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MiR-1271 as a tumor suppressor in breast cancer proliferation and progression via targeting SPIN1

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Abstract. – OBJECTIVE: As breast cancer has become the most common malignant tumor in women worldwide and several microRNAs involved in the mechanism of breast cancer development and progression have been identified, we aimed at investigating the role of miR-1271 in breast cancer.

PATIENTS AND METHODS: By quantitative Real-time polymerase chain reaction (qRT-PCR), miR-1271 expression levels in 94 pairs of breast cancer tissue samples and five breast cancer-derived cell lines were detected. Using miR-1271 mimics and inhibitors, the effects of miR-1271 over-expression and knockdown on the proliferation, invasion and migration of MCF-7 cells were analyzed, respectively. Dual-luciferase activity assay was recruited to examine the potential target gene SPIN1 that was predicted by several databases. Protein level was studied using Western blotting.

RESULTS: MiR-1271 was significantly lowly expressed in breast cancer tissue samples and cell lines. Over-expression of miR-1271 in MCF-7 cells significantly decreased the cell proliferation, invasion, and migration abilities while down-regulation of miR-1271 in MDA-MB-453 cells increased these abilities oppositely. Dual-luciferase and Western blotting were used to confirm SPIN1 as a target gene of miR-1271. Furthermore, up-regulation SPIN1 reserved the suppressive effect of miR-1271 over-expression on cell growth and progression.

CONCLUSIONS: miR-1271 can suppress breast cancer cell proliferation and progression via SPIN1, which may provide a potential therapeutic target in treatment for breast cancer.

Key Words

miR-1271, Breast cancer, SPIN1, Wnt/β-catenin.

Introduction

Breast cancer (BC) is the most common malignant tumor in women around the world[1,2]. Many researches[3,4] have revealed the underlying mechanism of breast cancer development and progression partially. However, more investigations are needed to explain the complicated molecular mechanism responsible for breast cancer, which may provide more targets for diagnosis and treatment strategies.

MicroRNAs (miRNAs) are a sort of single-strand, non-coding RNAs, which could suppress specific genes expression level by binding to their 3'-untranslated regions (3'-UTRs), thus regulating the cell activity at the post-translation level[5]. Along with the improvement of biological detection, miRNAs are found highly related to tumorigenicity and evolution[6,7]. Numerous miRNAs have been identified to have a role in breast cancer progress. MiR-203 and miR-96 could promote breast cancer tumor growth and invasion via targeting FGF 2 and PTPN9, while miR-198 and miR-500a-5p inhibited breast cancer proliferation by targeting CUB domain-containing protein 1 and PAK1[8-12]. In addition, miRNA-130b has been reported to regulate breast cancer cell proliferation and drug resistance though the PI3K/Akt pathway[13,14].

MiR-1271 has been shown to act as a tumor suppressor in several cancers via targeting different genes. In prostate cancer, miR-1271 inhibited cell growth by ETS-related gene (ERG). In gastric cancer, it decreases cell proliferation, metastasis and epithelial to mesenchymal transition (EMT) targeting FOXQ1, and miR-1271 decreases pancreatic cancer cell migration, invasion and EMT via ZEB1 and TWIST1[15-17]. However, whether miR-1271 abnormally expresses in breast cancer and how it affects BC is still unclear. The aim of this study was to investigate the expression of miR-1271 in breast cancer, and its underlying mechanisms.

Patients and Methods

Tissue Samples

94 patients with breast cancer had undergone surgery at our hospital and did not received any chemotherapy before the surgery. 94 pairs of BC

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and adjacent normal tissues were taken from the patients. All recruited patients signed the informed consents and the study was approved by the Medical Ethics Committee of Zaozhuang Municipal Hospital (Zaozhuang, China). The clinical features were collected according to the standard of the American Joint Committee on Cancer (AJCC).

Cell Lines and Cell Culture

Human breast cancer-derived cell lines MDA-MB-453, SK-BR3, MDA-MB-415, MDA-MB-468, MCF-7 and human breast epithelial cell line MC-F10A were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All six cell lines were inoculated with Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) high glucose medium containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), placed in 37°C, 5%, saturated humidity cell culture incubator.

Cells Transfection

The miR-1271 mimics and inhibitors were provided by GenePharma (Shanghai, China). Cells in the logarithmic growth phase were taken, digested with 0.25% trypsin, centrifuged and prepared into the single-cell suspension. The suspension was inoculated into a 6-well plate, and the cell density reached 70-90% on the second day. Next, cells were incubated with miR-1271 mimics, miR-1271 inhibitors or relative negative control in serum-free Dulbecco's Modified Eagle Medium (DMEM) containing lipofectamine 2000 for 48 h according to the specifications. Cells were planted into six-well plate and culture to density of 60-70%; after that, they were incubated with miR-1271 mimics, miR-1271 inhibitors or relative negative control in serum-free DMEM containing lipofectamine 3000 for 48 h according to the specifications. The transfection efficiency was measured by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

