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Original research paper

MicroRNA-140-5p inhibits cellular proliferation, migration and invasion by downregulating AKT/STAT3/NF-κB pathway in breast carcinoma cells

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ABSTRACT

MicroRNA-140-5p (miR-140-5p) plays a pivotal role in human cancers. However, its role and molecular mechanisms in breast carcinoma are not fully explored. Using miR-140-5p transfected breast cancer cell line MDA-MB-231, several *in vitro* experiments were performed and described in this paper. They consist of the cell proliferation assay, wound healing assay, transwell assay, colony formation assays and qRTPCR. Expression levels of target proteins were determined using Western blotting. In addition, experiments on animal models were performed to study the possible role of miR-140-5p in tumorigenesis of breast carcinoma cells. The induction of experimental breast tumor in mice model was achieved through the incorporation of MDA-MB-231 tumor cells subcutaneously into the middle left side of the mice. The results showed that miR-140-5p up-regulation significantly suppresses proliferation, cellular invasion and migration of breast carcinoma cells. Furthermore, miR-140-5p up-regulation stops breast cancer cells at G0/G1 phase. The results of the animal model indicated that up-regulation of miR-140-5p suppresses its tumorigenic ability. Moreover, we also found that miR-140-5p up-regulation reduces the phosphorylation level of STAT3, p65, and AKT. In addition, miR-140-5p overexpression significantly decreases CDK2 expression while increasing E-cadherin expression level. These data revealed that miR-140-5p suppressed tumor progression of breast carcinoma cells through inhibition of the AKT/STAT3/NF-κB pathway. Taken the present study results together, we can conclude that miR-140-5p may act as a novel target in microRNA-targeting anticancer strategy for the treatment of breast cancer.

Keywords: breast cancer, microRNA-140-5p, cell proliferation, tumor progression, AKT/STAT3/NF-κB

In the present times, the incidence of non-communicable diseases is alarmingly increasing each day (1, 2). Cancer is considered the major cause of death worldwide among non-communicable diseases. Around 19.3 million new cancer cases were reported in 2020

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alone, and the numbers are expected to increase in the future (3). Breast cancer accounts for about 25 % of all types of human cancers (3). Due to increased screening awareness programs among women worldwide, diagnosed cases of breast cancer are rising (4). Breast cancer initiation and progression is a multistep and complex process involving many genes and signaling molecules. Recently microRNAs have emerged as new molecules responsible for the initiation and progression of breast cancer.

MicroRNAs (miRNAs) are small RNA molecules that block translation or induce mRNA degradation (5). miRNAs regulate a broad array of cellular processes, including cell growth, differentiation, and death, by regulating multiple genes and their downstream networks (6). Of note, miR-140-5p is frequently dysregulated in different human tumors (7–9). miR-140-5p was shown to inhibit bone morphogenic protein-2 (BMP2) associated osteogenesis (10), and suppress acute lung injury-induced inflammation (11). Expression levels of miR-140-5p decrease in primary colorectal carcinomas and liver metastatic tissues (7). In addition, down-regulation of miR-140-5p has been observed in rat cerebral tissues following middle cerebral artery occlusion (12). Also, miR-140-5p showed inhibitory effects on hepatocellular carcinoma (13), and colorectal cancer (14). Yang *et al.* (15) reported that down-regulation of miR-140-5p overexpression increases proliferation and invasiveness in glioma cells. Song *et al.* (16) confirmed that miR-140-5p mimics significantly lead to G1 phase arrest and induce cell apoptosis in osteosarcoma and colon cancer cells. miR-140-5p has been reported to function as a tumor-suppressive agent in breast cancer by inhibiting cell invasion and migration (9, 17, 18).

Cyclin-dependent kinase (CDK) 2 is a crucial regulatory molecule essential for G1-S transition (19). The processes of tumor progression, in which tumor cells obtain more efficient invasive and migratory abilities, were called epithelial-to-mesenchymal transition (EMT). E-cadherin is one of the most common markers of EMT, which mainly mediates the loss of cell-cell adhesion and promotes cell invasion and migration (20). As the core of the PI3K/Akt pathway, Akt is essential for cell survival and growth, and Akt down-regulation could suppress tumor cell proliferation (21). STAT3 promotes tumor cell proliferation, survival and tumor invasion (22). Activation of NF-κB is associated with increased cell cycle progression, invasion and migration (23). The present study explores the role of miR-140-5p in tumor progression of breast carcinoma cells through AKT/STAT3/NF-kB pathway using *in vitro* and *in vivo* assays. It suggests miRNA-140-5p as a potentially novel target in anticancer therapeutic strategy.

