

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

Jin-Won Lee<sup>1</sup> | Wei Guan<sup>2</sup> | Sanghak Han<sup>3</sup> | Deok-Ki Hong<sup>4</sup> | Lee-Su Kim<sup>1</sup> | Haesung Kim<sup>1</sup> 

<sup>1</sup>Department of Surgery, Chuncheon Sacred Heart Hospital, College of Medicine, Hallym University, Chuncheon, South Korea

<sup>2</sup>Cancer Center, Daping Hospital and Research Institute of Surgery, Third Military Medical University, Chongqing, China

<sup>3</sup>Department of Pathology, Chuncheon Sacred Heart Hospital, College of Medicine, Hallym University, Chuncheon, South Korea

<sup>4</sup>Department of Biochemistry, Chuncheon Sacred Heart Hospital, College of Medicine, Hallym University, Chuncheon, South Korea

## Correspondence

Haesung Kim, Department of Surgery, Chuncheon Sacred Heart Hospital, College of Medicine, Hallym University, Chuncheon, South Korea.  
Email: kimhs70783@gmail.com

## Funding information

Hallym University Research Fund, Grant/Award Number: HURF-2014-40

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*. In contrast, overexpression of miR-708-3p dramatically inhibited breast cancer cell metastasis and enhanced the sensitivity of breast cancer cells to chemotherapy 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 miR-708-3p acts as a cancer suppressor miRNA and carries out its anticancer function by inhibiting EMT in breast cancer. In addition, 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.<sup>1</sup> 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.<sup>2</sup> Importantly, chemoresistance and metastasis are closely correlated with poor clinical outcome in patients with breast cancers,<sup>3</sup> 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

Jin-Won Lee and Wei Guan contributed equally to this work.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2018 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

carcinoma pathogenesis to drive epithelial cells towards a mesenchymal state.<sup>4</sup> 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.<sup>4</sup> 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.<sup>5</sup> 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<sup>6</sup> and inhibit cancer metastasis.<sup>7</sup>

Studies show that many factors, including microRNAs (miRNAs), are involved in the regulation of EMT.<sup>8-10</sup> 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.<sup>11</sup> 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.<sup>12-16</sup> Accumulating evidence shows that dysregulated miRNAs are also involved in the activation of EMT in cancer.<sup>10,17</sup> Studies show that dysregulated miRNAs induce EMT partly through targeting EMT stimulators, including ZEB1,<sup>9</sup> CDH2,<sup>18</sup> N-cadherin,<sup>19</sup> and vimentin.<sup>20</sup>

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, MTT, 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 to E-cadherin, CDH2, vimentin, ZEB1 and  $\beta$ -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% CO<sub>2</sub>.

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

**TABLE 1** Characteristics of patients with breast cancer in the present study

Variables	Low miR-708-3p		High miR-708-3p		P-value
	No.	%	No.	%	
Age (years)					
<50	11	52.4	13	44.8	.60
>50	10	47.6	16	55.2	
T status					
T1-T2	9	42.9	20	69.0	.06
T3-T4	12	57.1	9	31.0	
LN metastasis					
Yes	12	57.1	8	27.6	.04
No	9	42.9	21	72.4	
Clinical stage					
I-II	8	38.1	20	69.0	.02
III-IV	13	61.9	9	31.0	
ER status					
Negative	7	33.3	13	44.8	.41
Positive	14	66.7	16	55.2	
PR status					
Negative	8	38.1	14	48.3	.47
Positive	13	61.9	15	51.7	
HER2 status					
Negative	11	52.4	18	62.1	.49
Positive	10	47.6	11	37.9	

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  $2^{-\Delta Ct}$  method, and the miR-708-3p expression fold change in breast cancer samples matched to non-tumor control samples was evaluated using the  $2^{-\Delta\Delta Ct}$  method. On the basis of the mean fold change of miR-708-3p expression, patients were divided into high- (fold change > mean) and low- (fold change < mean) miR-708-3p expression groups.<sup>15</sup>

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'-GTCAGCAGAAGTTGAAGAAATAGTG-3' and reverse 5'-GCAAGTTGATTGGAGGGATG-3'; for vimentin, forward 5'-GACGC CATCAACACCGAGTT-3' and reverse 5'-CTTTGTCGTTGGTTAG CTGGT-3'; for ZEB1: forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; for actin, forward 5'-AG AGTACGAGCTGCC TGAC-3' and reverse, 5'-AGCACTGTGTTGG CGTACAG-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.<sup>21</sup>

## 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,  $1 \times 10^4$  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 mm<sup>3</sup>, 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  $\pm$  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