The plasmid pcDNA3.1-SPIN1 was synthesized by GenePharma (Shanghai, China). Cells were cultured for 24 h in six-well plate, and then incubated with pcDNA3.1-SPIN1 mixed with lipofectamine 3000 in serum-free DMEM.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted under aseptic RNase-free conditions in strict accordance with the instructions of RNA extraction kits (Invitrogen, Carlsbad, CA, USA). Then, extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to the instructions of Prime-Script™ reverse transcription kits (TaKaRa, Otsu, Shiga, Japan). miR-1271 expression level was detected by qRT-PCR reaction system, which was prepared according to the instructions of SYBR Green (Invitrogen, Carlsbad, CA, USA). After the reaction was completed, the cycle threshold (Ct) of each reaction tube was calculated, and the relative expression quantity of miR-1271 was calculated by $2^{-\Delta\Delta Ct}$ method. Each experiment was confirmed three times.

Cell Counting Kit-8 (CCK8) Assay

BC cell lines seeded in a density of $1*10^3$ cells with 100 uL medium were inoculated into a 96-well plate. 200 μL DMEM containing 10% fetal bovine serum (FBS) were added into each well. The cell proliferation was detected at 24, 48 and 72 h after transfection. During detection, 20 μL CCK-8 reagent (Dojindo, Kumamoto, Japan) were added into each well in the 96-well plate for incubation at 37°C for 2 h. The optical density (OD) value of each well was detected at a wavelength of 450 nm using a spectrophotometer.

Colony Formation Assay

To further investigate the cell growth of BC cells, cells were plated in 6-well plates at a density of 600 per well and maintained in a normal medium for 10 days. The colonies were fixed in 70% methanol for 20 min. Next, they were stained with 0.5% crystal violet for 10 min on ice; each well was washed 3 times with phosphate-buffered saline (PBS).

Wound-Healing Assay

Cells in the logarithmic growth phase were digested with trypsin, centrifuged and prepared into the single-cell suspension. The suspension was inoculated into the 6-well plate and cultured in 5% CO_2 at 37°C overnight. When 80-90% cells grew, a wound was gently made in the middle of the cell plate using a 10 μL sterile spearhead under the same strength, and the width of wound in each group should be the same. After the plate was washed twice with PBS, cells were treated with drugs. The width of different wounds in any three parts in each group was measured under a low-power microscope at 0 and 48 h after culture.

Transwell Assay

A total of 1 x 105 treated cells in 10% fetal bovine serum (FBS) medium were seeded into the top chamber of the insert, which had plated Matrigel (BD, Franklin Lakes, NJ, USA). The upper chamber surface of the bottom membrane of the transwell chamber was dried with 50 mg/L matrigel with 1:8 dilution, and dried at 4°C. A diluted matrigel (3.9 μg/μl) 60-80 ul was added to the polycarbonate film on the upper chamber, and the lower chamber was immersed in FBS-free medium. The matrigel was polymerized into a gel at 37°C for 30 min. After 24 h of transfection, the cells were digested by trypsin. The cell density was adjusted to $5x10^4$/ml. 6 ml FBS containing medium were added into the lower chamber. After 24 h of normal culture, the basement membrane of the lower chamber was removed. The cells stained were calculated after that the pictures were taken using a microscope in five random visions.

Dual-Luciferase Assay

Dual luciferase reporter plasmids inserted with wild-type and mutant SPIN1 -3'UTRs were established to test the activity of luciferase. MCF-7 cells were seeded into the 96-well plate and co-transfected with reporter plasmids and miR-1271 mimics or NC-mimics according to experimental methods described above. After 48 h of transfection, luciferase activity was determined using luminometer (Promega, Madison, WI, USA) and measured as the fold-change to the basic pGL3 vector relatively.

Protein Isolation and Western Blot

We measured the concentration of collected protein using a protein assay kit (Beyotime, Shanghai, China). By using a bicinchoninic acid (BCA) Kit (Thermo Fisher Scientific, Waltham, MA, USA), the protein collected from MCF-7 was detected. The antibodies against SPIN1, β-catenin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were collected from Santa Cruz Biotechnology Company (Santa Cruz, CA, USA). The secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Statistical Analysis

Statistical analysis was performed using STATA 12.0 (StataCorp., College Station, TX, USA), and presented with Graph PAD prism software (Version X; La Jolla, CA, USA). The results obtained from experiment *in vitro* assays are presented as mean ± SEM. One-way ANOVA test followed by LSD (Least Significant Difference) was used for comparison between groups. All the results were regarded as statistically significant at $p<0.05$.

Results

MiR-1271 Expressed Lower in BC Tissues and Cell Lines

To detect the relationship between the miR-1271 expression and breast cancer, we examined miR-1271 levels in 94 pairs of BC tissues and adjacent normal tissues as well as in five breast cancer cell lines MDA-MB-453, SK-BR3, MDA-MB-415, MDA-MB-468, MCF-7 and normal breast epithelial cell MCF10A. miR-1271 level was significantly lowly expressed in BC tissues than that in adjacent normal tissues (Figure 1A). We next divided the 94 patients into high and low miR-1271 level group to evaluate the correlation between several clinical pathological features and miR-1271. Lower miR-1271 expression level was related to larger tumor size, more lymph node metastasis and advanced TNM stage (Table I). In cell lines, miR-1271 level was lower in BC cell lines compared with MCF10A (Figure 1B). These results indicated that miR-1271 could inhibit BC progression.

