Original Article
MiR-300 regulate the malignancy of breast cancer by targeting p53

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Abstract: Objective: In this study, we investigated the role of miR-300 in regulating cell proliferation and invasion of breast cancer (BC) cells. Methods: MicroRNA and protein expression patterns were compared between breast cancer tissue and normal tissue and between two different prognostic groups. The up-regulation of miR-300 was confirmed by real-time reverse transcription polymerase chain reaction and its expression was analyzed in MCF-7 breast cancer cells. Results: We observed that miR-300 expression was frequently and dramatically up-regulated in human breast cancer tissues and cell lines compared with the matched adjacent normal tissues and cells. We further showed that transient and stable over-expression of miR-300 could promote cell proliferation and cell cycle progression. Moreover, p53, a key inhibitor of cell cycle, was verified as a direct target of miR-300, suggesting that miR-300 might promote breast cancer cell proliferation and invasion by regulating p53 expression. Conclusion: Our findings indicated that miR-300 up-regulation might exert some sort of antagonistic function by targeting p53 in breast cancer cell proliferation during breast tumorigenesis.

Keywords: MiR-300, p53, breast cancer, MCF-7, target therapy

Introduction

As the third leading culprit in cancer incidence worldwide [1], breast cancer (BC) continues to pose significant diagnostic, prognostic and therapeutic tribulations for clinicians. The American Joint Committee on Cancer (AJCC) TNM staging system is currently the only prognostic classification used in clinical practice to select patients for adjuvant chemotherapy [2-4]. However, the AJCC stage fails to predict recurrence accurately in many patients undergoing curative surgery for localized BC. This highlights the need for new biomarkers for a more precise prediction of high-risk patients with BC recurrence and consequently improved personalized cancer care.

MiRNAs are a novel class of small noncoding RNAs that typically inhibit the translation and stability of messenger RNAs (mRNAs) by binding to the 3'-untranslated regions (3'-UTR) of their target mRNAs [5]. MiRNAs have [6-8] nucleotides and are found in all multi-cellular eukaryotic cells. MiRNAs have important roles in various biological and pathological processes, such as development, cell proliferation, differentiation, apoptosis, inflammation, stress response and migration [7-9]. Increasing evidences have suggested that miRNAs are deregulated or upregulated in all types of cancers, acting either as tumor suppressors (e.g. miR-34, miR-15/16, let-7, miR 200 family) or as oncogenes (e.g. miR-155, miR-222/221, miR-17-5p, miR-21) [1, 3, 8, 10], in which the miRNAs play key roles in important aspects of tumorigenesis, such as cancer initiation, differentiation, growth and progression [3, 11-15], mainly by interfering with the expression of target genes involved in cell cycle, apoptosis, cell migration and invasion, angiogenesis.

This study was to determine the expression of miR-300 and association with breast cancer formation, progression and the underlying mechanisms. We found that miR-300 was significantly reduced in human breast cancer tissues and in breast cancer cell lines. Using the

approaches of miRNA array, systemic biology, in vitro manipulating expression of miR-300 and in vivo tumor-bearing mouse model, we found that miR-300 acted as a tumor promoter in breast cancer, which was through targeting p53.

Material and methods

Patients and samples

Based on tissue data availability, between 2010 and 2012, BC samples were included in the present study. Formalin-fixed paraffin-embedded tissues were collected from the Second and First Affiliated Hospitals of Jiangxi University of Chinese Medicine (Nangchang, China). This study was approved by the Ethics Committees of Jiangxi University of Chinese Medicine. Informed consent was obtained from all participants and the study was performed in accordance with the Declaration of Helsinki. Follow-up data were obtained from medical records and direct communication with the patients or their relatives. The follow-up period was defined as the time from the date of surgery to the date of patient mortality or the final follow-up in January 2014.

Cell culture and transfection

MDA-MB-231, MDA-MB-453 and MCF-7 cell lines were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI-1640 in 5% CO_2 at 37°C. NS siRNA and scrambled siRNA were purchased from Invitrogen Life Technologies and transfected using Lipofectamine® 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions.

