miR-139-3p suppresses the invasion and migration properties of breast cancer cells by targeting RAB1A

WEI ZHANG, JING XU, KE WANG, XIAOJIANG TANG and JIANJUN HE

Department of Breast Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061, P.R. China

Received December 23, 2018; Accepted June 24, 2019

DOI: 10.3892/or.2019.7297

Abstract. Accumulated evidence indicates that aberrant microRNAs (miRNAs) expression plays an important role in the initiation and progression of various cancers, including breast cancer. Previous studies suggested that miR-139-3p might serve as a tumor suppressor and is downregulated in several cancer types. However, the expression patterns and exact role of miR-139-3p in breast cancer remain to be elucidated. In this study, we aimed to analyze the effect of miR-139-3p on the progression of breast cancer and the mechanism involved. Through bioinformatics analysis and *in vitro* experimental studies, we found that miR-139-3p was decreased in breast cancer tissues and cell lines, and decreased miR-139-3p is associated with a poor prognosis in breast cancer patients. Overexpression of miR-139-3p by transfection significantly inhibits cell proliferation, migration, and invasion of breast cancer cells. Bioinformatics analysis and luciferase reporter gene assay confirmed that RAB1A was a potential target of miR-139-3p. Furthermore, overexpression of RAB1A counteracted the suppressing effects of miR-139-3p on breast cancer cell migration, invasion and epithelial-to-mesenchymal transition (EMT). Taken together, these data suggest that miR-139-3p plays a tumor suppressive role in breast cancer by targeting RAB1A and may serve as a potential new biomarker for breast cancer prognosis.

Introduction

Breast cancer is the most frequently diagnosed cancer and accounts for 30% of all new malignancies diagnosed in females (1). Although advances in screening and treatment have improved survival rates of patients with breast cancer, currently, breast cancer remains the leading cause of cancer-related death among females worldwide (2). Approximately 90% of breast cancer deaths are caused by local invasion and distant metastasis of tumor cells (3). The metastatic cascade represents a complex multistage process which includes local tumor cell invasion, entry into the blood or lymphatic system followed by the exit of cancer cells from the circulation and formation of metastases at the distal sites (4). Dissemination of cancer cells from the primary tumor is an essential step for cancer invasion and metastasis (5). Thus, identifying novel therapeutic targets to prevent these processes is of great importance for the treatment of breast cancer.

MicroRNAs (miRNAs) are a family of endogenous, evolutionarily conserved, non-coding RNAs that can post-transcriptionally affect gene expression by negatively regulating protein-coding mRNAs through base-pair matching within the target mRNA 3' untranslated region (UTR) (6). Abundant evidence has demonstrated that miRNAs play important regulatory roles in diverse physiological and pathological processes through regulating the expressions of target genes (7-9). Accumulated evidence indicates aberrant miRNA expression may profoundly influence cancer-related signaling pathways and play critical roles in almost all aspects of cancer biology such as cell differentiation, proliferation and apoptosis, migration, metabolism and stem cell renewal (10,11). Increasing evidence has suggested that the aberrant expression of miRNAs contributes to the initiation and progression of cancer by regulating the expression of their downstream target genes. In addition, it has been reported that the pattern of miRNA expression can be correlated with cancer type, stage, and other clinical variables, which suggests that miRNAs can be used as a potential novel biomarker for cancer detection and prognosis, as well as a promising target in cancer therapy (12).

Previous studies have shown that dysregulation of miRNA expression was implicated in the progression of breast cancer (13) and varies with lymph node metastasis and other clinicopathological features (14). These miRNAs can be classified as oncogenes (oncomirs) or tumor-suppressor genes according to their potential roles played in the pathogenesis and development of breast cancer (15). Generally, oncomirs are upregulated in cancer cells and exert tumor-promoting activity by targeting tumor-suppressor genes. Examples of breast cancer oncomirs are miR-10b, miR-21, miR-155, miR-373, and miR-520c (16). Tumor-suppressor miRNAs exhibit a lower expression in cancer cells and exert antitumor effect by suppressing oncogene expression. Emerging evidence

Correspondence to: Dr Jianjun He, Department of Breast Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, 277 West Yanta Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: tougaohjj@126.com

Key words: microRNAs, breast cancer, epithelial-to-mesenchymal transition, cell invasion

suggests that miR-139-3p may serve as a tumor suppressor with several types of cancer. It was reported that miR-139-3p level in colorectal cancer patient serum and cancer tissues was significantly lower than in control subjects, which suggests that miR-139-3p may be used as a biomarker for colorectal cancer diagnosis (17). In addition, lower levels of miR-139-3p were significantly associated with poor overall survival in colon cancer patients (18). Furthermore, miR-139-3p was significantly downregulated in bladder cancer tissues, and reduced miR-139-3p expression is associated with enhanced migration and invasion abilities of bladder cancer cells (19). By analyzing breast cancer tissue specimens, we found that miR-139-3p was downregulated in breast cancer tissues. However, the role and underlying mechanism of action of miR-139-3p in the progression of breast cancer remains unknown.

In the present study, we aimed to detect the expression of miR-139-3p in breast cancer, and analyzed the effect of miR-139-3p on the migration and invasion abilities of breast cancer cells, and further explored the underlying mechanism. We found that miR-139-3p was downregulated in breast cancer tissues, and decreased miR-139-3p expression was associated with a poor prognosis in breast cancer patients. *In vitro* experiments indicated that overexpression of miR-139-3p inhibited cell proliferation, migration and invasion of breast cancer. Further study revealed that RAB1A is a potential target of miR-139-3p. These findings suggest that miR-139-3p may serve as a tumor suppressor gene in breast cancer by targeting RAB1A.

Materials and methods

Ethics statement. This study was approved by the Academic Committee of the Ethical Committee of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an, China, and conducted according to the principles expressed in the Declaration of Helsinki. Specimens were obtained with informed consent from all patients.

Clinical tissues and cell culture. From January 2017 to December 2017, 86 pairs of breast cancer tissues and corresponding adjacent non-cancerous tissues were obtained from patients (ranging in age from 20 to 79 years with median age 52.0 years; including 11 intraductal carcinoma, 35 infiltrative ductal carcinoma, 33 infiltrative lobular carcinoma, and 7 other infiltrative carcinoma) who had been diagnosed with primary breast cancer and underwent surgical treatment at the Department of Breast Surgery in the First Affiliated Hospital of Xi'an Jiaotong University. Tissues and information of patients were gained with written informed consent. No chemotherapy or radiotherapy was applied before surgery in any of the included patients. After surgical removal, the tissues were collected and immediately frozen in liquid nitrogen, and stored at -80˚C or embedded in paraffin. Safety margin is defined as 2 cm from the margin of cancerous tissues. The non-cancerous tissues were confirmed by two independent experienced pathologists after H&E staining. The breast cancer cell lines MDA-MB-231, MCF-7, MDA-MB-468, MDA-MB-453, SK-BR-3 and T-47D were obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS, Shanghai, China). All cells were cultured in recommended medium containing 10% fetal bovine serum (FBS; BioWest, Nuaillé, France) and 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA) in a humidified incubator containing 5% CO_2 at 37˚C.