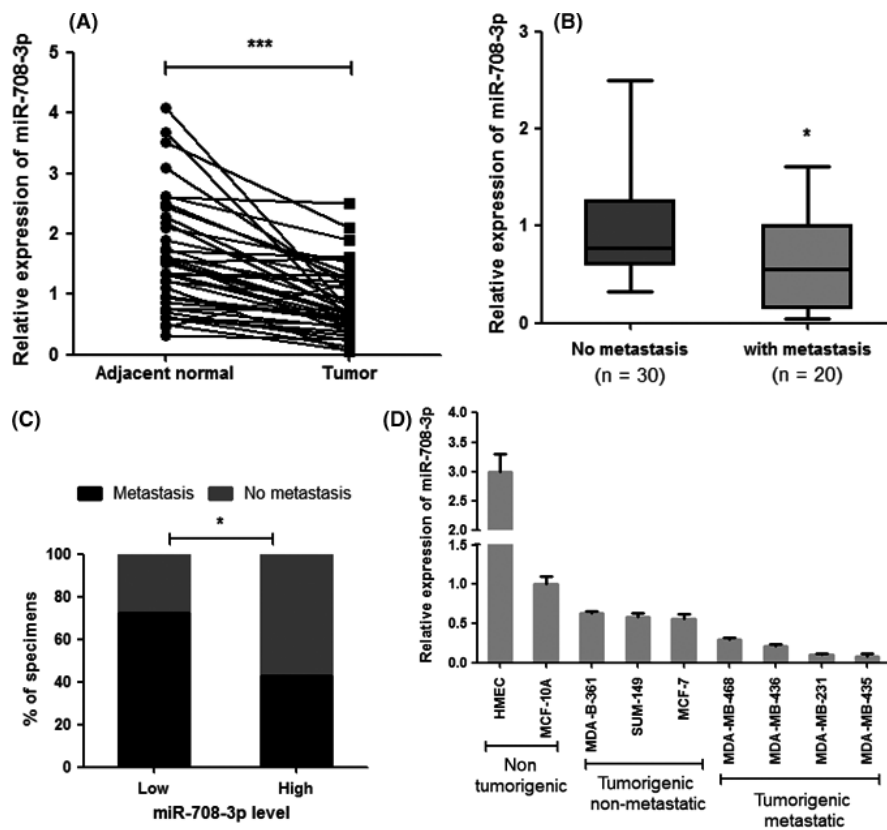
#### 3.1 | Decreased expression of miR-708-3p was correlated with metastasis in breast cancer

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.<sup>22</sup> 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-708-3p level (Table 1). Consistent

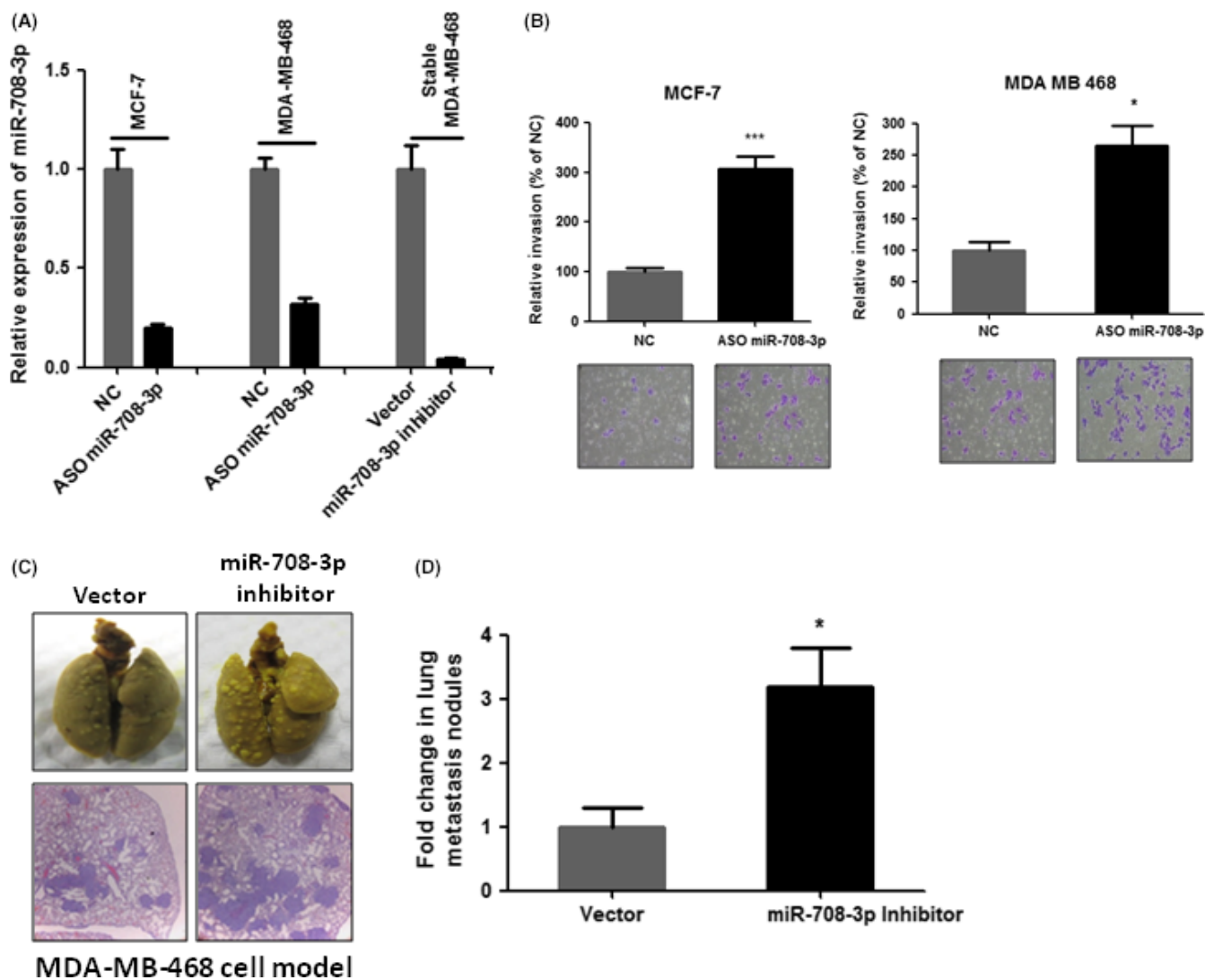
with clinical data, *in vitro* experiments also showed that miR-708-3p was significantly downregulated in aggressive breast cancer cell lines (Figure 1D). Taken together, these findings suggest that decreased expression of miR-708-3p was significantly correlated with breast cancer metastasis.