PCR

RNA isolation and reverse transcription were performed as previously described [22]. Oligonucleotide primer sequences were as follows: β-actin (264 bp), forward: 5'-GAG ACC TTC AAC ACC CCA GCC-3'; reverse: 5'-AAT GTC AC G CAC GATT TCC C-3'; p53 (201 bp), forward: 5'-TCC CCA TCG CCA TCC CC-3' reverse: 5'-CAC CAT GGC CTC GGC TGG-3'. For all the above genes, amplification was performed under the same cycling conditions (1 minute at 94°C, 50 seconds at 57°C, 1 minute at 72°C), except the

number of cycles that were specified for each gene (32 for p53).

Western blot and immunoprecipitation

MCF-7 were harvested at specific times after treatment with regents as indicated in each experiment. Cells were mixed with loading buffer and subject to electrophoresis. After electrophoresis, proteins were transferred to polyvinyl difluoride membranes (Pall Filtron) using a semidry blotting apparatus (Pharmacia) and probed with mouse mAbs, followed by incubation with peroxidase-labeled secondary antibodies. Detection was performed by the use of a chemiluminescence system (Amersham) according to the manufacturer's instructions. Then membrane was striped with elution buffer and re-probed with antibodies against the non-phosphorylated protein as a measure of loading control. Controls for the immunoprecipitation used the same procedure, except agarose beads contained only mouse IgG.

MTT assay

Cell viability was assessed using an MTT assay. Following transfection, cells were plated in 96-well plates and incubated for 24, 48 and 72 h. A total of 20 μl 5 mg/ml MTT (Sigma-Aldrich) was added to each corresponding test well and incubated for 4 h at 37°C. The supernatant was then discarded and 200 μl dimethyl sulfoxide was added to each well to dissolve the formazan. Optical density was assessed by measuring the absorbance of each well at 490 nm using a spectrophotometer (SpectraMax Plus384; Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate.

Cell viability assays

An Alamar blue assay was used to measure cell proliferation. This assay is based on the quantitative metabolic conversion of blue, non-fluorescent resazurin to pink, fluorescent resorufin by living cells. After 72 h of incubation, an Alamar blue (Invitrogen) stock solution was aseptically added to the wells to equal to 10% of the total incubation volume. The resazurin reduction in the cultures was determined after a 2-6 h incubation with Alamar blue by measuring the absorbances at 530-nm and 590-nm wavelengths on a Synergy HT Multi-Mode Microplate Reader (Bio-tek Instruments).
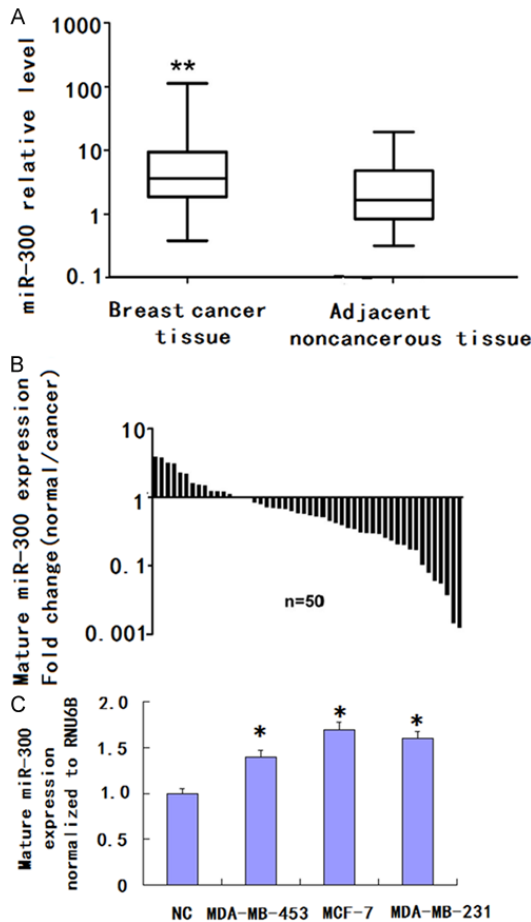
Figure 1. Up-regulation of miR-300 in breast cancer tissues and cell lines. A. In human breast cancer tissues compared with paired adjacent noncancerous (normal) breast tissues, the miR-300 was up-regulated. The expression of miR-300 was analyzed by real-time PCR and normalized to RNU6B. The results are displayed on a log scale. The statistical differences between samples were analyzed with the wilcoxon signed-rank test (n = 50). B. Relative expression of miR-300 in 50 breast cancer tissues compared with matched normal tissues. Data are shown as $2^{-\Delta\Delta CT}$ values. C. The expression of miR-300 in 3 breast cancer cell lines and one immortalized normal breast mucosal epithelial cell line (NS) was carried out by real-time PCR and normalized to RNU6B.