RT-qPCR. Total RNA was isolated from breast cancer cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using a reverse transcription kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. RT-qPCR products were amplified using a miScript SYBR-Green PCR Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Amplicons were detected by an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The following PCR conditions were used: One cycle at 94˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 5 sec and annealing and extension at 60˚C for 30 sec. Gene expression levels were calculated using the $\Delta\Delta$Cq method (20). GAPDH was selected as the reference gene. The PCR primer sequences used were: miR-139-3p, 5'-GGAGACGCGGCCCTGTTGGAGT-3'; RAB1A, 5'-GGAGCCCATGGCATCATA-3' (forward) and 5'-TTGAAGGACTCCTGATCTGTCA-3' (reverse); GAPDH: 5'-CTCTGATTTGGTCGTATTGGG-3' (forward) and 5'-TGGAAGATGGTGATGGGATT-3' (reverse). All primers used in this study were synthesized by DingGuo ChangSheng Biotechnology Co., Ltd. (Beijing, China). Expression of miR-139-3p was analyzed by relative quantity (RQ) using the equation $RQ=2^{-\Delta\Delta Cq}$ (Cq=quantification cycle to detect fluorescence) according to a previous study (21). The mean RQ was 2.32±1.19, and 65 (75.6%) patients were classified as low miR-139-3p (≤2.32) and 21 (24.4%) as high miR-139-3p (>2.32).

MTT assay. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. After the cells had been transfected for 24 h, they were seeded in 96-well microtiter plates (5,000 cells per well) and cultured for the indicated times. Then 20 μl MTT (5 mg/ml diluted in 1X PBS) was added to each well, and incubated for an additional 4 h at 37˚C. After incubation, 150 μl of dimethyl sulfoxide (DMSO) was added to dissolve the crystals. Finally, the absorbance of each well was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Tests were performed in triplicate.

Cell migration and invasion assays. Matrigel uncoated and coated Transwell inserts (8 μm pore size; Millipore, Billerica, MA, USA) were used to examine the migration and invasion abilities of breast cancer cell *in vitro*. Briefly, a total of $5x10^4$ transfected cells were suspended in 150 μl serum-free DMEM and added to the upper chamber of the Transwell chambers, and 600 μl of 20% FBS containing DMEM was loaded in the lower chamber. After 48 h, the non-migratory cells on the upper side were removed using a cotton swab, cells located on the lower surface of the chamber were fixed in 4% paraformaldehyde for 20 min and then stained with 0.1% crystal violet for 15 min. The mean number of migrated or invaded cells was counted by averaging the numbers of cells in 10 fields in both inserts under a microscope (Olympus, Tokyo, Japan). All experiments were performed in duplicate and repeated at least three times.

Western blot analysis. Total proteins were extracted from human breast cancer cells with RIPA lysis buffer and quantified with a BCA Protein Assay Kit (Qiagen, Valencia, CA, USA). Protein samples (30 μg) were run on 10% SDS-PAGE gels, and then were transferred to PVDF membranes (Millipore, Billerica, MA, USA). After being blocked with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with primary antibodies: Against E-cadherin (Cell Signaling Technology, Beverly, MA, USA; rabbit monoclonal Ab no. 3195, 1:1,000 dilution)/N-cadherin (Cell Signaling Technology; rabbit monoclonal Ab no. 13116, 1:1,000 dilution)/MMP-2 (Cell Signaling Technology; rabbit monoclonal Ab no. 40994, 1:800 dilution)/MMP-9 (Cell Signaling Technology; rabbit monoclonal Ab no. 13667, 1:750 dilution) and GAPDH (Abcam, Cambridge, MA, USA; rabbit monoclonal EPR16891, 1:750 dilution) overnight at 4˚C. After washing, the membranes were incubated with HRP-conjugated secondary antibodies (Abcam, Cambridge, MA, USA; goat anti-rabbit, ab7090) at room temperature for 2 h. After washing, the immunoreactive proteins were visualized using an enhancing chemiluminescence kit (Amersham, Little Chalfont, UK). GAPDH was used as a protein loading control.

Colony formation assay. For colony formation, 1,000 transfected cells were seeded into 35-mm petri dish and cultured for 2 weeks. At indicated time points (24, 48 and 72 h), colonies were fixed in 4% paraformaldehyde for 20 min and then stained with 0.1% crystal violet for 15 min. Images were captured under a microscope (Olympus, Tokyo, Japan) at a magnification of x400.

Immunohistochemical (IHC) analysis. The tissue sections were deparaffinized in xylene and rehydrated with ethanol. Endogenous peroxidase activity was blocked via incubating the sections with hydrogen peroxide (0.3% in methanol) at room temperature for 10 min. After blocking with 10% goat serum, the slides were incubated overnight at 4˚C with primary monoclonal antibodies for RAB1A (1:200, Cell Signaling Technology). After several rinses in phosphate-buffered saline, the slides were incubated in the biotinylated secondary antibodies, and antibodies were fixed using the streptavidin-peroxidase (SP) immunohistochemical method (22). The semi-quantitative results were analyzed according to the staining intensity and percentage of positively labeled cells.

Cell transfection. miR-139-3p mimics/inhibitors and corresponding control vectors were obtained from GenePharma (Shanghai, China). The RAB1A overexpression plasmids (pcDNA3.1-RAB1A) were synthesized by Bioworld Biotech Co., Ltd. (Shanghai, China). Breast cancer cells were seeded in 24-well plates, and once cells had grown to 70% confluence, transfection with the above vectors was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were harvested after 48 h for subsequent analysis.

Luciferase reporter assay. The 3'-UTR sequence of RAB1A is predicted to interact with miR-139-3p by binding to the corresponding target sequence or a mutated sequence within the predicted target site. The normal and mutated 3'-UTRs were therefore inserted into the pGL3 reporter luciferase vector (GeneChem). The breast cancer MDA-MB-231 and MCF-7 cells were seeded in 24-well plates. Aliquots of 50 nM of miR-139-3p mimic or control miRNA were co-transfected with 0.1 mg of the pGL3-30UTR wild or mutant RAB1A plasmid DNA using the Lipofectamine 2000 reagent (Invitrogen). After transfection for 48 h, luciferase activity was measured using the dual luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The relative luciferase activity was normalized to *Renilla* luciferase activity. The experiment was performed in triplicate.

Bioinformatics prediction. TargetScan Human 7.0 (http://www.targetscan.org/) was used to predict the potential targets of miR-139-3p.