#### 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. \* $P < .05$ ; \*\*\* $P < .001$



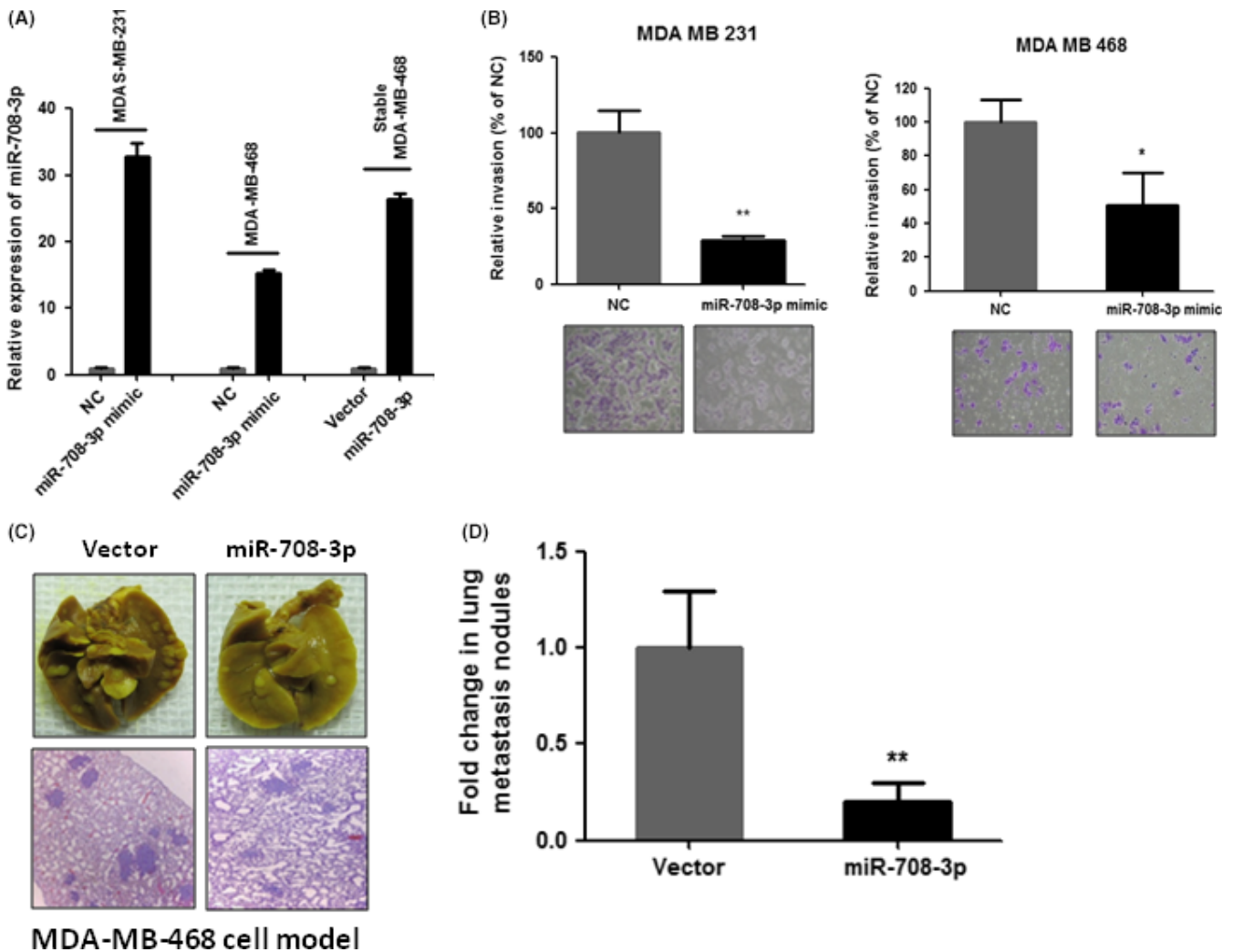
**FIGURE 2** Inhibition of microRNA-708-3p (miR-708-3p) significantly stimulated breast cancer cell metastasis. A, miR-708-3p expression was significantly decreased in miR-708-3p antisense nucleotide (ASO miR-708-3p)-transfected or stably expressing miR-708-3p antisense breast cancer cells. The indicated cells were transfected with indicated nucleotides or plasmid and, then, the expression of miR-708-3p was measured by RT-qPCR after 72 h of transfection. B, Inhibition of miR-708-3p significantly stimulated cell invasion in both MCF-7 and MDA-MB-468 breast cancer cells. Indicated cells were transfected with negative control oligonucleotides (NC) or ASO miR-708-3p. After 48 h of transfection, the cells were subjected to invasion assays. C, Inhibition of miR-708-3p significantly stimulated MDA-MB-468 cell lung metastasis. Stably expressing miR-708-3p antisense breast cancer cells or their vector control cells were injected into nude mice through the tail vein. After 1 mo of cell injection, the mice were killed, and their lungs were collected. D, Tumor nodules on the lung surface were counted. \* $P < .05$ ; \*\*\* $P < .001$

