (Invitrogen) according to the manufacturer’s protocol and transfected into the indicated cells for selection of stable miR-708-3p-expressing cells. After 48 hours of transfection, cells were incubated with 10 mg/mL blasticidin for 2 weeks. To construct stably expressing miR-708-3p-antisense cells, a miR-708-3p-antisense expression vector was transfected into the indicated cells. After 48 hours of transfection, cells were incubated with 2 mg/mL puromycin for 1 week. Then, cells were frozen in aliquots for later use.

2.9 Animal experiments

Stably expressing miR-708-3p or miR-708-3p-antisense cells and their vector control cells were used to generate the animal model. For the subcutaneous tumor growth assay, $2 \times 10^6$ of the indicated cells in 0.1 mL PBS were s.c. injected into 6-week-old female nude mice (5 mice per group). When tumors reached a size of approximately 100 mm³, the mice were started on a treatment of either PBS or doxorubicin (5 mg/kg body weight) twice a week. Tumor volume was measured every week and the mice were killed after 4 weeks of doxorubicin treatment. For the lung metastasis experiment, $5 \times 10^5$ of the indicated cells were suspended in 0.1 mL PBS and injected into the lateral tail vein of 6-week-old female nude mice (5 mice per group). At 4 weeks after injection, all mice were killed, and the lung surface tumor foci were counted. All animal care and experimentation was conducted according to the guidelines of the Institutional Animal Care and Use Committee of the Chuncheon Sacred Heart Hospital.

2.10 Statistical analysis

All data are presented as the mean ± standard deviation (SD), and significant differences between treatment groups were analyzed by Student’s t test or one-way analysis of variance (ANOVA) and Duncan’s multiple range test using SAS statistical software version 6.12 (SAS Institute). Differences were considered statistically significant at a P-value of $<.05$. 
3 | RESULTS

3.1 | Decreased expression of miR-708-3p was correlated with metastasis in breast cancer

Solexa (Illumina) deep-sequencing data show that miR-708-3p expression was decreased in the metastatic breast cancer cell line MDA-MB-231 compared to that in the non-cancerous mammary epithelial cell line MCF-10A.22 However, the function and expression level of miR-708-3p in breast cancer patients are unknown. Thus, we first investigated the expression of miR-708-3p in human breast tumors. Our data showed that miR-708-3p expression was significantly decreased in breast cancer specimens compared to that in their matched adjacent normal tissues (Figure 1A). In addition, we found that miR-708-3p expression was more significantly decreased in specimens from breast cancer patients with lymph node metastasis compared to those from breast cancer patients with no metastasis (Figure 1B,C; Table 1). However, clinicopathological features, such as progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor-2 (HER2) status, were not significantly associated with miR-308-3p level (Table 1). Consistent with clinical data, in vitro experiments also showed that miR-708-3p was significantly downregulated in aggressive breast cancer cell lines (Figure 1D). Taken together, these findings suggest that decreased expression of miR-708-3p was significantly correlated with breast cancer metastasis.

3.2 | Inhibition of miR-708-3p stimulates breast cancer cell metastasis

To investigate whether decreased expression of miR-708-3p was involved in breast cancer cell metastasis, we inhibited miR-708-3p using antisense nucleotides of miR-708-3p (ASO miR-708-3p) in a cell line with high expression of miR-708-3p (MCF-7) (Figure 2A) and then carried out invasion assays. Our results showed that inhibition of miR-708-3p significantly stimulated MCF-7 cell invasion (Figure 2B). This result was confirmed using another cell line, MDA-MB-468, and results similar to those seen in the MCF-7 cell line were observed (Figure 2B). Furthermore, to confirm these in vitro results in vivo, we used MDA-MB-468 cells that stably express antisense miR-708-3p to generate a breast cancer lung metastasis model (Figure 2A) and determined the number of tumor nodules on the

FIGURE 1 MicroRNA-708-3p (miR-708-3p) expression level was negatively correlated with metastasis in breast cancer. A, miR-708-3p expression was significantly decreased in human breast tumor tissues compared to that in matched adjacent normal tissues (n = 30). B, miR-708-3p expression was significantly decreased in specimens from breast cancer patients with metastasis (n = 20) compared to that in specimens from breast cancer patients with no metastasis (n = 30). C, Correlation of miR-708-3p expression level with metastasis in breast cancer patients. D, miR-708-3p expression level was negatively correlated with invasiveness in breast cancer cell lines. miR-708-3p expression was measured using RT-qPCR in specimens and breast cancer cell lines. *P < .05; ***P < .001
As expected, our results showed that inhibition of miR-708-3p significantly increased the number of lung surface tumor nodules (Figure 2C,D). Taken together, these findings suggest that decreased expression of miR-708-3p significantly contributes to breast cancer cell metastasis.