Cell cycle analyses

DNA contents of cells were analyzed using flow cytometry as described previously. Control and transfected cells were harvested and washed twice with PBS (Phosphate Buffer Saline), fixed in 70% ethanol and kept at -20°C until analysis. Then the cells were stained with 20 μg/ml PI containing 20 μg/ml RNase (DNase free) for 2 h. The stained cells were analyzed by flow cytometry (Partec Pas, Germany). The popula-

tion of G0/G1, S, G2/M and sub-G1 cells was determined using Mulicycle Cell Cycle Software. The results are expressed as percentage of the cells in each phase.

In vivo tumorigenesis study

Twenty four BALB/c nude mice (female, 6-9 wk, Guangzhou Laboratory Animal Center, Chinese Academy of Sciences, Guangzhou, China) were used in the study. Mice were maintained in a special pathogen-free (SPF) house with 12 h alternating light and dark cycles, and were given adequate nutrition and water *ad libitum*. 6×10^6 of cells suspended in FBS-free DMEM were injected into each side of the posterior flank of nude mice subcutaneously. Mice were sacrificed and tumors were collected 30 days after implantation. Tumor volume was calculated as follows: volume = 0.5 × length × width². Experimental protocols were reviewed and approved by the Animal Ethics Committee of Guangzhou Medical College.

Statistical analysis

Results are expressed as mean ± standard deviation. Data were analyzed using the unpaired two-tailed student's t test and the log rank test. *P* values of p < 0.05 were considered significant.

Results

miR-300 is frequently up-regulated in human breast cancer

Firstly, to determine whether the miR-300 is differentially expressed in human primary breast cancers, the expression level of the mature miR-300 was examined using real-time PCR in 50 pairs of human breast cancer tissues and pair-matched adjacent non-cancerous breast tissues. Our results showed that the expression level of miR-300 was significantly increased in breast cancer tissues in comparison with the adjacent non-cancerous breast tissues (**Figure 1A**). About 72% of tumor samples were higher expressed with miR-300 (**Figure 1B**). Using $2^{-\Delta\Delta CT}$ values, fold change of miR-300 < 1.0 was considered as low, while it > 1.0 was regarded as high expression [18]. miR-300 expression was also evaluated in breast cancer cell lines and one immortalized normal breast mucosal epithelial cell line (NS).
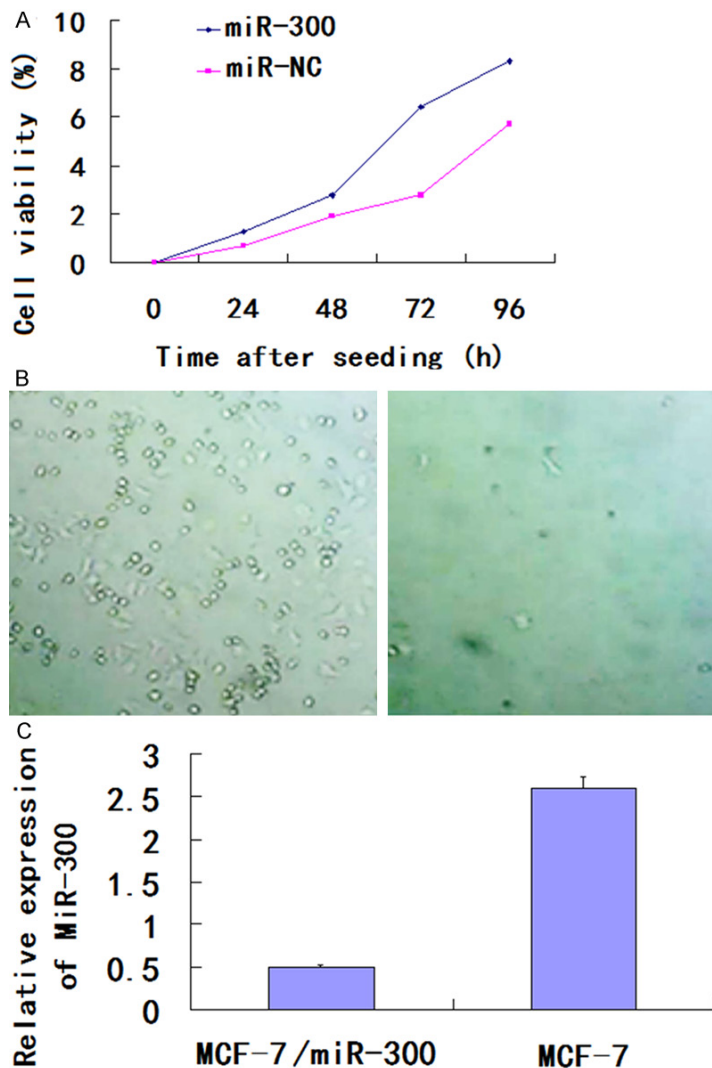
Figure 2. Upregulation of miR-300 promoted growth of breast cancers. A. The upregulation of miR-300 promoted MCF-7 (human breast cancer cells) cell growth. Cell viability was measured using a WST-1 kit at indicated time points. Data are presented as mean ± SD from three independent experiments performed in sextuple; B. MiR-300 over-expression induced colony formation of MCF-7 cells. 103 cells were mixed with agarose and seeded in 9-well plates for two weeks; C. The expression levels of miR-300 in the parental MCF-7 and the MCF-7/miR-300 stable cell line. Data are presented as mean ± SD. *P < 0.05 vs control.