lung surface. 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.



**FIGURE 3** Overexpression of microRNA-708-3p (miR-708-3p) significantly inhibited breast cancer cell metastasis. A, miR-708-3p expression was significantly increased in miR-708-3p mimic transfected or stably expressing miR-708-3p breast cancer cells. The indicated cells were transfected with negative control nucleotides (NC) or miR-708-3p mimics and, then, the expression of miR-708-3p was measured using RT-qPCR after 72 h of transfection. B, Overexpression of miR-708-3p significantly inhibited cell invasion in both MDA-MB-231 and MDA-MB-468 breast cancer cells. The indicated cells were transfected with NC or miR-708-3p mimics. After 48 h of transfection, the cells were subjected to invasion assays. C, Overexpression of miR-708-3p significantly inhibited MDA-MB-468 cell lung metastasis. Stably expressing miR-708-3p breast cancer cells or their vector control cells were injected into nude mice through the tail vein. After 1 mo of cell injection, the mice were killed, and their lungs were collected. D, Tumor nodules on the lung surface were counted. \* $P < .05$ ; \*\* $P < .01$

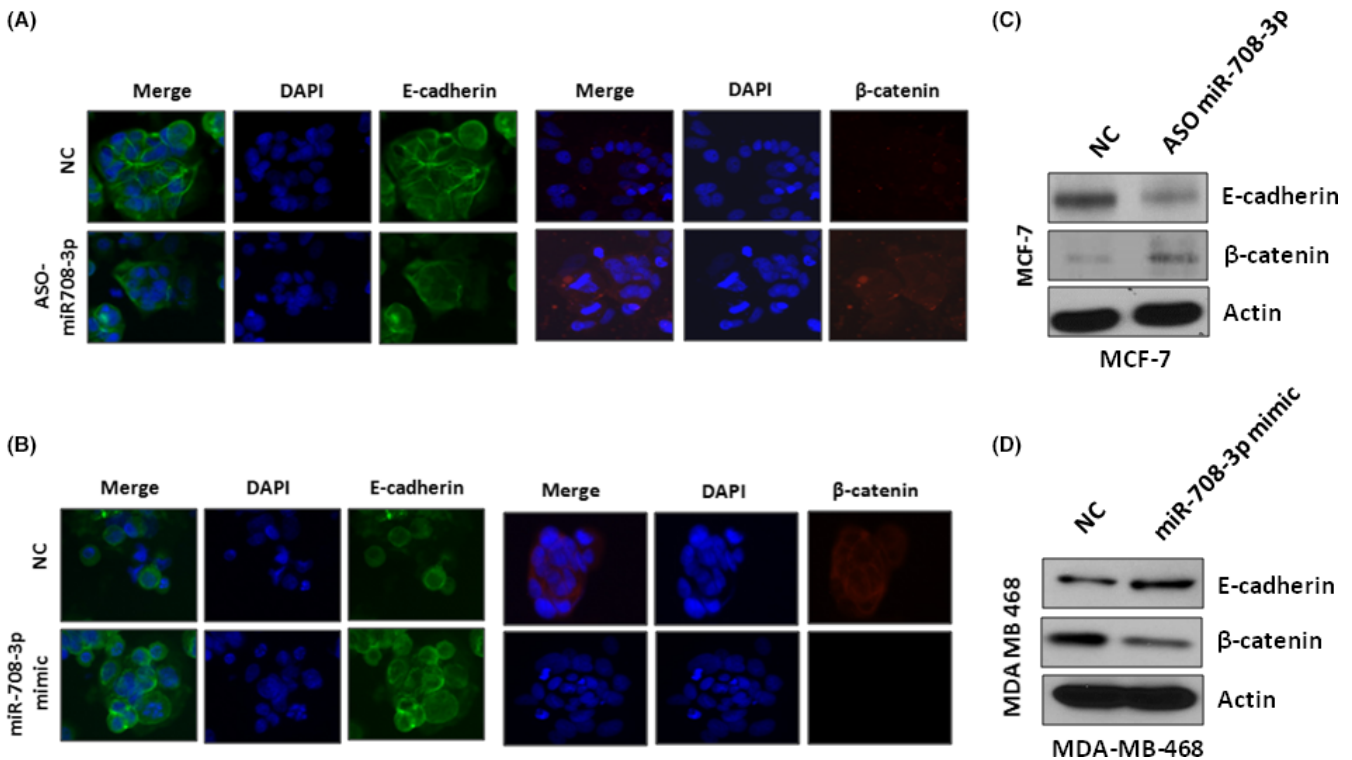
### 3.4 | miR-708-3p inhibits EMT in breast cancer cells