3.3 Overexpression of miR-708-3p dramatically inhibits breast cancer cell lung metastasis

Our observations that the inhibition of miR-708-3p stimulates breast cancer cell lung metastasis, in turn, prompted us to investigate whether miR-708-3p overexpression could suppress breast cancer cell lung metastasis. Our in vitro trans-well results clearly showed that overexpression of miR-708-3p (Figure 3A) significantly inhibited breast cancer cell invasion in both MDA-MB-231 and MDA-MB-468 cells (Figure 3B). These results were further confirmed using animal models. Consistent with our in vitro experimental results, overexpression of miR-708-3p suppressed breast cancer cell MDA-MB-468 lung metastasis in nude mice (Figure 3C, D). These data suggest that restoration of miR-708-3p may be a useful strategy for the treatment of breast cancer cell lung metastasis.
3.4 | miR-708-3p inhibits EMT in breast cancer cells

Epithelial-to-mesenchymal transition plays an important role in breast cancer cell metastasis. Thus, herein, we investigated whether miR-708-3p is involved in the regulation of breast cancer cell EMT. Our immunofluorescence staining results (IF) showed that inhibition of miR-708-3p significantly suppressed expression of E-cadherin, a negative regulator of EMT, but increased expression of β-catenin, an EMT activator, in miR-708-3p high-expressing cell line MCF-7 (Figure 4A). In contrast, overexpression of miR-708-3p stimulated E-cadherin expression but inhibited β-catenin expression in MDA-MB-468 breast cancer cells (Figure 4B). These IF results were confirmed using western blot analysis, and similar results were observed (Figure 4C,D). These findings suggest that miR-708-3p is negatively correlated with EMT progression in breast cancer cells.

3.5 | miR-708-3p inhibits EMT by targeting EMT stimulators

To investigate the underlying mechanism of the effect of miR-708-3p on breast cancer cell EMT, we used miRNA target prediction algorithms (targetsdb.org and mirdb.org) to screen for miR-708-3p target genes, and we identified ZEB1, CDH2 and vimentin as tentative targets of miR-708-3p. ZEB1, CDH2 and vimentin are well-defined stimulators of EMT in breast cancer, and our target prediction results showed that these genes contain the binding sequence of miR-708-3p (Figure 5A). To investigate the association...
between miR-708-3p and target gene expression, we examined the expression levels of these target genes in miR-708-3p-overexpressing or miR-708-3p-inhibited MDA-MB-468 breast cancer cells. As shown in Figure 5B,C, the expression of ZEB1, CDH2 and vimentin was significantly decreased in miR-708-3p-overexpressing cells at both the mRNA and protein levels compared to that in vector control cells. In contrast, inhibition of miR-708-3p led to an increase in the expression of ZEB1, CDH2 and vimentin at both the mRNA and protein levels (Figure 5B,C). Next, to determine whether the regulation of the expression of target luciferase reporter genes depended on binding of their complementary 3′-UTR sequences to the miR-708-3p seed sequence, a 3-nucleotide mutation was inserted into the 3′-UTR of the target genes, as indicated in Figure 5A. Our data showed that overexpression or inhibition of miR-708-3p significantly repressed or increased the luciferase activity associated with the wild-type 3′-UTR, respectively. However, the 3′-UTR mutation completely abrogated the effect of miR-708-3p overexpression or inhibition on luciferase activity in MDA-MB-468 cells (Figure 5D). Cumulatively, these data suggest that miR-708-3p negatively regulates the expression of ZEB1, CDH2 and vimentin in breast cancer cells by directly targeting their 3′-UTR sequence.