To further investigate the effect of miR-1271 in BC, we next chose MCF-7 cells to over-express while MDA-MB-453 cells to knockdown miR-1271 according to their relative expression level. Transfected with miR-1271 mimics or inhibitors, expression of miR-1271 in MCF-7 cells was markedly increased while decreased in MDA-MB-453 cells (Figure 1C-D).

MiR-1271 Over-Expression Inhibited the Proliferation of BC Cells

CCK8 and colony formation assay were used to explore the miR-1271 effects in cell proliferation. MCF-7 cells treated with miR-1271 mimics performed reduced proliferation activity than negative control group (Figure 2A), while MDA-MB-453 cells transfected with miR-1271 inhibitors displayed increased growth ability compared with that in the control group (Figure 2B). In addition, MCF-7 cells formed lesser colonies after miR-1271 mimics treatment (Figure 2C-D), and MDA-MB-453 cells formed more colonies after miR-1271 inhibitors transfection compared to control group, relatively (Figure 2E-F). All these data suggested that miR-1271 could inhibit BC cells proliferation.

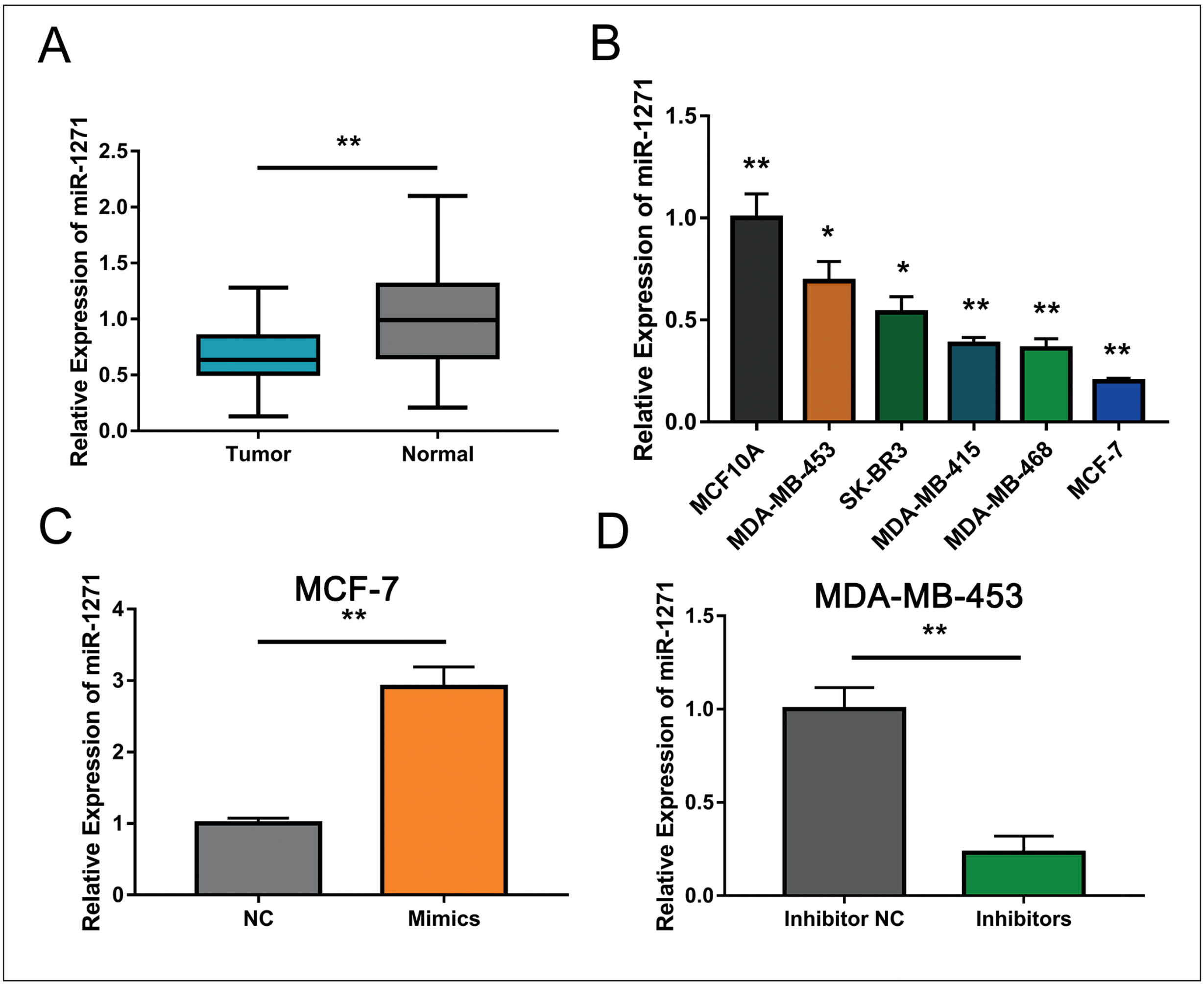

Figure 1. MiR-1271 was down-regulated in BC tissues and cell lines. **A**, Analysis of the expression level of miR-1271 in 94 pairs BC tumor and adjacent tissues. **B**, Analysis of miR-1271 expression level in BC cell lines (MDA-MB-453, SK-BR3, MDA-MB-415, MDA-MB-468, MCF-7) and breast epithelial cell line (MCF10A). **C**, Expression of miR-1271 in miR-1271 mimics treated MCF-7 cells. **D**, Expression of miR-1271 in miR-1271 inhibitors treated MDA-MB-453 cells. MiR-1271 was detected by qRT-PCR and U6 was used as an internal control. Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$.

Up-Regulation of miR-1271 Inhibited the BC Cell Migration and Invasion

We measured the miR-1271 influence on BC cells migration abilities using wound-healing assay. Over-expression miR-1271 in MCF-7 cells significantly decreased the migration ability (Figure 3A-B). On contrast, knockdown miR-1271 in MDA-MB-453 cells increased the wound-healing ability compared with control cells (Figure 3C-D). In addition, to evaluating the influence of miR-1271 in cell invasion, we carried out transwell invasion assay and found sharp decline after mir-1271 overexpression (Figure 3E-F) but markedly enhancement after miR-1271 inhibition in cell invasion ability (Figure 3G-H). These results illustrated miR-1271 suppressed BC cell migration and invasion.

SPIN1 Was a Target Gene of miR-1271