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  $\beta$ -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  $\beta$ -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 (targetscan.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,<sup>23</sup> CDH2<sup>24</sup> and vimentin<sup>25</sup> 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



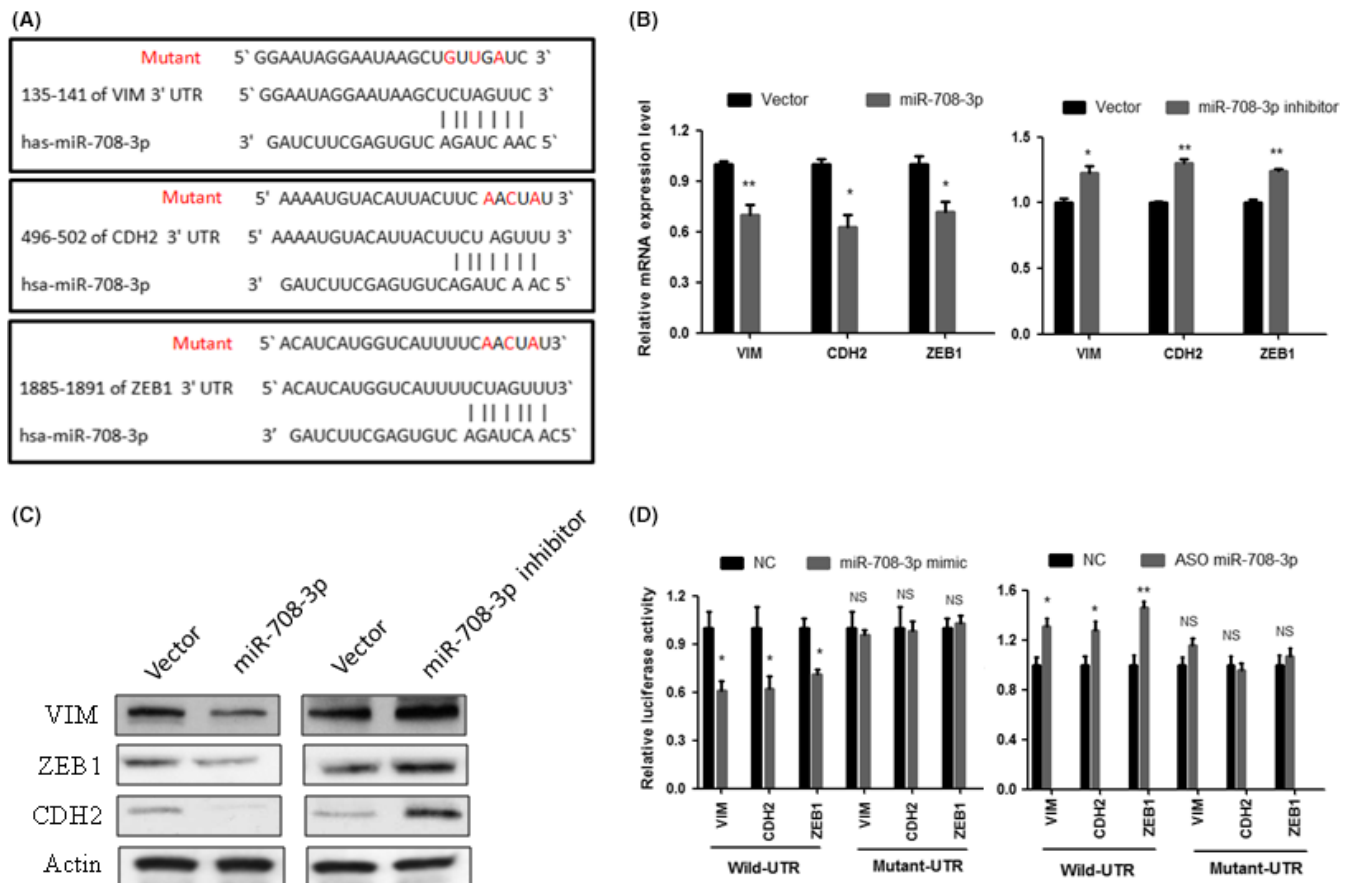
**FIGURE 4** MicroRNA-708-3p (miR-708-3p) negatively regulated epithelial-to-mesenchymal transition (EMT) in breast cancer cells. A, Inhibition of miR-708-3p suppressed E-cadherin expression and increased  $\beta$ -catenin expression in MCF-7 cells. MCF-7 cells were transfected with negative control oligonucleotides (NC) or miR-708-3p antisense oligonucleotide (ASO miR-708-3p). After 72 h of transfection, the cells were subjected to immunofluorescence assay. B, Overexpression of miR-708-3p increased E-cadherin expression and decreased  $\beta$ -catenin expression in MDA-MB-468 cells. The indicated cells were transfected with negative control oligonucleotides (NC) or miR-708-3p mimics. After 72 h of transfection, the cells were subjected to immunofluorescence assay. C, Western blot analysis showed that inhibition of miR-708-3p suppressed E-cadherin expression but increased  $\beta$ -catenin expression in MCF-7 cells. MCF-7 cells were transfected with the indicated nucleotides for 72 h and then subjected to analysis. D, Western blot analysis showed that overexpression of miR-708-3p increased E-cadherin expression and decreased  $\beta$ -catenin expression in MDA-MB-468 cells. The indicated cells were transfected with the indicated nucleotides. After 72 h of transfection, the cells were subjected to analysis