EXPERIMENTAL

Reagents

Primary antibodies like anti-CDK2, anti-E-cadherin, anti-p-AKT, anti-p-STAT3, anti-p-p65 and anti-GAPDH were obtained from Cell Signaling Technology (USA). All other chemicals used were acquired from Sigma-Aldrich (USA) unless mentioned differently.

Culture and transfection

MDA-MB-231 cells were obtained from ATCC and grown in an incubator set at 5 % CO_2 and 37 °C using MEM/EBSS medium. miR-140-5p mimic and control mimic were

purchased from ThermoFisher Scientific. MDA-MB-231 cells were added into six-well plates (2×10^5 per well) and either transfected with miR-140-5p mimics (50 nmol L^{-1}) or control miRNA mimic (miR-NC) (50 nmol L^{-1}) using Lipofectamine 2000 or left un-transfected (control). The efficacy of transfection was checked using qRTPCR.

Cell proliferation

The proliferation rate of cells was evaluated through the MTT assay. MDA-MB-231 cells were grown in 96-well plates at a concentration of 6×10^3 cells/well. Cells were cultured for 24 h followed by transfection. 48 h post-transfection, media was aspirated from both transfected (miR-140-5p or miR-NC) and control cells, and each well was filled with 10 μL MTT stock solution (5 mg mL^{-1}). Then the cells were left untouched for about 3 h followed by the dissolving of formazan crystals with DMSO. Finally, absorbance was measured at 600 nm.

Colony formation experiment

MDA-MB-231 cells (500 cells per well) were added into six-well plates and transfected (miR-140-5p or miR-NC) or left un-transfected (control). Two weeks later, the colonies were fixed using 4 % paraformaldehyde and then stained for 30 minutes with crystal violet (0.1 %). Subsequently, single colonies were counted within three random fields.

Cell cycle analysis

Control, miR-NC and miR-140-5p transfected cells after 48 h incubation were washed with PBS, fixed in 75 % ethanol (cold), and left overnight at 4 °C. The cells were then washed with PBS and incubated for 30 minutes with 100 μL propidium iodide solutions in darkness. The proportion of cells at different cell cycle stages was detected using a Flow Cytometer (BD Biosciences).

Cell adhesion assay

The MDA-MB-231 cells (1×10^4) were added into Matrigel pre-coated 96-well plates (2 μg per well). After incubation for 24 h miR-140-5p and miR-NC transfected cells and controls were washed, and the cells were fixed with 4 % formaldehyde and stained with crystal violet (0.1 %). Finally, cell adhesion rates were determined based on the absorption at 595 nm using an enzyme-labelling reader (Bio-Tek Instruments, USA).

Wound healing experiment

For wound healing assay purposes, the control, miR-NC and miR-140-5p transfected cells were cultured in a 12-well plate overnight. Using a 10 μL tip, a scratch was created on the cell monolayer. 24 h after forming scratch, cells were washed once with culture media to remove floating cells. Images of fresh scratches were captured immediately with the help of a digital camera. The scratch area was determined using Image-Pro software. Cell migration was determined by calculating the scratch closure.

Transwell assay

The control, miR-NC and miR-140-5p transfected cells were adjusted to a density of 5×10^4 cells/mL using MEM without serum and then added into the upper chamber (Merck Millipore, USA) pre-coated with 100 μL/well Matrigel. In the lower chamber, 600 μL of MEM was placed. After 24 h, the cells that remained in the upper chamber were rinsed and migrated cells in the lower chamber were fixed and stained and then photographed under a microscope.

qRTPCR

RNA from MDA-MB-231 cells (mR-140-5p- and mR-NC-transfected, and the control) was obtained using the TRIzol method and then reversed to cDNA with RT Kit. Quantitative PCR was carried out using SYBRGreen (Takara) with appropriate primers designed by Primer 5.0 according to the conditions, including an initial step of 10 min at 95 °C, and then 40 cycles of amplification, which includes 10 s at 95 °C, 20 s in 55 °C and 25 s in 72 °C. Quantification was determined by $2^{-\Delta\Delta CT}$ (24). The internal control used was GAPDH.