To further explore the molecular mechanism of miR-1271 involved in BC, we next searched three databases TargetScan, miRwalk, and PicTar. After comprehensive analysis, we found that SPIN1 was a candidate target gene of miR-1271. To confirm the prediction, we employed dual-luciferase assay using conducted wild-type or mutant SPIN1 3'-UTR vector (Figure 4A). The result of dual-luciferase assay displayed a significant activity decrease in the WT group but no difference in mutant group (Figure 4B). The

Table I. Correlation between miR-1271 level and clinicopathological features.

Variables	Total	miR-1271 expression Low	miR-1271 expression High	*p*-value
Age				
< 50 years	46	21	25	0.538
≥ 50 years	48	26	22	
Tumor size				
< 3 cm	55	17	38	0.0001*
≥ 3 cm	39	30	9	
Lymph node metastasis				
Yes	73	42	31	0.013*
No	21	5	16	
T stage				
T1	25	6	19	0.0107*
T2	54	30	24	
T3	13	10	3	
T4	2	1	1	
Distance metastasis				
Yes	87	45	42	0.431
No	7	2	5	
TNM stage				
I	6	0	6	0.0053*
II	35	16	19	
III	46	38	18	
IV	7	3	4	

The expression level of miR-1271 was cut off by median expression level and *indicated $p<0.05$.

protein expression of SPIN1 in miR-1271 mimics or inhibitor treated cells was measured by Western blot analysis. As reported by several studies, wnt/β-catenin was the downstream signal pathway of SPIN1, and we also tested β-catenin protein level[18]. Notably, up-regulation of miR-191 reduced the SPIN1 level in MCF-7 cells (Figure 4C-D) while down-regulation of miR-1271 in MDA-MB-453 cells increased the SPIN1 expression (Figure 4E-F). These above results suggested that SPIN1 was a direct target of miR-1271.

SPIN1 Over-Expression Counteracted the Effect of miR-1271 Up-Regulation

As we speculated that miR-1271 suppressed BC cells proliferation, migration and invasion via down-regulating SPIN1, we established plasmid pcDNA3.1-SPIN1 to reverse the effect of miR-1271 mimics to further confirm these results. CCK8 assay showed that SPIN1 over-expression in miR-191 mimics treated MCF-7 cells significantly rescued the cell proliferation ability (Figure 5A). Furthermore, the decrease of invasion activity caused by miR-1271 mimics was reversed by SPIN1 up-regulation (Figure 5B-C). Then, Western blotting assay showed that SPIN1 and β-catenin levels were rescued by SPIN1 up-regulation (Figure 5D-E). These data indicated that miR-1271 suppressed BC cells proliferation and progression via SPIN1 and its downstream Wnt/β-catenin pathway.

Discussion

We analyzed the expression level in BC tissues for the first time and found lower miR-1271 expression in BC tissue samples compared to the normal group. In addition, study of the correlation between miR-1271 and BC clinical features suggested that lower miR-1271 indicates advanced BC progression. These findings emphasized miR-1271 functioned as a tumor inhibitor in BC as well as in several other cancers including prostate cancer, hepatocellular carcinoma, and pancreatic cancer[15,16,19].