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,<sup>26</sup> 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



**FIGURE 5** MicroRNA-708-3p (miR-708-3p) targeted ZEB1, CDH2 and vimentin in breast cancer cells. A, Sequence alignment of miR-708-3p with the 3'-UTR of ZEB1, CDH2 and vimentin. B,C, miR-708-3p negatively regulated the expression of ZEB1, CDH2 and vimentin in MDA-MB-468 cells at both mRNA and protein levels. Stably expressing miR-708-3p or miR-708-3p-antisense MDA-MB-468 cells were subjected to RT-qPCR and western blot analysis. D, MDA-MB-468 cells were cotransfected with the 3'-UTR luciferase reporter construct (wild-type or mutant type) of the indicated genes and the indicated nucleotides. After 48 h of transfection, the cells were subjected to luciferase activity assay. \* $P < .05$ ; \*\* $P < .01$ . NC, negative control oligonucleotides

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-231 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.

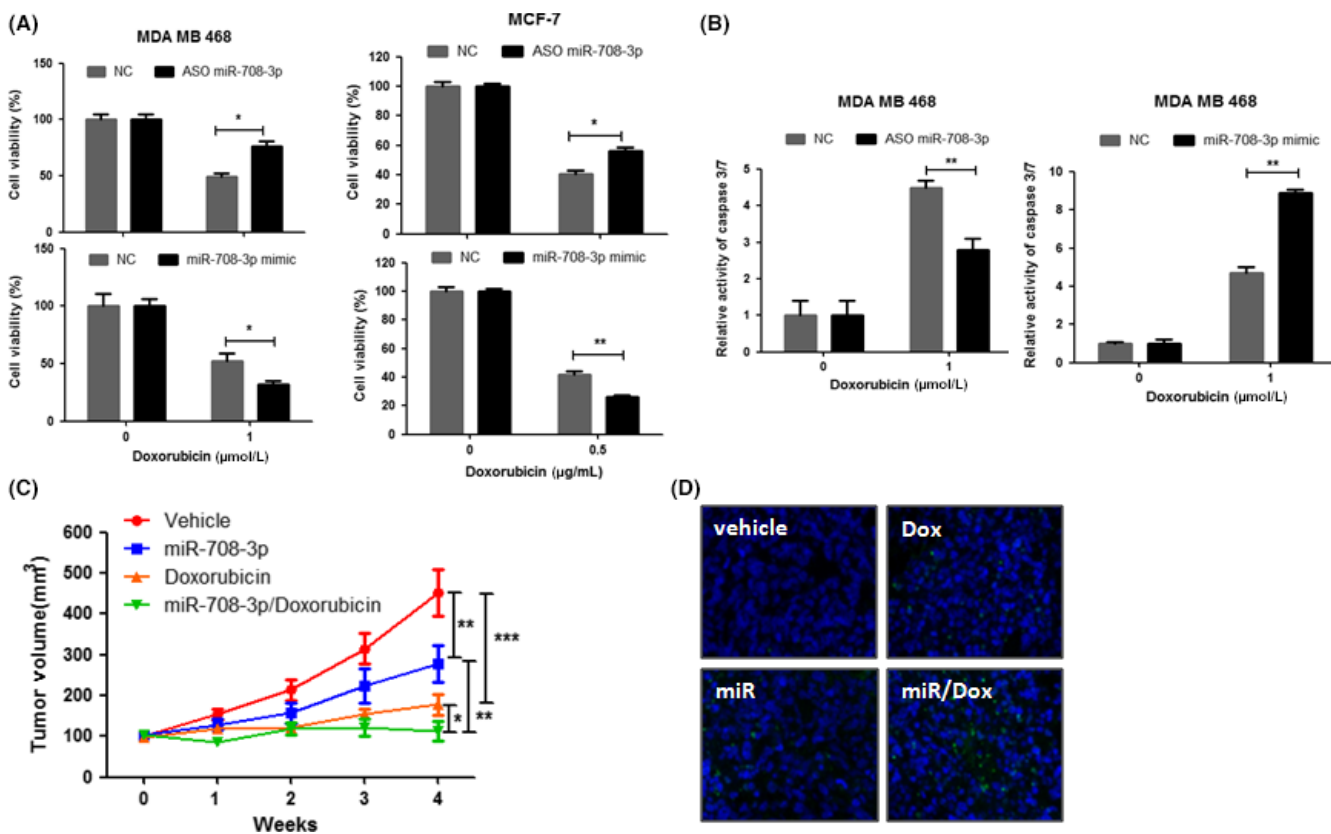
## 4 | DISCUSSION

Metastasis and chemoresistance are important prognostic factors and are associated with poor survival in patients with breast cancer.<sup>27</sup> 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.<sup>28</sup> 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 with no 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 metastasis and enhanced the sensitivity of chemotherapy in breast cancer both in vitro and in vivo, thus suggesting that ectopic expression of miR-708-3p may be a novel strategy for inhibiting metastasis and enhancing the chemosensitivity of breast cancer.

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