Table I. Relationship between the expression of miR-139-3p and the clinical pathological characteristics in breast cancer tissues.

		miR-139-3p expression		
Parameters	n	High (n=21)	Low (n=65)	P-value
Age				0.78
≤50	35	8	27	
>50	51	13	38	
Tumor size				0.38[a]
≤2 cm	15	4	11	
>2 cm, <5 cm	67	15	52	
>5 cm	4	2	2	
TNM stage				**0.02**
Early stage (I/II)	39	14	25	
Late stage (III/IV)	47	7	40	
ER status				
Negative	41	11	30	0.62
Positive	45	10	35	
PR status				0.45
Negative	43	9	34	
Positive	43	12	31	
HER status				0.72
Negative	64	15	49	
Positive	22	6	16	
Lymph node metastasis				**0.001**
Negative	35	15	20	
Positive	51	6	45	
Distant metastasis				
No	73	20	53	0.17[a]
Yes	13	1	12	

[a]Fisher's exact test. All other P-values were derived from a Chi-square test. Values in bold fond indicate a statistically significant difference (P<0.05). ER, estrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor receptor.

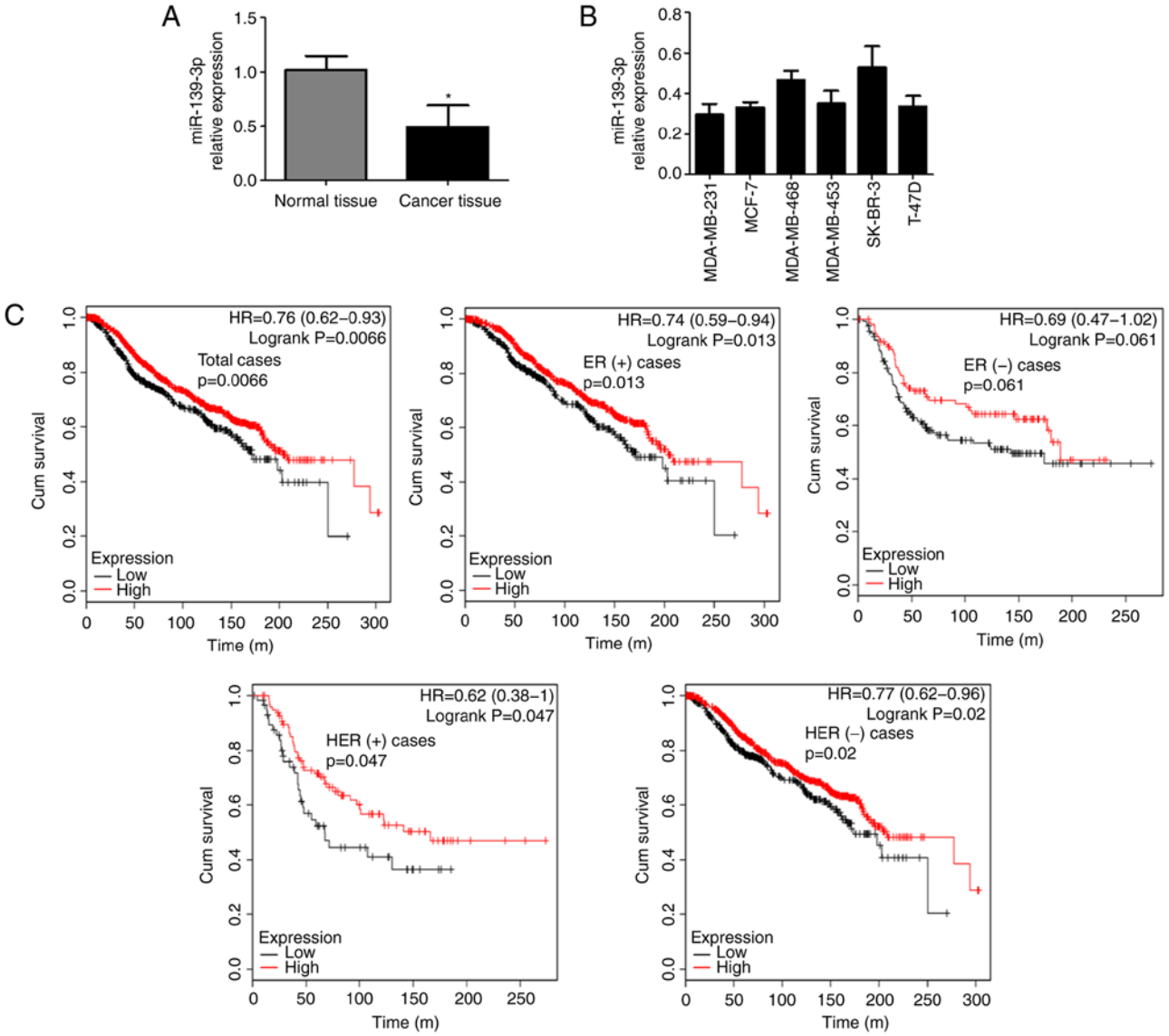

Figure 1. miR-139-3p is downregulated in breast cancer and indicated a poor prognosis of breast cancer. (A) The expression level of miR-139-3p in breast cancer tissues and corresponding adjacent tissues was measured by RT-qPCR, *P<0.05 vs. normal tissues based on the Student's t-test. (B) The expression of miR-139-3p in breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468, MDA-MB-453, SK-BR-3 and T-47D) was measured by RT-qPCR. (C) The prognostic value of miR-139-3p in breast cancer patients was evaluated using an online database Kaplan-Meier Plotter (http://kmplot.com/analysis/).

The Kaplan-Meier plotter. The prognostic value of miR-139-3p expression was evaluated using an online database, Kaplan-Meier Plotter (http://kmplot.com/analysis/), according to a previous study (23). To analyze the overall survival (OS) of patients with breast cancer, patient samples were split into two groups by median expression (high vs. low expression) and assessed by a Kaplan-Meier survival plot. The hazard ratio (HR), 95% confidence intervals (CI), and log-rank P-values were calculated and displayed.

Statistical analysis. All statistical analyses were conducted using SPSS 16.0 software (SPSS; Inc., Chicago, IL, USA) and data were presented as the mean ± standard deviation. Student's t-test was performed to assess difference between two groups. Differences between multiple groups were assessed using one-way analysis of variance (ANOVA) with Dunnett's test for post hoc analysis. The relationship between miR-139-3p expression level and clinicopathological features of the patients was analyzed using the Chi-square test. Each experiment was performed at least for three times. Differences were defined as significant at P<0.05.