As shown in **Figure 1C**, miR-300 was significantly high expression in all cancer cell lines compared with NS. Taken together, these results provide strong evidence that miR-300 was up-regulated in breast cancer.

Up-regulation of miR-300 increased in the proliferation of breast cancer

MCF-7 breast cell line with a stable over-expression of miR-300 were established by transfect-ing miR-300 plasmids, meanwhile, the MCF-7/miR-NC cell line was established as a control. The over-expression of miR-300 in MCF-7/miR-300 cells was confirmed by RT-PCR (**Figure 2C**). Cell growth was measured using WST-1 kit at indicated time points. The results showed that upregulation of miR-300 markedly promoted the proliferation of MCF-7 cells (**Figure 2A**). The anchorage independent colony formation assay also demonstrated the same results (**Figure 2B**).

miR-300 promoted breast cell migration in MCF-7 cells

Scratch wound-healing assay was conducted in miR-300-transfected MCF-7 cells and control cells. Migration distance was measured at 0, 24, 48 hours after cells were scratched. The results indicated that miR-300 promoted breast cell migration in MCF-7 cells (**Figure 3**).

Correlation between p53 and miR-300 expression

Analysis of microRNA and mRNA expression data from breast cancer tissue samples obtained from patients in Group 1 showed that P53 mRNA expression was positively correlated with miR-300 expression (**Figure 4A**; r = 0.712, P < 0.001). P53 expression was up-regulated in breast cancer compared with non-cancer tissue and was higher in C1 compared with C2 patients (**Figure 4B**). Over-expression of miR-300 was significantly associated with shorter overall survival (OS; P = 0.018; **Figure 4C-a**). Over-expression of P53 exhibited a tendency to poor OS (P = 0.215; **Figure 4C-b**).