Tumor xenograft model

Female 4–5-week-BALB/c nude mice were commercially obtained and were put under pathogen-free conditions. Then, 5×10^6 MDA-MB-231 cells transfected with either miR-140-5p or miR-NC or un-transfected were injected into the middle left side of mice subcutaneously. On the 28th day, the volume of tumors was calculated following this formula: $0.5 \times \text{length} \times \text{width}^2$ (mm^3). After sacrificing mice, tumors were obtained and were stored at –80 °C for further experiments.

Western blot

The miR-140-5p and miR-NC transfected and control cells or homogenized mass of breast cancer cells were treated with RIPA lysis buffer for the isolation of total cellular proteins and the protein concentration was determined with the Bradford method. From each sample, 45 μg of proteins were loaded and run on SDS-PAGE gels which were processed for blotting to PVDF membranes followed by exposure to primary and secondary antibodies and finally the protein bands of interest were visualized with the help of an efficient chemiluminescence reagent. Primary antibodies were directed against CDK2, E-cadherin, p-AKT, p-STAT3, p-p65 and beta-actin (all from Abcam, Cambridge, UK). The human β-actin gene was used as an internal control.

Statistical analysis

GraphPad Prism 6 was used to perform data analyses. Each experiment was performed thrice and in triplicates. For each experimental test condition, the statistical differences in comparison to controls were compared using a student's *t*-test or one-way analysis of variance (ANOVA). The experiment results were shown as mean ± SD ($n = 3$). The statistically significant standard was $p < 0.05$.

RESULTS AND DISCUSSION

miR-140-5p up-regulation decreases cell proliferation, arrests cell cycle and increases cell adhesion

The aberrant expression of miR-140-5p is a common event in different cancers (7–9, 17, 18) and therefore suggests its essential role in tumor development. We, therefore, set an experiment to look at the possible role of miR-140-5p in breast cancer.

The cellular proliferation was significantly decreased by miR-140-5p up-regulation compared to control (Fig. 1a). The colony formation ability was decreased after the up-regulation of miR-140-5p (Fig. 1b and c). As shown in Fig. 1d, miR-140-5p up-regulation stops breast cancer cells at G0/G1 phase. Cell adhesion rate was found significantly increased in cells in which miR-140-5p was over-expressed (Fig. 1e). Therefore, miR-140-5p up-regulation