Results

miR-139-3p was down-regulated in breast cancer and decreased miR-139-3p expression is associated with a poor prognosis in breast cancer patients. Firstly, we examined the miR-139-3p expression in breast cancer. In total, 86 pairs of breast cancer tissues and adjacent non-cancerous tissues were collected and subjected to RT-qPCR analysis. As shown in Fig. 1A, the expression levels of miR-139-3p in breast cancer tissues were significantly decreased compared with those in non-cancerous breast tissues. By analyzing the relationship between clinicopathological features and miR-139-3p expression, we found that low miR-139-3p expression was significantly associated with TNM stage (P=0.02) and lymph

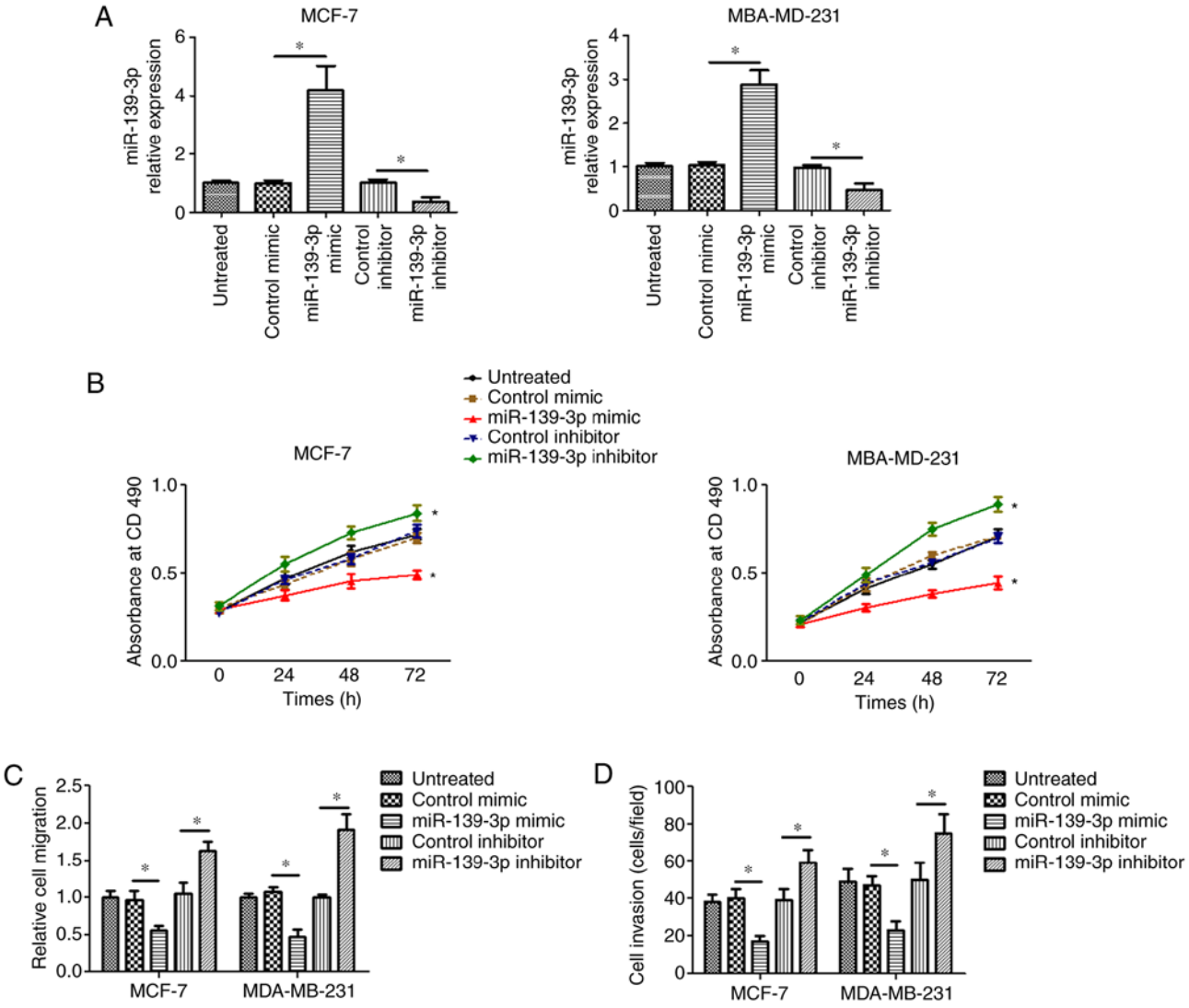

Figure 2. Ectopically expressing miR-139-3p inhibited cell proliferation, migration and invasion of breast cancer cells. (A) The expression level of miR-139-3p was measured by RT-qPCR after breast cancer cells MCF-7 and MDA-MB-231 were transfected with miR-139-3p mimic/inhibitor or corresponding controls. *P<0.05. (B) MTT assays showing the cell growth curves for MCF-7 and MDA-MB-231 after cells were transfected with miR-139-3p mimic/inhibitor for 0, 24, 48 and 72 h. *P<0.05 vs. untreated group. (C and D) The effect of miR-139-3p on the migration and invasion of MCF-7 and MDA-MB-231 cells was measured by Transwell assay after cells were transfected with miR-139-3p mimic/inhibitor, *P<0.05.

node metastasis (P=0.001), but not with patients' age, tumor size, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor (HER) status and distant metastasis (P>0.05, Table I). We then detected miR-139-3p expression in breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468, MDA-MB-453, SK-BR-3 and T-47D). The results showed that miR-139-3p was widely expressed in all tested breast cancer cell lines (Fig. 1B). We further evaluated the relationships of miR-139-3p expression with OS of breast cancer patients using an online database (http://kmplot.com/analysis/). The Kaplan-Meier curve and log-rank test analyses revealed that low miR-139-3p expression was associated with poorer overall survival (OS) in breast cancer patients (Fig. 1C). Although there was no statistical difference in ER (-) population, there was still a trend that cases with low miR-139-3p expression experienced a worse prognosis regardless of ER or HER status. These results suggest that miR-139-3p is decreased in breast cancer and associated with worse prognosis of breast cancer patients, which indicated that miR-139-3p may exert anti-tumor effect in breast cancer.

Overexpression of miR-139-3p inhibited cell proliferation, migration and invasion of breast cancer. Next, we investigated the effect of miR-139-3p on the proliferation, migration and invasion of breast cancer cells. miR-139-3p mimic or inhibitor was transfected into two breast cancer cell lines with different ER status [MCF-7, ER (+); MBA-MD-231, ER (-)] to increase or decrease miR-139-3p expression in these two cell lines separately (Fig. 2A). The MTT method was used to investigate the effect of miR-139-3p on the proliferation of breast cancer cells. As shown in Fig. 2B, overexpression of miR-139-3p with miR-139-3p mimic significantly suppressed the proliferative ability of MCF-7 and MBA-MD-231 cells. In contrast, down-regulation of miR-139-3p with miR-139-3p inhibitor increased the proliferative ability of MCF-7 and MBA-MD-231 cells. In cell migration experiments (Fig. 2C), overexpression of miR-139-3p with miR-139-3p mimic significantly decreased