p53 was a direct target of miR-300 in breast cancer cells

To determine the mechanisms by which miR-300 promotes breast cell proliferation, bioinformatics analysis was performed to search for miR-300 targets. CDNK1B (encoding p53), a
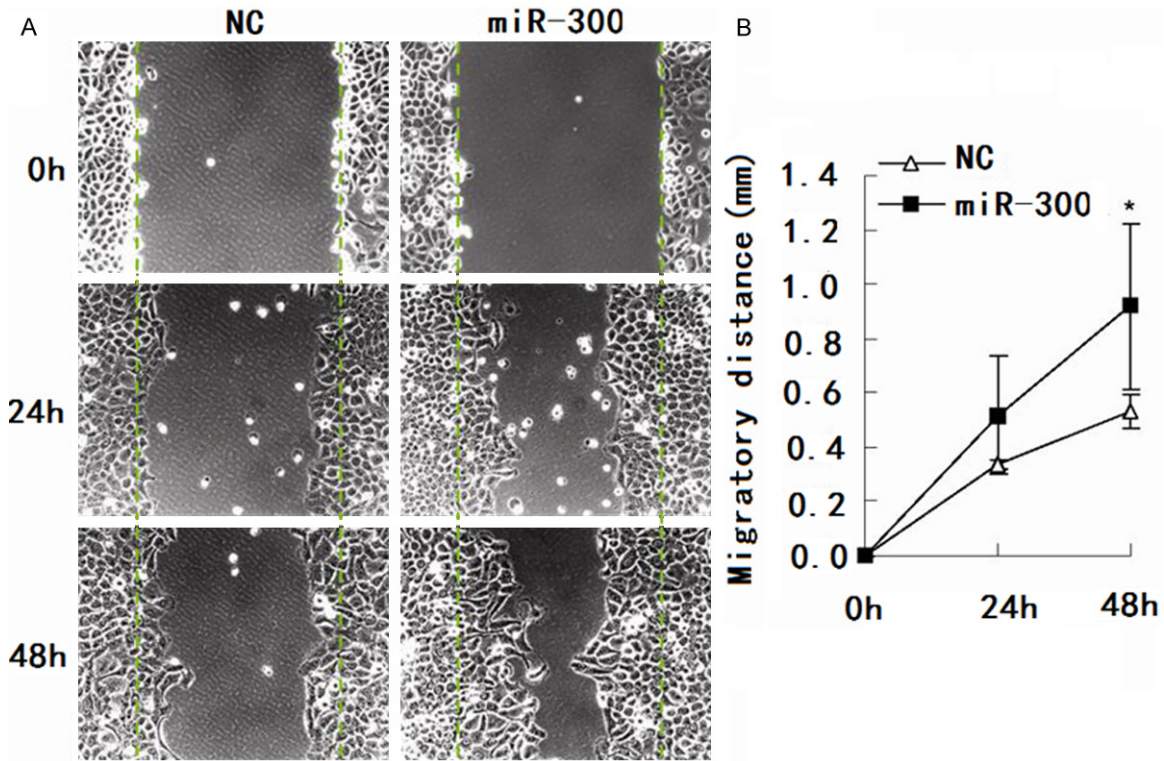
Figure 3. miR-300 promoted breast cell migration in MCF-7 cells. Scratch wound-healing assay was conducted in miR-300-transfected MCF-7 cells and control cells. Migration distance was measured at 0, 24, 48 hours after cells were scratched. Values represent mean ± SD. n = 3. *P < 0.05.

gene critical for cell cycle [15], was predicted as a putative target of miR-300. p53 mRNA 3'UTR has a single predicted binding site that is highly conserved in mammals, and in chicken. To determine whether miR-300 could directly regulate p53, p53 mRNA 3'UTR encompassing the predicted binding site with or without mutation that would disrupt miRNA interaction, were inserted to the downstream of luciferase open reading frame in pGL3-CM reporter vector. When introduced into MCF-7 cells, the wild type p53 3'UTR reporter showed a 39% reduction in luciferase activity in the miR-300-transfected cells compared with that in scrambled-miRNA-transfected cells (**Figure 5A**, p < 0.01). Mutation of miR-300 binding site abrogated the repression of luciferase activity caused by miR-300 over-expression (**Figure 5A**). Next, we pursued the ability of miR-300 to regulate the expression of endogenous p53. miR-300 overexpression with mimic in MCF-7 cells dramatically repressed p53 expression (**Figure 5B**). Conversely, miR-300 knockdown by AS dramatically enhanced p53 expression in MCF-7 cells

(**Figure 3B**), indicating that endogenous miR-300 could regulate p53 abundance. To examine the functional interrelationship between miR-300 and p53, MCF-7 cells was simultaneously transfected with miR-300 mimic and p53-expressing vector (CMV-p53). As expected, over-expression of p53 dramatically rescued miR-300-induced growth promotion (**Figure 5C** and **5D**, P< 0.01), indicating that miR-300 enhanced cell proliferation possibly by repressing p53.