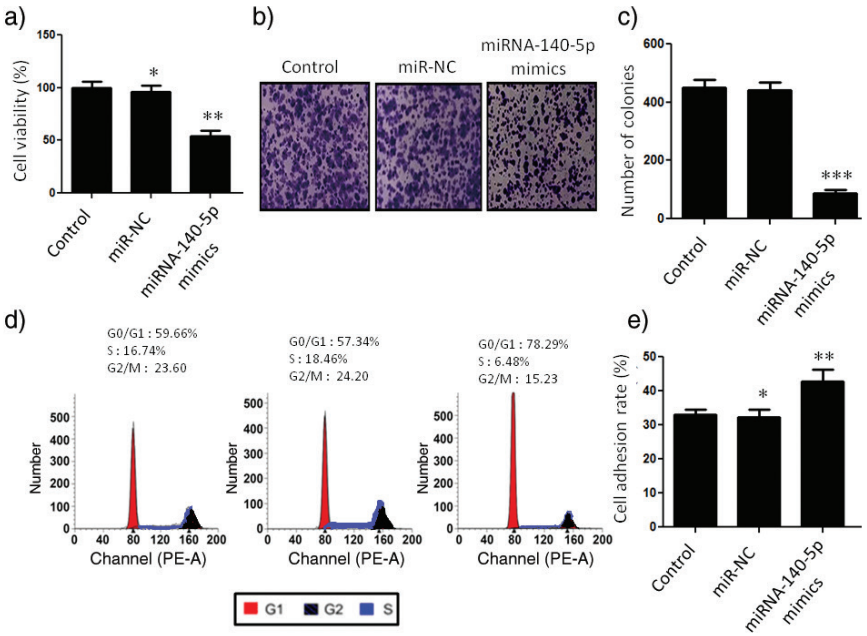

Fig. 1. Results of miR-140-5p up-regulation on MDA-MB-231 cell proliferation, cell cycle distribution and cell adhesion. a) The proliferation of control (non-transfected cells), negative control (cells transfected with miR-NC) and miR-140-5p transfected MDA-MB-231 cells detected using the MTT method; b) and c) The colony formation ability in control, miR-NC and miR-140-5p transfected MDA-MB-231 cells; d) The proportion of cells at different stages of the cell cycle of control, miR-NC and miR-140-5p transfected MDA-MB-231 cells determined using flow cytometry; e) Cell adhesion rate of control, miR-NC and miR-140-5p transfected MDA-MB-231 cells. Data are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Where control: un-transfected cells, miR-NC: cells transfected with control miRNA mimic and miR-140-5p mimic: cells transfected with miR-140-5p mimic. Data from control was compared to data from miR-NC, while miR-140-5p mimic data was compared to miR-NC data.

inhibits MDA-MB-231 cell proliferation, G1/S transition in the cell cycle, and migration *in vitro,* while the cell adhesion rate increases. The aberrant expression of miR-140-5p is a frequent event in various cancers, suggesting an important role for miR-140-5p in tumor initiation and progression (13–15). Consistent with our data, miR-140-5p mimic transfection has inhibited proliferation, invasion and migration of gastric carcinoma cells (25). The current study reports that miR-140-5p performs a significant function as the tumor-suppressive gene in breast cancer cell invasion and migration, consistent with earlier studies (9, 17, 18).

miR-140-5p up-regulation suppressed invasion and migration

In addition to proliferation, metastasis is another important characteristic of breast cancer cells. Next, we explore the function of miR-140-5p on MDA-MB-231 cell invasion and migration ability. The results from the wound healing experiment indicated that the wound area decreased 24 h after transfection with miR-140-5p mimic. However, the wound area was larger in comparison with control and miR-NC (Fig. 2a). In other words, miR-140-5p overexpression inhibits the migration distance of MDA-MB-231 cells. Consistently, invasive cell number was significantly decreased after miR-140-5p overexpression (Fig. 2b). Therefore, miR-140-5p up-regulation inhibited the invasion and migration ability of breast carcinoma cells.

miR-140-5p up-regulation increases E-cadherin expression while suppressing the CDK 2 and AKT/STAT3/NF-κB pathway-related proteins

CDK 2 is a crucial regulatory molecule essential for G1-S transition (19). In this study, miR-140-5p overexpression suppressed the expression level of CDK 2 (Fig. 3a-c). E-cadherin is one of the most common markers of EMT, which mainly mediates the loss of cell-cell adhesion and promotes cell invasion and migration (20). Here, the increased E-cadherin

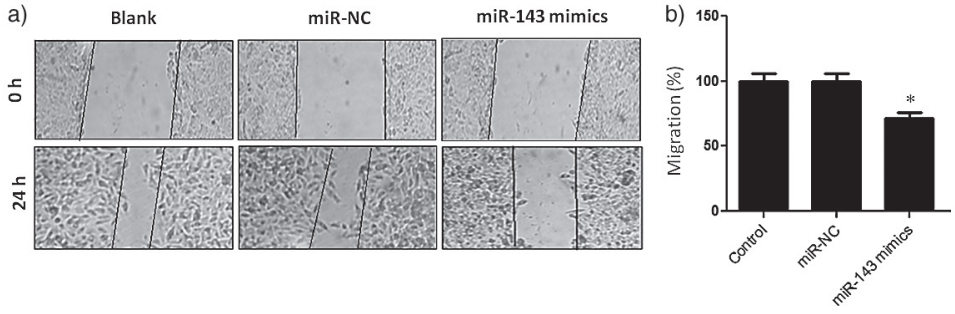

Fig. 2. Influence of miR-140-5p overexpression on MDA-MB-231 cell invasion and migration. a) Scratch wound healing experiment results of control (non-transfected cells), miR-NC transfected cells (negative control) and miR-140-5p transfected MDA-MB-231 cells; b) Migration experiment results of control, miR-NC and miR-140-5p transfected MDA-MB-231 cells. Data were shown as mean ± SD. * $p < 0.05$. Where control: un-transfected cells, miR-NC: cells transfected with control miRNA mimic and miR-140-5p mimic: cells transfected with miR-140-5p mimic. Data from control was compared to data from miR-NC, while miR-140-5p mimic data was compared to miR-NC data.