**FIGURE 6** MicroRNA-708-3p (miR-708-3p) enhanced doxorubicin-induced apoptosis in breast cancer. A, Inhibition of miR-708-3p protected cells from doxorubicin-induced inhibition of cell viability, whereas overexpression of miR-708-3p enhanced doxorubicin-induced inhibition of cell viability in both MDA-MB-468 and MCF-7 breast cancer cell lines. B, Inhibition of miR-708-3p inhibited doxorubicin-induced caspase-3/7 activity, whereas overexpression of miR-708-3p enhanced doxorubicin-induced caspase-3/7 activity in MDA-MB-468 breast cancer cells. C, Combination of miR-708-3p and doxorubicin significantly suppressed tumor growth in an MDA-MB-231 xenograft model compared to vehicle and single treatments. MDA-MB-231 cells, which stably express miR-708-3p, and their vector control cells were injected s.c. into nude mice. After the mean tumor volume reached approximately 100 mm<sup>3</sup>, treatment of the mice with doxorubicin for 1 mo was started. D, Combination of miR-708-3p and doxorubicin more significantly induced apoptosis than vehicle control and single drug treatments in xenograft tumors. TUNEL assay was carried out for the detection of apoptotic cells in tumor tissues from the MDA-MB-231 xenograft model. \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ . NC, negative control oligonucleotides

mesenchymal phenotype, facilitating their entry into blood circulation and migration to distant locations.<sup>28,29</sup> Studies show that breast cancer is an aggressive cancer with a high EMT-associated metastatic ability.<sup>28,30</sup> 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,<sup>31</sup> 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.<sup>32,33</sup> Interestingly, recent studies show that overexpression of EMT stimulators is caused by the downregulation of tumor suppressor miRNAs.<sup>34,35</sup> 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.

#### ACKNOWLEDGMENT

This research was supported by Hallym University Research Fund (HURF-2014-40).

## CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

## ORCID

Haesung Kim  <http://orcid.org/0000-0002-7047-3810>

## REFERENCES

- DeSantis C, Ma J, Bryan L, Jemal A. Breast cancer statistics, 2013. *CA Cancer J Clin* 2014;64:52-62.
- Chen LP, Cai SM, Fan JX, Li ZT. PEBA regimen (cisplatin, etoposide, bleomycin, and adriamycin) in the treatment of drug-resistant choriocarcinoma. *Gynecol Oncol*. 1995;56:231-234.
- Matysiak M, Kapka-Skrzypczak L, Jodlowska-Jedrych B, Kruszewski M. EMT promoting transcription factors as prognostic markers in human breast cancer. *Arch Gynecol Obstet*. 2017;295:817-825.
- Aiello NM, Brabletz T, Kang YB, Nieto MA, Weinberg RA, Stanger BZ. Upholding a role for EMT in pancreatic cancer metastasis. *Nature*. 2017;547:E7-E8.
- Zhang PJ, Sun YT, Ma L. ZEB1: at the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. *Cell Cycle*. 2015;14:481-487.
- Piskareva O, Harvey H, Nolan J, et al. The development of cisplatin resistance in neuroblastoma is accompanied by epithelial to mesenchymal transition in vitro. *Cancer Lett*. 2015;364:142-155.
- Xu Q, Sun Q, Zhang JJ, Yu JS, Chen WT, Zhang ZY. Downregulation of miR-153 contributes to epithelial-mesenchymal transition and tumor metastasis in human epithelial cancer. *Carcinogenesis*. 2013;34:539-549.
- Roy SS, Gonugunta VK, Bandyopadhyay A, et al. Significance of PELP1/HDAC2/miR-200 regulatory network in EMT and metastasis of breast cancer. *Oncogene*. 2014;33:3707-3716.
- Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol*. 2008;10:593-601.
- Iliopoulos D, Polytaichou C, Hatzia Apostolou M, et al. MicroRNAs differentially regulated by Akt isoforms control EMT and stem cell renewal in cancer cells. *Sci Signal*. 2009;2:ra62.
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281-297.
- Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM. MicroRNA expression and function in cancer. *Trends Mol Med*. 2006;12:580-587.
- Ueda T, Volinia S, Okumura H, et al. Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol*. 2010;11:136-146.
- Korpala M, Eli BJ, Buffa FM, et al. Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nat Med*. 2011;17:1101-1108.
- Wang RT, Xu M, Xu CX, Song ZG, Jin H. Decreased expression of miR216a contributes to non-small-cell lung cancer progression. *Clin Cancer Res*. 2014;20:4705-4716.
- Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA*. 2006;103:2257-2261.
- Burk U, Schubert J, Wellner U, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep*. 2008;9:582-589.
- Zhao Z, Fan X, Jiang L, et al. miR-503-3p promotes epithelial-mesenchymal transition in breast cancer by directly targeting SMAD2 and E-cadherin. *J Genet Genomics*. 2017;44:75-84.
- Ma T, Zhao Y, Wei K, et al. MicroRNA-124 functions as a tumor suppressor by regulating CDH2 and epithelial-mesenchymal transition in non-small cell lung cancer. *Cell Physiol Biochem*. 2016;38:1563-1574.
- Zhu Y, Zhang Y, Sui Z, Zhang Y, Liu M, Tang H. USP14 de-ubiquitinates vimentin and miR-320a modulates USP14 and vimentin to contribute to malignancy in gastric cancer cells. *Oncotarget*. 2017;8:48725-48736.
- Xu CX, Jere D, Jin H, et al. Poly(ester amine)-mediated, aerosol-delivered Akt1 small interfering RNA suppresses lung tumorigenesis. *Am J Respir Crit Care Med*. 2008;178:60-73.
- Zhou W, Fong MY, Min Y, et al. Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell*. 2014;25:501-515.
- Aigner K, Dampier B, Descovich L, et al. The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. *Oncogene*. 2007;26:6979-6988.
- Wu Y, Sarkissyan M, Vadgama JV. Epithelial-mesenchymal transition and breast cancer. *J Clin Med*. 2016;5:13.
- Vuoriluoto K, Haugen H, Kiviluoto S, et al. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. *Oncogene*. 2011;30:1436-1448.
- Sciacovelli M, Goncalves E, Johnson TI, et al. Fumarate is an epigenetic modifier that elicits epithelial-to-mesenchymal transition. *Nature*. 2016;537:544-547.
- Hu GH, Chong RA, Yang QF, et al. MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. *Cancer Cell*. 2009;15:9-20.
- Zare M, Bastami M, Solali S, Alivand MR. Aberrant miRNA promoter methylation and EMT-involving miRNAs in breast cancer metastasis: diagnosis and therapeutic implications. *J Cell Physiol*. 2018;233:3729-3744.
- Bill R, Christofori G. The relevance of EMT in breast cancer metastasis: correlation or causality? *FEBS Lett*. 2015;589:1577-1587.
- Smith BN, Bhowmick NA. Role of EMT in metastasis and therapy resistance. *J Clin Med*. 2016;5:17.
- Zheng XF, Carstens JL, Kim J, et al. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature*. 2015;527:525-530.
- Gerashchenko TS, Zavyalova MV, Denisov EV, et al. Intratumoral morphological heterogeneity of breast cancer as an indicator of the metastatic potential and tumor chemosensitivity. *Acta Naturae*. 2017;9:56-67.
- Korsching E, Packeisen J, Liedtke C, et al. The origin of vimentin expression in invasive breast cancer: epithelial-mesenchymal transition, myoepithelial histogenesis or histogenesis from progenitor cells with bilinear differentiation potential? *J Pathol*. 2005;206:451-457.
- Ma ZH, Li Y, Xu JC, Ren QZ, Yao JH, Tian XF. MicroRNA-409-3p regulates cell invasion and metastasis by targeting ZEB1 in breast cancer. *IUBMB Life*. 2016;68:394-402.
- Zhang J, Liu D, Feng Z, et al. MicroRNA-138 modulates metastasis and EMT in breast cancer cells by targeting vimentin. *Biomed Pharmacother*. 2016;77:135-141.

**How to cite this article:** Lee J-W, Guan W, Han S, Hong D-K, Kim L-S, Kim H. MicroRNA-708-3p mediates metastasis and chemoresistance through inhibition of epithelial-to-mesenchymal transition in breast cancer. *Cancer Sci*. 2018;109:1404-1413. <https://doi.org/10.1111/cas.13588>