Next, to confirm the function of miR-1271 in BC, we conducted loss- and gain- function exper-

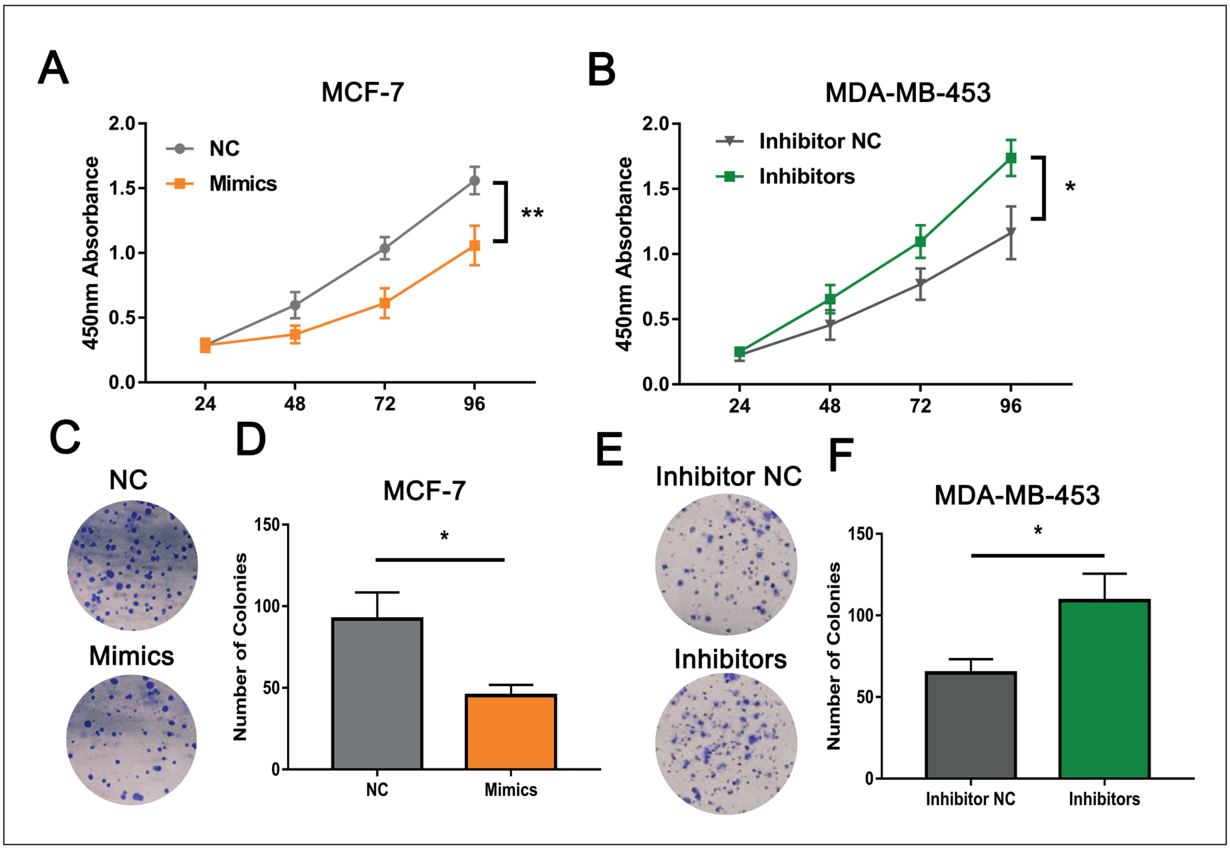

Figure 2. MiR-1271 effected the proliferation of BC cells. **A-B**, CCK8 assay was performed to determine proliferation of MCF-7 (**A**) or MDA-MB-453 (**B**) cells treating with miR-1271 mimics or inhibitors compared to each negative control. **C-F**, Colony formation assay was performed to determine the growth of MCF-7 (**C**, **D**) or MDA-MB-453 (E,F) cells transfected with mimics or inhibitors, respectively. Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$.

iments using miR-1271 mimics and inhibitors. By CCK8 assay and colony formation assay, we studied the proliferation activity of established cell lines.

The growth ability of MCF-7 cells was significantly reduced after miR-1271 up-regulation, while MDA-MB-453 cells treated with miR-1271 inhibitors showed increased cell proliferation ability compared to relative control group. These results suggested that miR-1271 can inhibit BC cell proliferation, which is presented in neuroglioma and hepatocellular carcinoma[20,21]. Furthermore, cell migration and invasion abilities changes were visualized. As reported in pancreatic cancer cells[15], miR-1271 inhibits cell migration and invasion, miR-1271 over-expression reduced MCF-7 cell migration and invasion while down-regulation of miR-1271 promoted these activities in MDA-MB-453 cells. These results clearly showed miR-1271 inhibited BC progression. As far as we know, this is the first report explaining miR-1271 function in BC. To detect the molecular mechanism of miR-1271, we speculated SPIN1 as a potential target gene due to several databases. Then, we used dual-luciferase assay to verify our assumption by co-transfecting vector containing wild type or mutant SPIN1 3'-UTR and miR-1271 mimics in MCF-7 cells. The WT group performed significantly decrease of luciferase activity while mutant group showed no difference, which demonstrated SPIN1 was direct target of miR-1271. Western blot further confirmed that SPIN1 was a downstream molecular of miR-1271. SPIN1 was defined as a nuclear protein involved in the NIH3T3 cell transformation for the first time, acting as an ovarian cancer-related protein[22,23]. It promoted cancer cell proliferation through Wnt pathway activation[18,24,25]. We next detected a key marker of Wnt pathway β-catenin. Besides, β-catenin showed the same alteration trends with SPIN1 expression level in treated cells. Furthermore, SPIN1 was reported to be exerted by miRNA-29b-1-5p in triple negative breast cancer, and mediated PI3K/Akt pathway