MiR-218 induced in vivo tumor growth

The role of miR-300 was further explored using ectopic transplantation in a nude mice model. Stable cell lines, MCF-7/miR-300, and MCF-7/miR-NC were injected into nude mice subcutaneously. Tumor growth in the miR-300 group was increased significantly when compared to the control group (**Figure 6A** and **6B**). The tumor weights of the xenograft in the miR-300 group were lesser than those in the miR-NC group (**Figure 6C**). These data indicated the promoting role of miR-300 in breast cancer.
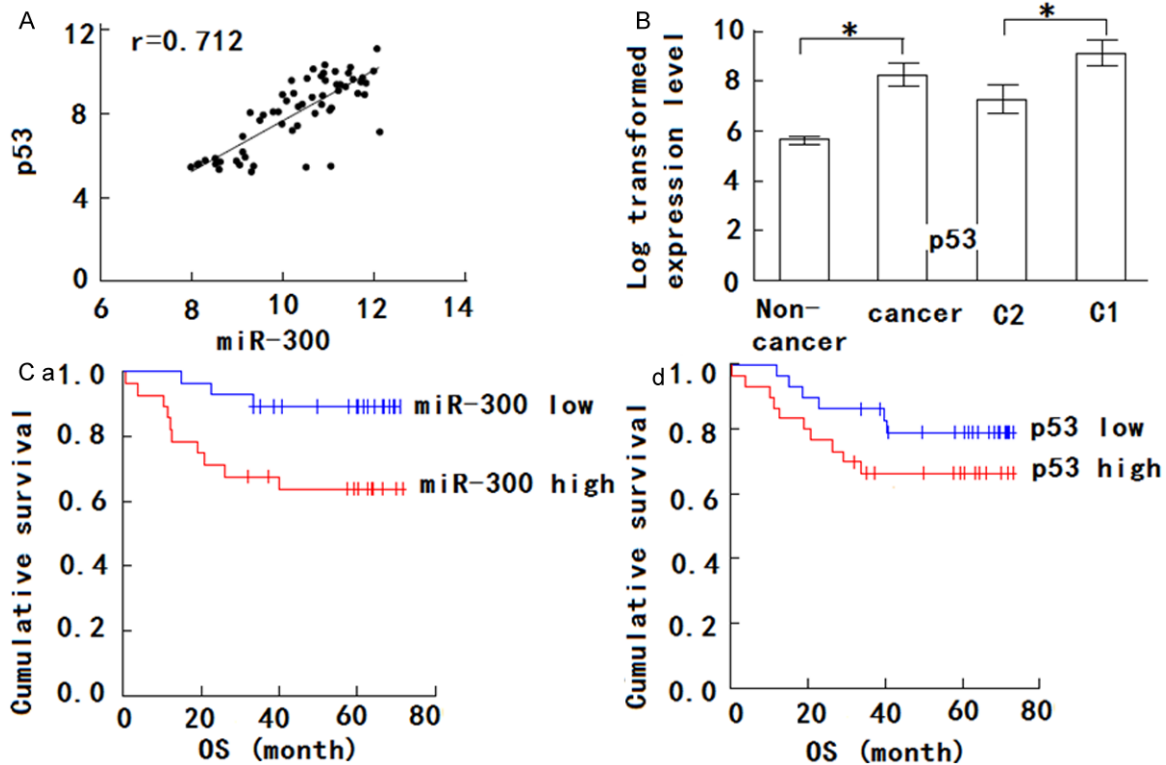
Figure 4. The correlation between miR-300 and p53 expression in breast cancer. A. Expression of miR-300 and p53 were positively correlated (r = 0.712; P < 0.001). B. p53 expression level in breast cancer vs non-cancer tissues and in the C1 vs the C2 group. Results are presented as means with 95% CI; C. Kaplan-Meier plot of overall survival according to the expression level of miR-300 and p53.

Discussion

Recently, miRNAs have been reported to promote [16, 17] or suppress [18, 19] tumor metastasis, providing a new perspective on the metastatic process. Nonetheless, the role of miRNAs in BC metastasis is lacking. In this report, we explored and obtained for the first time metastasis-related miRNAs in BC based on a well-established metastasis cell model. The finding that miR-300 was up-regulated in metastatic BC is intriguing, as decreased miR-300 levels have been reported in several types of solid tumors [20-23], indicating that the loss of miR-300 may be a common event in tumorigenesis. In the present study, we focused on the effect of miR-300 on BC metastasis and demonstrated that miR-300 acts as a tumor promoter in BC metastasis. Restoration of miR-300 induced cell migration and invasion in vitro and tumor metastasis *in vivo*. To obtain stable cell lines that over-expressed miR-300, we transfected MCF-7-M cells with miR-300 plasmids and screened by G418. We selected

twelve cell colonies in the miR-300-transfected group and found 10 out of 12 colonies exhibited remarkably uniform, stable and high-level expression of miR-300. Furthermore, three randomly chosen monoclonal cell lines exhibited similar reduction in invasive ability [24]. However, plasmid transfection strategies often result in lower integration efficiency compared to viral expression leading to the possibility of stochastic selection of rare functionally heterogeneous variants from the initial bulk population [25]. Therefore the future use of viral expression systems should create a more unbiased starting population to test our hypothesis.