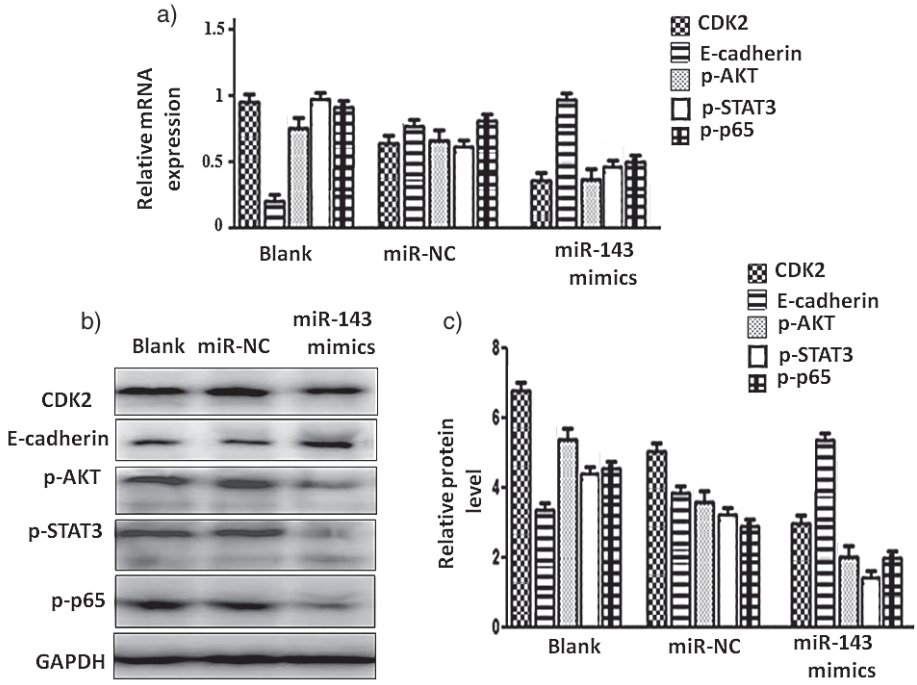

Fig. 3. Influences of miR-140-5p overexpression on AKT/STAT3/NF-κB pathway in MDA-MB-231 cells. a) The mRNA levels of CDK2, E-cadherin, AKT, STAT3 and NF-κB in control (non-transfected cells), miR-NC transfected cells (negative control) and miR-140-5p transfected MDA-MB-231 cells determined with qRTPCR; b) The protein levels of CDK2, E-cadherin, p-AKT, p-STAT3 and p-p65 in control, miR-NC and miR-140-5p transfected MDA-MB-231 analysed by Western blot; c) Densitometry analysis of Western blot analysis. Where control: un-transfected cells, miR-NC: cells transfected with control miRNA mimic and miR-140-5p mimic: cells transfected with miR-140-5p mimic. Data from control was compared to data from miR-NC, while miR-140-5p mimic data was compared to miR-NC data.

induced by miR-140-5p up-regulation is observed and might be correlated with suppressed cell invasion and migration ability in miR-140-5p mimics-transfected MDA-MB-231 cells. Therefore, except for the G0/G1 phase arrest and impaired cell migration, Western blotting analysis indicated a decrease in CDK2 expression and an increase in E-cadherin after miR-140-5p transfection.

Moreover, we next studied the impact of miR-140-5p overexpression on the AKT/STAT3/NF-κB pathway (Fig. 3a-c). As compared to the other two groups, the phosphorylation of the AKT, STAT3 and p65 in MDA-MB-231 cells transfected with miR-140-5p mimics were lower at protein levels. As the core of the PI3K/Akt pathway, Akt is essential for cell survival and growth, and down-regulation could suppress tumor cell proliferation (21). STAT3 promotes tumor cell proliferation, survival and tumor invasion (22). Activation of NF-κB is associated with increased cell cycle progression, cell invasion and migration (23).