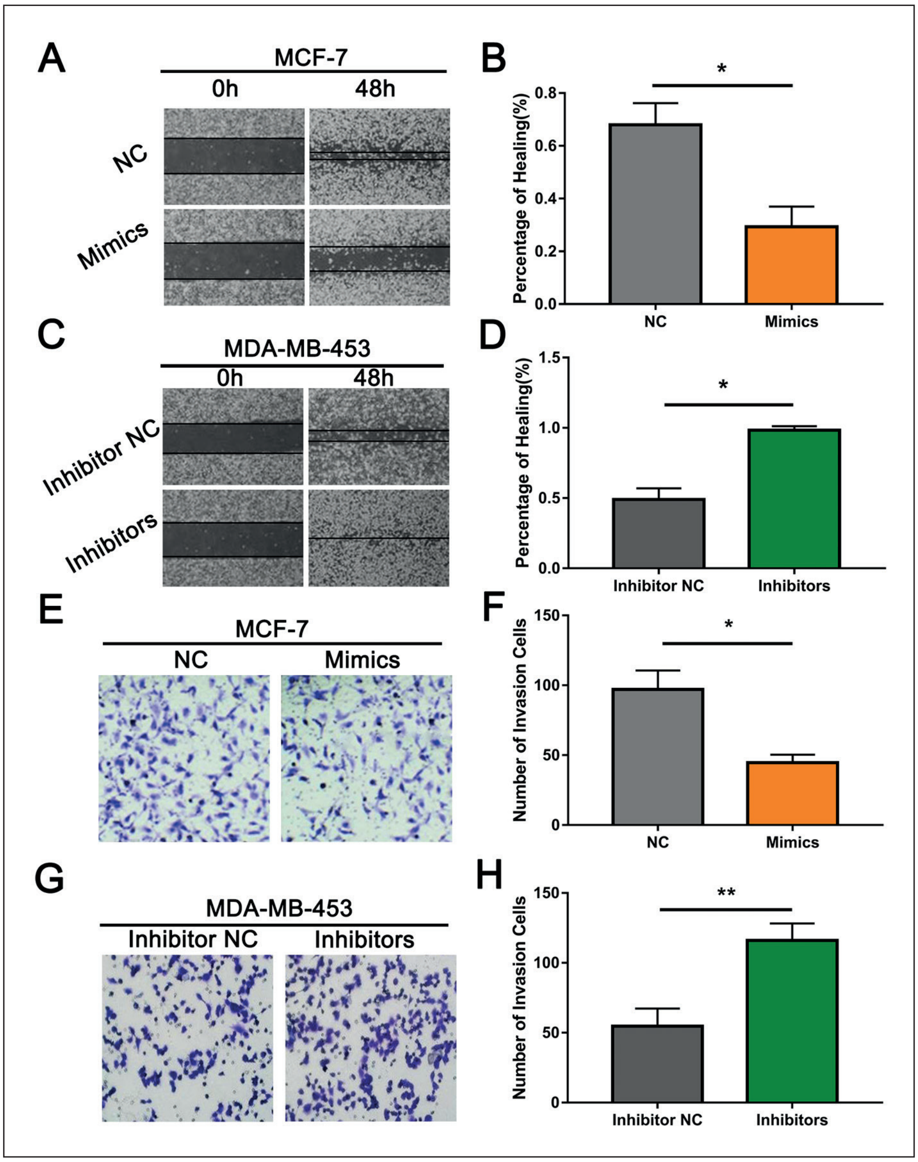

Figure 3. MiR-1271 effected the migration and invasion of BC cells. (**A**, **D**) Wound-healing assay was performed to determine proliferation of MCF-7 (**A**, **B**) or MDA-MB-453 (**C**, **D**) cells treating with miR-1271 mimics or inhibitors compared to each negative control. (**E**-**H**) Transwell invasion assay was used to detect the invasion ability of miR-1271 mimics treated MCF-7 cells (**E**, **F**) or miR-1271 inhibitors treated MDA-MB-453 cells (**G**, **H**). Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$.

to increase chemosensitivity in breast cancer. In combination with our research, SPIN1 played an important role in BC progression especially in miRNAs regulation network[26,27]. In addition, to analyze the function of SPIN1, it was found that over-expressed SPIN1 in miR-1271 up-regulated MCF-7 cells, and SPIN1 rescued the inhibition function of miR-1271 in cell proliferation and invasion. All these data demonstrated miR-1271 inhibited BC cell growth and progression via SPIN1 and its downstream Wnt/β-catenin signaling pathway. Though we studied the miR-1271 *in vitro*, more studies *in vivo* are still needed to further explore the miR-1271 function in BC.