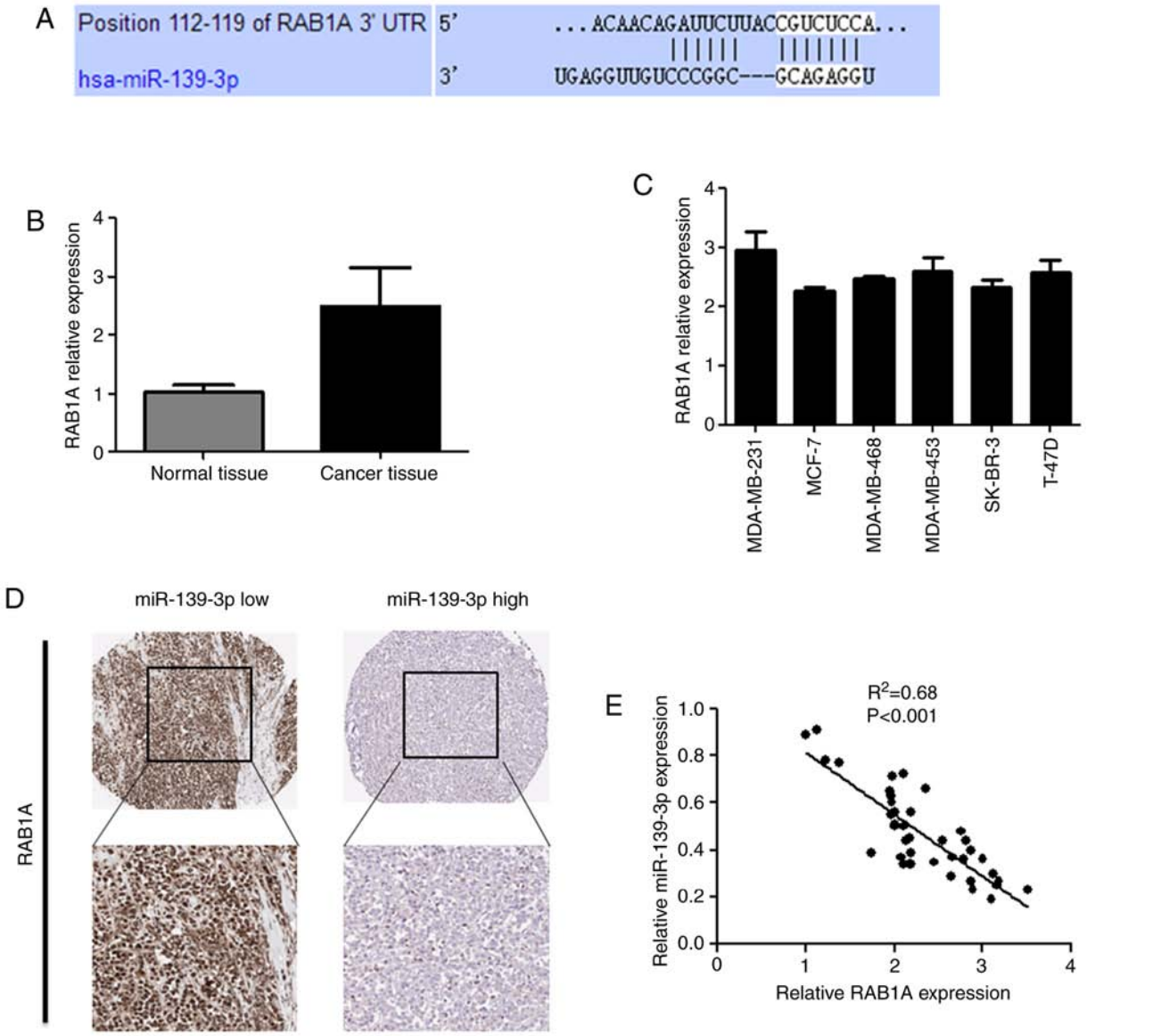

Figure 3. RAB1A is a potential target of miR-139-3p and inversely correlated with miR-139-3p expression in breast cancer tissues. (A) The putative miR-139-3p binding sequence in the 3'UTR of RAB1A was predicted using TargetScan Human 7.0. (B) The expression level of RAB1A in breast cancer tissues and corresponding adjacent normal tissues was measured by RT-qPCR. *P<0.05 vs. normal tissues based on the Student's t-test. (C) The expression of RAB1A in breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468, MDA-MB-453, SK-BR-3 and T-47D) was measured by RT-qPCR. (D) Representative results showing IHC staining for RAB1A in miR-139-3p low and high breast cancer tissues. (E) The expression of RAB1A was inversely correlated with the miR-139-3p level in breast cancer tissues.

the migratory ability in MCF-7 and MBA-MD-231 cells. While the migratory ability of MCF-7 and MBA-MD-231 cells were significantly increased after silencing miR-139-3p. The Transwell assay (Fig. 2D) showed that the invasive ability of breast cancer cells was decreased when cells were transfected with miR-139-3p mimic. By contrast, silencing miR-139-3p expression by miR-139-3p inhibitor enhanced the invasive ability of breast cancer cells. These results indicate that miR-139-3p may play a tumor suppression role in breast cancer progression.

RAB1A is a potential target of miR-139-3p and is inversely correlated with miR-139-3p expression in breast cancer tissues. We performed a target search using TargetScan (TargetScan Human 7.0 software) and predicted that RAB1A is a direct target of miR-139-3p (Fig. 3A). We next detected the expression of RAB1A in breast cancer tissues by RT-qPCR. The result showed that the expression level of RAB1A was significantly increased in breast cancer tissues compared with the normal breast tissues (Fig. 3B). Similarly, higher expression levels of RAB1A were detected in breast cancer cell lines (Fig. 3C). Moreover, we could see a negative correlation between RAB1A expression and miR-139-3p expression from immunohistochemical staining for RAB1A (Fig. 3D). This was further confirmed by a linear correlation analysis, which showed that RAB1A expression was inversely proportional to miR-139-3p expression (Fig. 3E).

To verify that RAB1A is a potential target of miR-139-3p, we analyzed the relationship between miR-139-3p and RAB1A. Both real-time PCR (Fig. 4A) and western blot analysis (Fig. 4B) showed that overexpression of miR-139-3p with miR-139-3p mimic led to a corresponding decrease of RAB1A