Changes in miRNA expression have been found to contribute to the initiation and progression of cancers [26]. The relationship between miRNAs and tumors has suggested that miRNAs may be altered by treatment in patients with BC [27]. In the present study, we focused on the effect of miR-300 on BC metastasis and found that miR-300 was a tumor promoter in BC
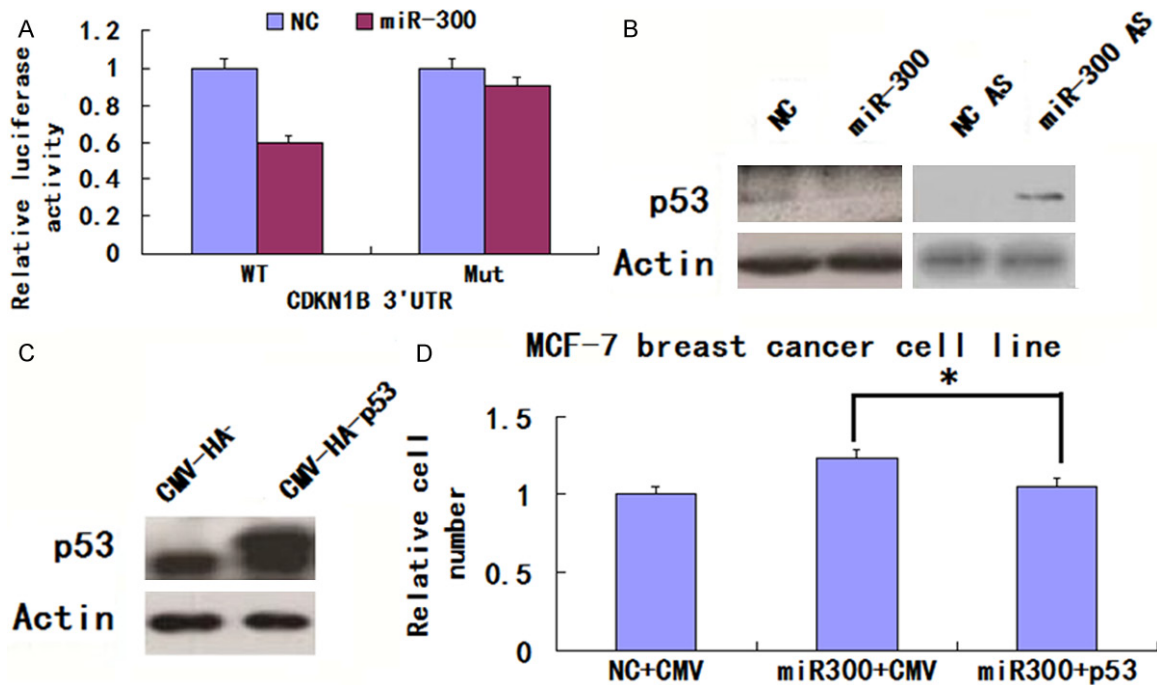
Figure 5. p53 was a direct target of miR-300 in breast cancer cells. A. The possible miR-300 binding site in CDNK1B (p53) mRNA 3'UTR was predicted by bioinformatic analyses. The mutant seed sequence was underlined. p53 had a single predicted binding site in mRNA 3'UTR that was highly conserved in mammals, and in chicken (the complementary sequences of seed sequence of miR-300 were highlighted). B. Luciferase activities were measured in MCF-7 cells co-transfected with the reporter constructs containing 3'UTR of p53 with or without mutant (underlined in A) and miR-300 mimic or negative-control miRNA. C. Western blot assays confirmed that p53 expression were down-regulated by miR-300 mimic in NS cells and up-regulated by miR-300 AS in MCF-7 cells. D. p53 expression was significantly increased in p53-transfected Sw-620 cells (CMV-HA-p53) compared with that in control cells (CMV-HA). The numbers of viable MCF-7 cells were determined by cell count 72 hours after cotransfection with miR-300 mimic and CMV-HA-p53 vector. Values rep-resent mean ± SD. n = 3. **P < 0.01.

metastasis. Some evidence has shown that miR-300 is associated with migration, metastasis, and drug resistance in some tumor types [28-32], indicating that miR-300 up-regulation might contribute to these biological processes in solid tumors. In fact, we also found that miR-300 over-expression with mimic could promote breast cell migration.