Conclusions

We firstly demonstrated that miR-1271 expression was decreased in BC and its over-expression inhibited BC cell proliferation, migration and invasion. Further, we elucidated miR-1271 function as a tumor suppressor via targeting SPIN1 and its downstream Wnt/β-catenin signaling pathway. These results can provide a potential target for BC diagnosis and therapy in the future.

Conflict of Interest

The authors declared no conflict of interest.

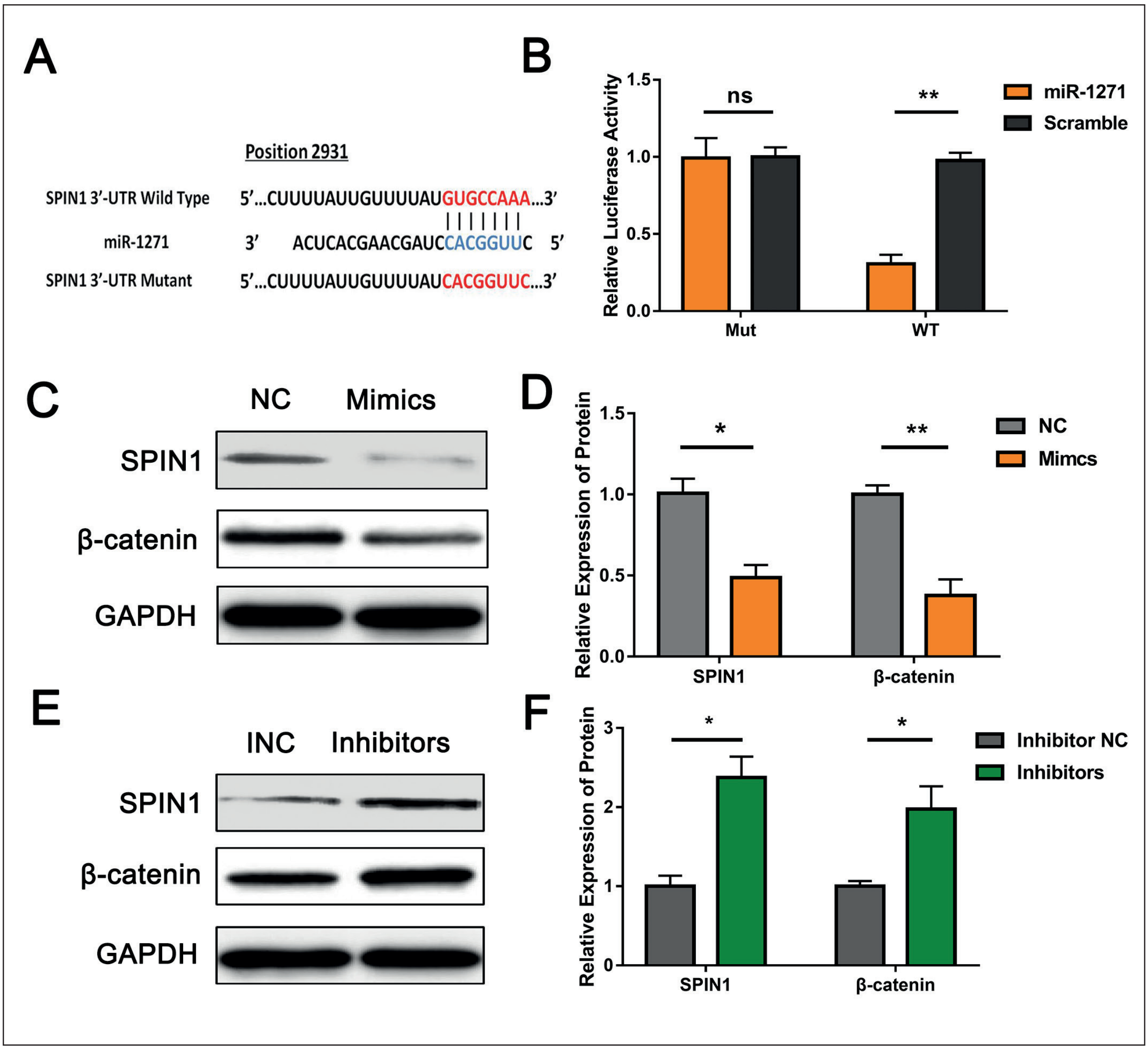

Figure 4. SPIN1 was a direct target of miR-1271. **A**, The predicted binding sites of miR-1271 in the 3'-UTR of SPIN1. **B**, Dual-luciferase reporter assay was used to determine the binding site. MCF-7 cells treated by mimics or NC were transfected with pGL3 construct containing the WT or mutant SPIN1 3'-UTR site. **C**, **E**, Levels of SPIN1 and β-catenin protein measured by Western-blot in miR-1271 overexpression MCF-7 cells (**C**) and miR-1271 knockdown MDA-MB-453 cells (**E**). **E**, **F**, The relative protein level of SPIN1 and β-catenin. The protein levels were normalized to that of GAPDH. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ns: non-sense.