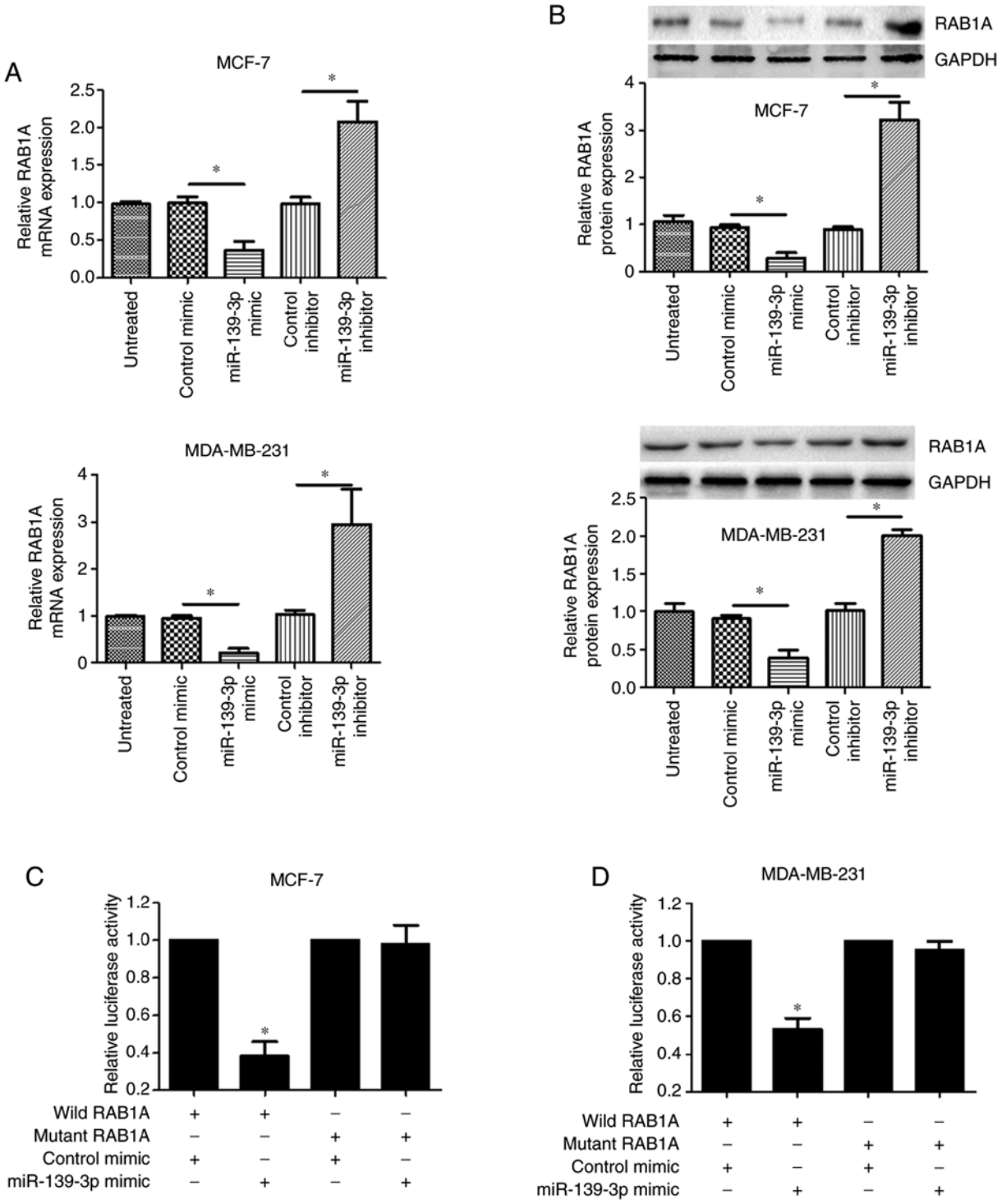

Figure 4. RAB1A is a direct target of miR-139-3p. (A and B) The expression of RAB1A was measured by RT-qPCR and western blot analysis after MCF-7 and MDA-MB-231 cells were transfected with miR-139-3p mimic/inhibitor, *P<0.05 vs. untreated group. (C and D) Relative luciferase activity of RAB1A in MCF-7 and MBA-MD-231 breast cancer cells that were co-transfected with miR-139-3p mimic as well as with wild or mutant of the RAB1A 3'-UTR reporter genes was assayed at 48 h post-transfection. The luciferase activity of each sample was normalized to *Renilla* luciferase activity. *P<0.05 vs. Control mimics + wild RAB1A group.

expression in MCF-7 and MDA-MB-231 cells. Conversely, RAB1A expression was upregulated in MCF-7 cells with reduced levels of miR-139-3p by miR-139-3p inhibitor. To further detect whether RAB1A was indeed directly regulated by miR-139-3p in breast cancer cells, a dual luciferase assay was conducted. The result showed that the luciferase activity in the wild RAB1A-transfected cells, was significantly decreased compared to the luciferase activity in the mutant cells (Fig. 4C and D). Collectively, these data suggest that RAB1A is a direct target gene of miR-139- 3p in breast cancer.

miR-139-3p suppresses cell migration, invasion and EMT by targeting RAB1A in breast cancer. To further investigate whether miR-139-3p suppresses cell migration and invasion by