As part of our research on how the increase of miR-300 affects BC metastasis, we demonstrated that p53 was a critical downstream target of miR-300. In the present study, miR-300 and p53 were co-activated in breast cancer samples, especially in the poor-prognosis group. miR-300 and p53 are also closely related to the development and progression of hematopoietic stem cells. This suggests that breast cancer co-activated by miR-300 and p53 might share the same pathogenesis as leukemia. Chronic inflammation is associated with breast carcinogenesis and there is recent evi-

dence that *H. pylori* infection recruits bone marrow-derived cells in the breast epithelial mucosa that participate in breast pre-neoplasia [33-35]. Over-expression of miR-300 and P53 may mediate the development of hematopoietic progenitor cells and breast cancer; thus, it is necessary to elucidate the mechanism and function of the co-activation of miR-300 and P53 in breast cancer.

The present study investigated the correlation between miR-300 and p53 expression with the malignant characteristics in patients with breast cancer. The key finding in our study is that miR-300 promoted the proliferation of tumor cells by targeting p53 in breast cancer. Significant correlations were observed between miR-300 and p53. Furthermore, these correlations were found to be independent of other patient characteristics. These findings indicate that high miR-300 expression may be a useful therapeutic target for breast cancer.
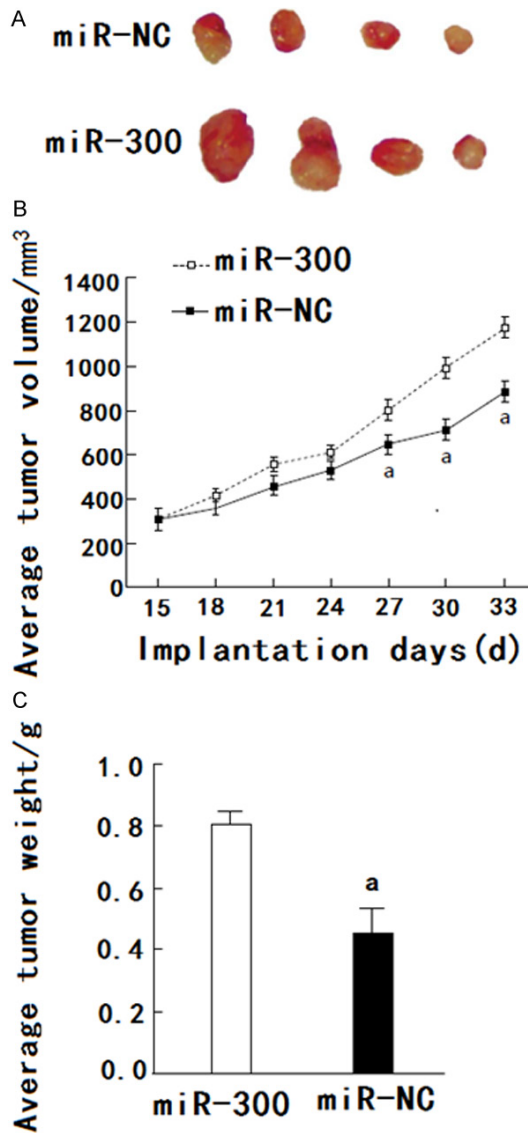
Figure 6. Upregulation of miR-300 promoted breast tumor growth *in vivo*. Cells stably expressing miR-300 or miR-NC were incubated in Dulbecco's modified Eagle's medium and subcutaneously injected into each side of the posterior flank of nude mice (n = 24). Thirty-three days after injection, mice were sacrificed and tumors were removed. A. Tumor volume at 33 d; B. Tumor volumes were detected every three days from the time they were obvious; C. Average tumor weights. [a]Denoted statistical significance between the two groups of miR-300 and control. [a]P < 0.05 vs control.

Disclosure of conflict of interest

None.

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