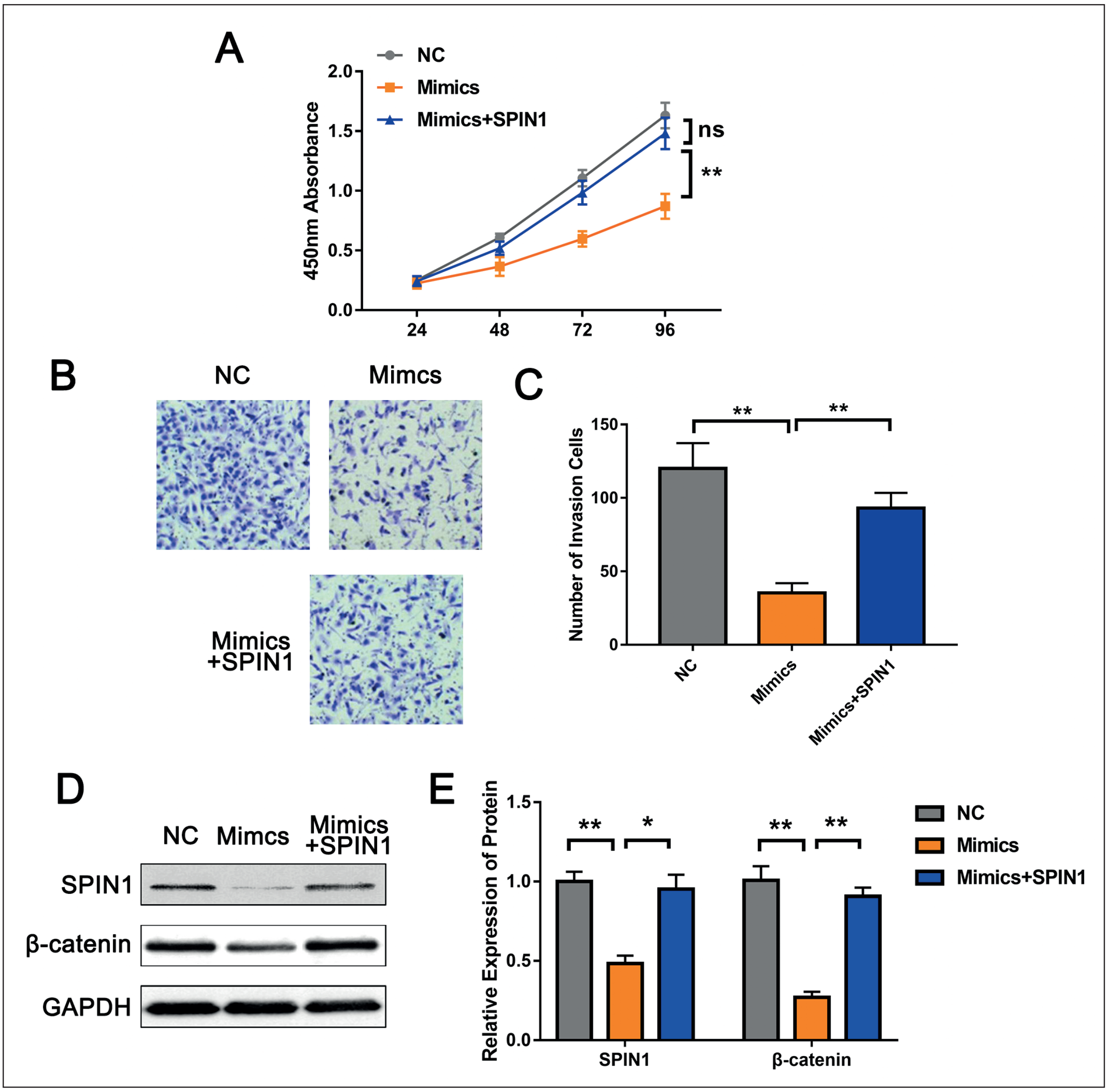

Figure 5. SPIN1 rescued the effects of miR-1271 mimics in MCF-7 cells. **A**, Analysis of the cell proliferation ability by CCK8 assay in miR-1271 NC, mimics, or mimics+SPIN1 treated MCF-7 cells. **B**, **C**, Cell invasion ability was measured by transwell assay. **D**, Western-blot analyses of SPIN1 and β-catenin expression level. GAPDH was used as an internal control. **E**, Relative protein band densities of SPIN1 and β-catenin. Data are represented as the mean ± SD of three replicates. $^*p<0.05$, $^{**}p<0.01$.

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