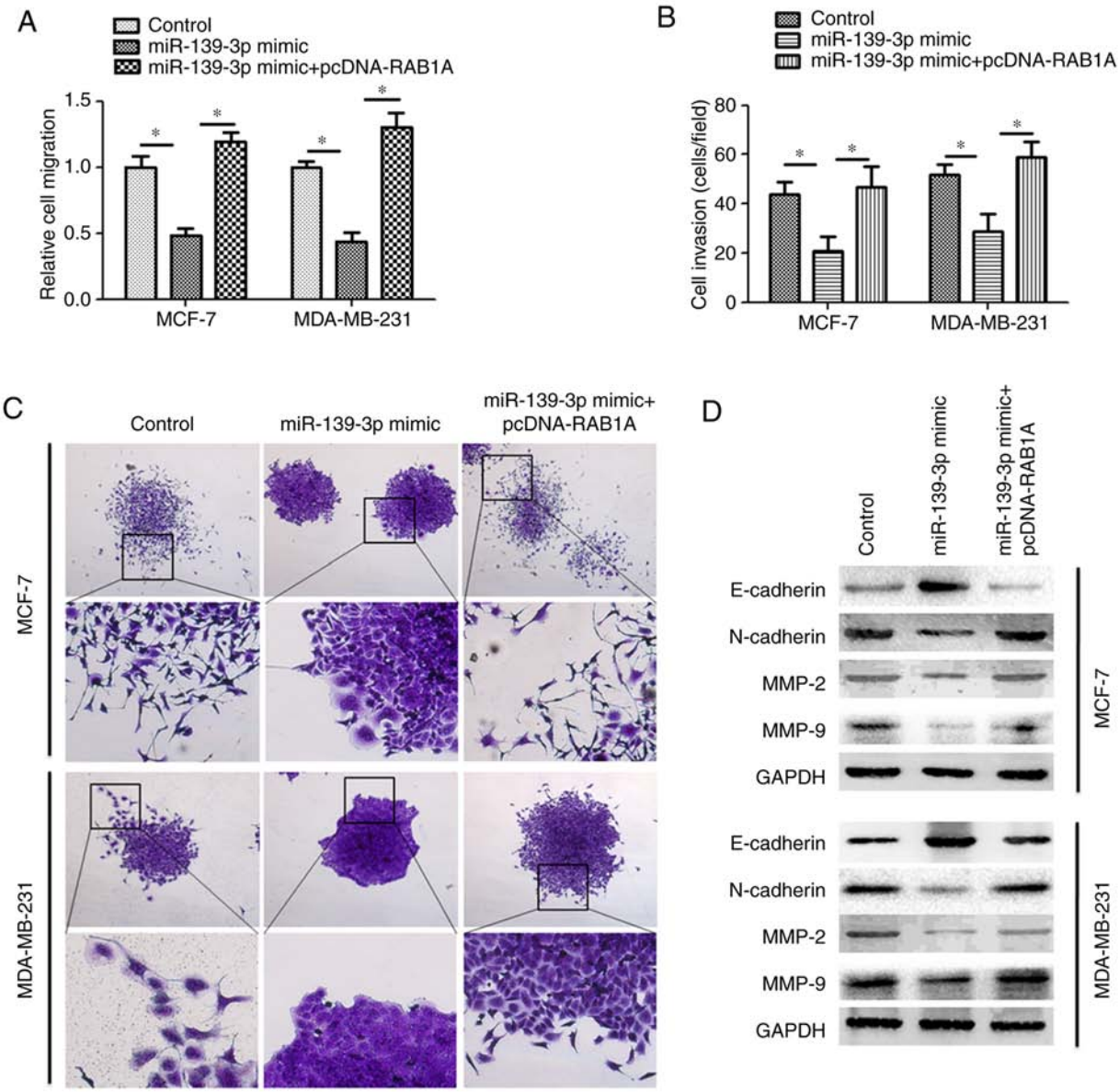

Figure 5. Ectopically expressing RAB1A restores miR-139-3p-reduced cell migration and invasion in MCF-7 cells. (A) The expression of RAB1A was measured by RT-qPCR after MCF-7 cells were co-transfected with miR-139-3p mimic and pcDNA-RAB1A for 48 h. *P<0.05. (B) The invasion of MCF-7 cells was measured by Transwell assay after cells were co-transfected with miR-139-3p mimic and pcDNA-RAB1A, *P<0.05. (C) Representative images showing morphology of MCF-7 cells after co-transfection with miR-139-3p mimic and pcDNA-RAB1A using colony formation assay. Images were taken under a microscope at a magnification of x400. (D) The expression level of MMP-2/MMP-9/E-cadherin/N-cadherin in MCF-7 and MBA-MD-231 cells was detected by western blot analysis after cells were co-transfected with miR-139-3p mimic and pcDNA- RAB1A for 48 h.

targeting RAB1A, we overexpressed miR-139-3p and RAB1A in MCF-7 cells by co-transfecting pcDNA-RAB1A and miR-139-3p mimic into MCF-7 cells. Consistently, the results (Fig. 5A and B) showed that MCF-7 cells only transfected with miR-139-3p mimic showed reduced cell migration and invasion abilities. However, when MCF-7 cells were co-transfected with miR-139-3p mimic and pcDNA-RAB1A, the migration and invasion properties were significantly increased as compared with the cells transfected with miR-139-3p mimic only. When conducting clone formation experiments, it was found that cancer cells tended to form tightly connected clones when ectopically overexpressing miR-139-3p in MCF-7 and MDA-MB-231 cells (Fig. 5C). By contrast, cancer cells co-transfected with miR-139-3p mimic and pcDNA-RAB1A exhibited dispersed and highly scattered growth patterns and adopted a spindle-shaped morphology during clone formation experiments. This is similar to the processes occurring during cancer invasion and metastasis termed as epithelial-to-mesenchymal transition (EMT) (24,25). Western blotting was performed to detect the expression of MMP-2, MMP-9, E-cadherin and N-cadherin. As expected, both MCF-7 and MDA-MB-231 cells showed reduced protein expression of MMP-2 and MMP-9 when transfected with miR-139-3p mimic. However, this effect was counteracted by co-transfection of MCF-7 and MDA-MB-231 cells with miR-139-3p mimic and pcDNA-RAB1A. More importantly, both MCF-7 and MDA-MB-231 cells showed increased E-cadherin and reduced N-cadherin when transfected with miR-139-3p mimic, while this cadherin switching was prevented by overexpressing RAB1A ectopically (Fig. 5D). These data indicate

that RAB1A plays an important role in miR-139-3p-induced cell migration, invasion and EMT in breast cancer cells.

Discussion

In this study, we investigated the expression, function, and mechanisms underlying the activities of miR-139-3p in breast cancer. Through bioinformatics analysis and *in vitro* experimental studies, we found that miR-139-3p was downregulated in breast cancer tissues and cell lines, and decreased miR-139-3p is associated with a poor prognosis in breast cancer patients. Overexpression of miR-139-3p significantly inhibits cell migration, invasion, and EMT by suppressing RAB1A expression. These data suggested that miR-139-3p might play a tumor suppressive role during breast cancer progression.

miR-139-3p has been found to be significantly downregulated in HPV-positive HNC (head and neck cancer) and cervical cancer cases. Further study revealed that HPV-16 mediated downregulation of miR-139-3p by promoter methylation created a favorable environment for viral replication and oncogenesis via activating pro-oncogenic pathways. In contrast, miR-139-3p overexpression decreased cell proliferation, migration, and increased the sensitivity of HPV-16-positive cells to chemotherapy (26). Moreover, reduced miR-139-3p expression in cervical cancer tissues and cell lines has been confirmed by another study (27), which revealed that overexpression of miR-139-3p suppressed cell proliferation, migration, invasion and induced apoptosis via downregulation of NOB1 expression. In the present study, we found that miR-139-3p was downregulated in breast cancer tissues and cell lines, and decreased miR-139-3p level is associated with a poor prognosis in breast cancer patients. *In vitro* experiments indicated that miR-139-3p suppresses cell migration, invasion and EMT by targeting RAB1A in breast cancer. Taken together, the above evidence supports the tumor suppressive role of miR-139-3p in various cancer types.

RAB1A is a small GTPase which belongs to the Ras oncogene family (28). In previous studies, aberrant expression of RAB1A in human cancer has been observed (29-32). By using microarray analysis, it was found that RAB1A was overexpressed in about 98% of tongue squamous cell carcinomas and 93% of premalignant lesions, which indicated that RAB1A is a potential biomarker for tongue carcinogenesis (33). In addition, it was reported that RAB1A is overexpressed in colorectal cancer, breast and liver tumors (28). More importantly, overexpression of RAB1A is correlated with tumor invasion, progression, poor prognosis, and rapamycin sensitivity in colorectal cancer. Further *in vitro* experiment indicated RAB1A overexpression is sufficient to transform immortalized fibroblasts and promote malignant growth of established tumor cells via activation of mTORC1 signaling, whereas, RAB1A knockdown selectively attenuates oncogenic growth of RAB1A-overexpressing cancer cells. In addition, another study indicated that RAB1A also acts as an oncogene in breast cancer, and downregulation of RAB1A inhibited the growth, migration, invasion and EMT of breast cancer cells (32). These results suggest that overexpression of RAB1A is a general phenomenon in human malignancies and can promote oncogenic transformation and malignant growth. However, to the best of our knowledge, how RAB1A can be regulated in cancer has never been investigated. In the present study, we found that RAB1A is a direct target gene of miR-139-3p, which was downregulated in breast cancer. Furthermore, overexpression of RAB1A promotes the migration, invasion, and EMT of breast cancer. These results confirm the oncogenic role of RAB1A in cancer and suggest that RAB1A could be a critical therapeutic target for breast cancer.

In summary, we provide the first data demonstrating that miR-139-3p plays a tumor suppressive role in breast cancer by downregulation of RAB1A. Our findings suggest that miR-139-3p may be a novel prognostic indicator of and potential therapeutic target for breast cancer.

Acknowledgements

Not applicable.