The result of our experiments indicated that miR-140-5p up-regulation significantly down-regulates the level of p-AKT, p-STAT3 and p-p65. Thus, we propose that miR-140-5p suppresses MDA-MB-231 cells proliferation and migration by down-regulating the phosphorylation level of AKT, STAT3 and p65.

miR-140-5p up-regulation decreased tumor growth in MDA-MB-231 xenografts and suppressed AKT/STAT3/NF-κB pathway

We established xenograft models using transfected MDA-MB-231 cells and normal control cells to elucidate the tumor-suppressive role of miR-140-5p. Image of the tumors in the nude mice showed that the tumor size of mice treated with miR-140-5p mimics was smaller than that tumor of other groups (Fig. 4a). The result also showed that tumor

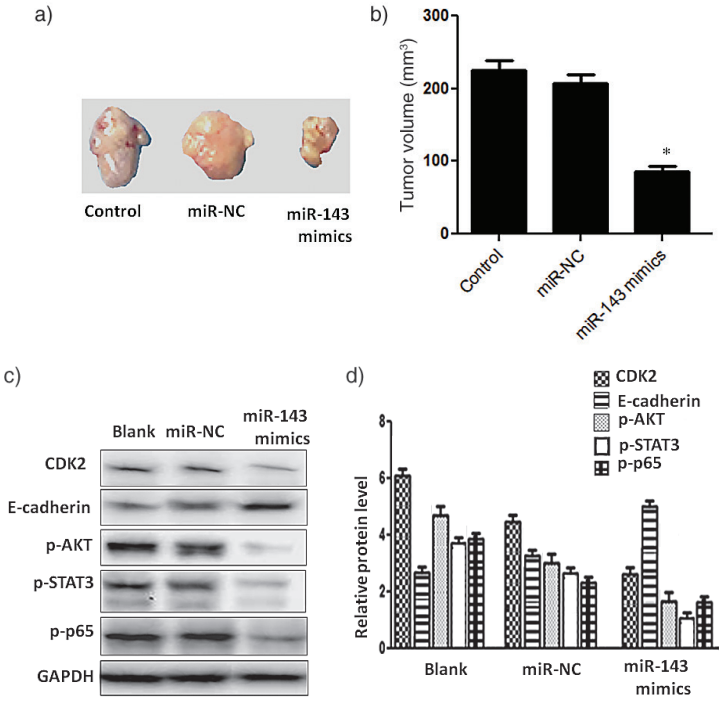

Fig. 4. Tumor xenotransplantation model was used to confirm tumor suppression function of miR-140-5p and its influence on the AKT/STAT3/NF-κB pathway. a) Representative images of subcutaneous tumors for three groups; b) The volume of subcutaneous tumors for three groups; c) Western blotting was applied to determine the expression levels of CDK2, E-cadherin, p-AKT, p-STAT3 and p-p65 in xenograft tumors derived from control, miR-NC and miR-140-5p transfected MDA-MB-231 cells; d) Shows densitometry analysis of Western blot. Data were shown as mean ± SD; * $p < 0.05$. Where control: un-transfected cells, miR-NC: cells transfected with control miRNA mimic and miR-140-5p mimic: cells transfected with miR-140-5p mimic. Data from control was compared to data from miR-NC, while miR-140-5p mimic data was compared to miR-NC data.

growth was suppressed after miR-140-5p transfection, as reflected by decreased tumor volume in miR-140-5p mimics group compared to other groups (Fig. 4b).

In tumor tissues (obtained from MDA-MB-231 xenograft), expression levels of CDK2, E-cadherin, p-AKT, p-STAT3 and p-p65 were determined. According to the results of Western blotting, miR-140-5p up-regulation down-regulated the protein level of CDK2, p-AKT, p-STAT3 and p-p65, while up-regulated E-cadherin protein expression (Fig. 4c). We concluded that miR-140-5p inhibited breast tumor cell growth by down-regulating the AKT/STAT3/NF-κB pathway.

CONCLUSIONS

In conclusion, we presented the evidence that miR-140-5p up-regulation significantly down-regulates the invasion and proliferation ability of MDA-MB-231 cells triggering G1/S-phase cell cycle arrest. AKT/STAT3/NF-κB pathway was suppressed by miR-140-5p up-regulation in MDA-MB-231 cells and MDA-MB-231 xenograft mice. Furthermore, miR-140-5p up-regulation decreased tumor growth in MDA-MB-231 xenograft mice. Taken together, we can conclude that miR-140-5p may act as a novel target in microRNA-targeting anticancer strategy for breast cancer treatment.

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Authors contributions. – Conceptualization, methodology and investigation, L.H. and Q.L; original draft preparation, Y.Z. and H.W., review and editing, Q.M. and F.Y. All the authors have read and agreed to the published version of the manuscript.

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