3.6 | miR-708-3p enhances chemosensitivity of breast cancer cells

Previous studies have shown that EMT plays a key role in the chemoresistance of breast cancer, and our data showed that miR-708-3p is involved in the regulation of EMT. Thus, herein, we investigated the effects of miR-708-3p on the chemosensitivity of breast cancer cells. As shown Figure 6A, inhibition of miR-708-3p suppressed doxorubicin-induced inhibition of cell viability in both MDA-MB-468 and MCF-7 cells compared to controls. In contrast, overexpression of miR-708-3p significantly enhanced doxorubicin-induced inhibition of cell viability in both MDA-MB-468 and MCF-7 cells compared to control cells (Figure 6A). Consistent with these results, caspase-3/7 activity assay results showed that inhibition of miR-708-3p decreased, whereas overexpression of miR-708-3p increased, doxorubicin-induced caspase-3/7 activity in MDA-MB-468 cells.
compared to controls (Figure 6B). Furthermore, we confirmed these in vitro experimental results using an animal xenograft model. Our animal experimental results clearly showed that the combination treatment of miR-708-3p and doxorubicin more dramatically inhibited tumor growth compared to vehicle control and single drug treatment in an MDA-MB-468 xenograft model (Figure 6C). TUNEL assay results also showed that combination treatment more significantly induced apoptosis than other treatments (Figure 6D). Taken together, these findings suggest that miR-708-3p is negatively correlated with chemoresistance in breast cancer and that the restoration of miR-708-3p may be a useful strategy for enhancing chemosensitivity for the treatment of breast cancer.

4 | DISCUSSION

Metastasis and chemoresistance are important prognostic factors and are associated with poor survival in patients with breast cancer.27 Regarding breast cancer, although extensive studies concerning its intricate molecular mechanisms are ongoing, development of novel therapies and more accurate diagnostic and prognostic approaches is still a challenge.28 Herein, we identified that the expression of miR-708-3p was remarkably decreased in specimens from breast cancer patients with metastasis compared to specimens from patients without metastasis. In addition, in vitro data showed that the expression level of miR-708-3p was negatively correlated with invasiveness in breast cancer cell lines, suggesting that miR-708-3p expression level may be a candidate biomarker for predicting breast cancer metastasis. Additionally, we demonstrated a crucial role for miR-708-3p in breast cancer metastasis and chemosensitivity. Our in vitro and in vivo experimental data clearly suggested that decreased expression of miR-708-3p significantly stimulated breast cancer cell metastasis and the development of chemoresistance. Notably, our investigation further discovered that ectopic expression of miR-708-3p significantly inhibited breast cancer cell metastasis and the development of chemoresistance. In the present study, we also clarified the tumor inhibition mechanism of miR-708-3p in breast cancer. EMT occurs when epithelial cells lose their connections with their environment and achieve a
mesenchymal phenotype, facilitating their entry into blood circulation and migration to distant locations.\(^{28,29}\) Studies show that breast cancer is an aggressive cancer with a high EMT-associated metastatic ability.\(^{28,30}\) Interestingly, recent studies show that EMT is also closely related with the development of chemoresistance and that inhibition of EMT can abrogate chemoresistance in breast cancer,\(^{31}\) suggesting that EMT is an important therapeutic target for breast cancer metastasis and chemoresistance. EMT can be activated by several stimulators, including CDH2, ZEB1 and vimentin, and studies show that these EMT stimulators are also closely correlated with metastasis and chemoresistance in breast cancer.\(^{32,33}\) Interestingly, recent studies show that overexpression of EMT stimulators is caused by the downregulation of tumor suppressor miRNAs.\(^{34,35}\) In the present study, for the first time, we found that miR-708-3p negatively regulates EMT in breast cancer cells. Furthermore, we identified ZEB1, CDH2 and vimentin as novel targets of miR-708-3p in breast cancer cells. Herein, our data showed that restoration of miR-708-3p expression in breast cancer cells lead to the suppression of ZEB1, CDH2 and vimentin expression; conversely, inhibition of miR-708-3p further upregulates ZEB1, CDH2 and vimentin expression. Luciferase reporter gene experiments showed that miR-708-3p directly targets the 3'-UTR of ZEB1, CDH2 and vimentin. These findings clearly suggest that miR-708-3p mediates breast cancer cell metastasis and chemoresistance by inhibition of EMT by targeting ZEB1, CDH2 and vimentin.

In summary, we identified a novel anticancer miRNA, miR-708-3p, in breast cancer, and the miR-708-3p level was negatively correlated with breast cancer metastasis and chemoresistance. Restoration of miR-708-3p inhibits metastasis and enhances the chemosensitivity of breast cancer cells by inhibition of EMT by targeting CDH2, ZEB1 and vimentin. Our findings may help to establish new strategies for improving therapeutic options for breast cancer patients with metastasis or chemoresistance.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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