Funding

This study was supported by the National Key Research and Development Program of Shaanxi (2018SF_156) and the Science Foundation of the First Affiliated Hospital of Xi'an Jiaotong University (2017QN-16).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WZ and JH conceived and designed the experiments. WZ, JX and KW performed the experiments and provided assistance for data acquisition, data analysis and statistical analysis. WZ and XT contributed reagents/materials/analysis tools and analyzed the data. WZ wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Academic Committee of the Ethical Committee of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an, China, and conducted according to the principles expressed in the Declaration of Helsinki. Specimens were obtained with informed consent from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2018. CA Cancer J Clin 68: 7-30, 2018.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.

3. Wang Y and Zhou BP: Epithelial-mesenchymal transition in breast cancer progression and metastasis. Chin J Cancer 30: 603-611, 2011.
4. van Zijl F, Krupitza G and Mikulits W: Initial steps of metastasis: Cell invasion and endothelial transmigration. Mutat Res 728: 23-34, 2011.
5. Chambers AF, Groom AC and MacDonald IC: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2: 563-572, 2002.
6. Abak A, Amini S, Sakhinia E and Abhari A: MicroRNA-221: Biogenesis, function and signatures in human cancers. Eur Rev Med Pharmacol Sci 22: 3094-3117, 2018.
7. Ardekani AM and Naeini MM: The Role of MicroRNAs in human diseases. Avicenna J Med Biotechnol 2: 161-179, 2010.
8. Ullah S, John P and Bhatti A: MicroRNAs with a role in gene regulation and in human diseases. Mol Biol Rep 41: 225-232, 2014.
9. Tüfekci KU, Oner MG, Meuwissen RL and Genc S: The role of microRNAs in human diseases. Methods Mol Biol 1107: 33-50, 2014.
10. Waldman SA and Terzic A: Applications of microRNA in cancer: Exploring the advantages of miRNA. Clin Transl Sci 2: 248-249, 2009.
11. Reddy KB: MicroRNA (miRNA) in cancer. Cancer Cell Int 15: 38, 2015.
12. Lee YS and Dutta A: MicroRNAs in cancer. Annu Rev Pathol 4: 199-227, 2009.
13. Chen L, Li Y, Fu Y, Peng J, Mo MH, Stamatakos M, Teal CB, Brem RF, Stojadinovic A, Grinkemeyer M, *et al*: Role of deregulated microRNAs in breast cancer progression using FFPE tissue. PLoS One 8: e54213, 2013.
14. Wang B, Li J, Sun M, Sun L and Zhang X: miRNA expression in breast cancer varies with lymph node metastasis and other clinicopathologic features. IUBMB Life 66: 371-377, 2014.
15. Tang J, Ahmad A and Sarkar FH: The role of microRNAs in breast cancer migration, invasion and metastasis. Int J Mol Sci 13: 13414-13437, 2012.
16. van Schooneveld E, Wildiers H, Vergote I, Vermeulen PB, Dirix LY and Van Laere SJ: Dysregulation of microRNAs in breast cancer and their potential role as prognostic and predictive biomarkers in patient management. Breast Cancer Res 17: 21, 2015.
17. Ng L, Wan TM, Man JH, Chow AK, Iyer D, Chen G, Yau TC, Lo OS, Foo DC, Poon JT, *et al*: Identification of serum miR-139-3p as a non-invasive biomarker for colorectal cancer. Oncotarget 8: 27393-27400, 2017.
18. Liu X, Duan B, Dong Y, He C, Zhou H, Sheng H, Gao H and Zhang X: MicroRNA-139-3p indicates a poor prognosis of colon cancer. Int J Clin Exp Pathol 7: 8046-8052, 2014.
19. Yonemori M, Seki N, Yoshino H, Matsushita R, Miyamoto K, Nakagawa M and Enokida H: Dual tumor-suppressors miR-139-5p and miR-139-3p targeting matrix metalloprotease 11 in bladder cancer. Cancer Sci 107: 1233-1242, 2016.
20. Schmittgen TD and Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101-1108, 2008.
21. Lee JA, Lee HY, Lee ES, Kim I and Bae JW: Prognostic implications of MicroRNA-21 overexpression in invasive ductal carcinomas of the breast. J Breast Cancer 14: 269-275, 2011.
22. Zhai J, Yang X, Zhang Y, Qi Q, Hu J and Wang Q: Reduced expression levels of the death-associated protein kinase and E-cadherin are correlated with the development of esophageal squamous cell carcinoma. Exp Ther Med 5: 972-976, 2013.
23. Lánczky A, Nagy Á, Bottai G, Munkácsy G, Szabó A, Santarpia L and Győrffy B: miRpower: A web-tool to validate survival-associated miRNAs utilizing expression data from 2178 breast cancer patients. Breast Cancer Res Treat 160: 439-446, 2016.
24. Dongre A, Rashidian M, Reinhardt F, Bagnato A, Keckesova Z, Ploegh HL and Weinberg RA: Epithelial-to-mesenchymal transition contributes to immunosuppression in breast carcinomas. Cancer Res 77: 3982-3989, 2017.
25. Bill R and Christofori G: The relevance of EMT in breast cancer metastasis: Correlation or causality. FEBS Lett 589: 1577-1587, 2015.
26. Sannigrahi MK, Sharma R, Singh V, Panda NK, Rattan V and Khullar M: Role of host miRNA Hsa-miR-139-3p in HPV-16-induced carcinomas. Clin Cancer Res 23: 3884-3895, 2017.
27. Huang P, Xi J and Liu S: miR-139-3p induces cell apoptosis and inhibits metastasis of cervical cancer by targeting NOB1. Biomed Pharmacother 83: 850-856, 2016.
28. Jin M, Saucan L, Farquhar MG and Palade GE: Rab1a and multiple other Rab proteins are associated with the transcytotic pathway in rat liver. J Biol Chem 271: 30105-30113, 1996.
29. Tanaka M, Mun S, Harada A, Ohkawa Y, Inagaki A, Sano S, Takahashi K, Izumi Y, Osada-Oka M, Wanibuchi H, *et al*: Hsc70 contributes to cancer cell survival by preventing Rab1A degradation under stress conditions. PLoS One 9: e96785, 2014.
30. Thomas JD, Zhang YJ, Wei YH, Cho JH, Morris LE, Wang HY and Zheng XFS: Rab1A is an mTORC1 activator and a colorectal oncogene. Cancer Cell 30: 181-182, 2016.
31. Wang X, Liu F, Qin X, Huang T, Huang B, Zhang Y and Jiang B: Expression of Rab1A is upregulated in human lung cancer and associated with tumor size and T stage. Aging (Albany NY) 8: 2790-2798, 2016.
32. Xu H, Qian M, Zhao B, Wu C, Maskey N, Song H, Li D, Song J, Hua K and Fang L: Inhibition of RAB1A suppresses epithelial-mesenchymal transition and proliferation of triple-negative breast cancer cells. Oncol Rep 37: 1619-1626, 2017.
33. Shimada K, Uzawa K, Kato M, Endo Y, Shiiba M, Bukawa H, Yokoe H, Seki N and Tanzawa H: Aberrant expression of RAB1A in human tongue cancer. Br J Cancer 92: 1915-1921, 2005.