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MicroRNA-574 enhances doxorubicin resistance through down-regulating SMAD4 in breast cancer cells

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Abstract. – OBJECTIVE: Drug resistance has become an important factor that threatens the survival and prognosis of patients with breast cancer, especially in patients with advanced breast cancer. Several microRNAs have been proved to participate in the resistant process; however, the role of miR-574 in doxorubicin (Dox) resistant breast cancer is still unclear.

PATIENTS AND METHODS: Quantitative Real-time poly chain reaction (qRT-PCR) was employed to detect the expression level of miR-574 in breast cancer Dox-resistant MCF-7/Adr cell line and parental MCF-7 cell line. Using miR-574 mimics and inhibitors, miR-574 level was up- or down- regulated. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was handled to detect the IC50, and flow cytometric analysis was employed to measure the apoptosis and cell circle. Dual-luciferase and Western-blot experiments were applied to verify the direct target gene of miR-574.

RESULTS: miR-574 expression level was significantly higher in MCF-7/Adr cells compared to normal MCF-7 cells. Up-regulation of miR-574 level in MCF-7 cells promoted the cell growth and G0/G1-to-S phase transition but inhibited cell apoptosis. However, knockdown of miR-574 in MCF-7/Adr cells decreased the IC50 and cell growth. Using luciferase assay, SMAD4 was confirmed to be a potential target of miR-574, and the expression of SMAD4 protein was regulated by miR-574. In blood samples of patients, the miR-574 level before chemotherapy was higher than that after chemotherapy.

CONCLUSIONS: We revealed miR-574 could promote doxorubicin resistance of breast cancer MCF-7 cells via down-regulating SMAD4, thus providing a novel target for advancing breast cancer chemotherapy.

Key Words:
MRNA-574, Doxorubicin, Resistance, SMAD4, Breast cancer.

Introduction

In recent decades, breast cancer remains to be one of the most common malignant tumors in women, which seriously threats women's quality of life and physical and mental health[1]. Combination of chemotherapy and drugs represented by doxorubicin is the standard treatment for adjuvant breast cancer at present[2]. However, the prognosis of patients with advanced breast cancer is still unclear[3]. One of reasons for the treatment failure is the emergence of chemotherapy resistance[4].

MicroRNA (miRNA) is a group of tiny non-coding RNA consisting of 18-25 nucleotides, which is highly conserved in true nuclear biology[5]. MiRNAs could bind with target genes by their 3'-untranslated regions, which resulted in degradation of their target genes or inhibition of target gene transcription[6]. They played important roles in the development of organisms, cell differentiation, apoptosis, tumor formation and other physiological and pathological processes[7]. Many studies have revealed that miRNAs were involved in the mediation of chemosensitivity of several cancers including breast cancer. Such as, miR-21 enhanced drug resistance via multi-genes in pancreatic and colon cancers; miRNA-137 could chemosensitize colon cancer cells to oxaliplatin via DCLK1 and miR-155 knockdown in lung cancer A549/Dox cells reversed its doxorubicin resistance[8-10]. In breast cancer, miR-20a regulated breast cancer chemoresistance through a MAPK1/c-Myc feedback loop; miR-760 mediated drug resistance via inhibiting epithelial to mesenchymal transition. Also, miR-542-3p could regulate doxorubicin efficiency in triple negative breast cancer therapy[11-13]. MiR-574 could act as an oncogene in prostate cancer and identify ectopic expressed in formalin-fixed par-

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affin-embedded breast cancer tissues[14,15]. However, the specific role of miR-574 in chemoresistance of breast cancer remains unclear.

We investigated the expression level of miR-574 in established Dox-resistant MCF-7/Adr and normal MCF-7 cell lines. Also, the miR-574 expression was regulated using the mimics or inhibitors. IC50 of these cells were calculated and cell proliferation and apoptosis were studied. Furthermore, SMAD4 was proved to be a direct target for miR-574 and regulated by ectopic miR-574 level. All these data indicated miR-574 enhanced doxorubicin resistance through down-regulating SMAD4 in breast cancer cells.

Patients and Methods

MCF-7 and MCF/Adr Cell Lines and cell Culture

The MCF-7 and MCF-7/Adr cell lines were purchased from KeyGENE (Nanjing, China). The doxorubicin used in the culture was obtained from Beyotime (Shanghai, China). Cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) mixed with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin and streptomycin (KeyGENE, Nanjing, China) in the atmosphere of humid air containing 5% CO_2 at 37°C.

Blood Samples

All the blood samples of 30 patients were collected from Yantai Yuhuangding Hospital between 2010 and 2014. All the patients have received doxorubicin-based chemotherapy due to advanced breast cancer. Blood sample was collected before and after chemotherapy, respectively. Written informed consents were obtained from the patients and the study conformed to the standards of the Declaration of Helsinki. This study obtained the approval from the Ethical Committee of Yantai Yuhuangding Hospital (Yantai, China).

Transfection of miR-574 Mimics and Inhibitors

MiR-574 level was up-regulated or down-regulated in MCF-7 or MCF-7/Adr cells using miR-574 mimics or inhibitors synthesized by Genepharma (Shanghai, China). Cells were plated in six-well plates and cultured to a density of 60%; then, the miR-574 mimics, inhibitors, mimics NC, or inhibitors NC were added into the plates. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was added into the culture medium to enhance the transfection according to the manufactures instruction. After cultured for 24 h, the transfection efficiency of miR-574 was confirmed using qRT-PCR.

RNA Isolation and qRT-PCR

Total RNA of blood samples or cells was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). miRNA Reverse Kit (TaKaRa, Dalian, China) was used to reverse the RNA sequence to cDNA. The miR-574 levels were detected by ABI 7900 (ABI, Waltham, MA, USA) using SYBR Premix kits (TaKaRa, Dalian, China) and U6 was used as internal control. The reaction condition of PCR was: 95°C for 30 s, 95°C for 5 s, 60°C for 30 s, and 72°C for 10 s × 40 circles. The relative expression levels of miR-574 to U6 were measured using the $2^{-\Delta\Delta}$CT method based on three times repeats.

MTT Assay

The sensitivity of the Dox-resistant MCF-7/Adr and MCF-7 cells to doxorubicin was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For IC 50 detection, a total of $3.5x10^3$ cells were put into 96well plates per well and co-cultured with different concentrations of doxorubicin (0, 1, 5, 10, 20, 30, 40, 50, 80 and 100 μg/mL). At different time lines (0, 24, 48, 72 and 96 h) post-doxorubicin treatment, cell activity was measured by using 0.5 mg/mL MTT buffer (Thermo Fisher, Waltham, MA, USA). Next, the absorbance at 570 nm was detected using a spectrophotometer (Bio-Rad, Hercules, CA, USA) after being cultured in dark for 2 h.

For cell proliferation ability detection, cells were treated with miR-574 mimics or inhibitors before seeding into 96well plates. Then, at 0, 24, 48, and 72 h, cell activity was measured by using MTT solution. Each experiment was repeated at least three times.

Cell Apoptosis and Cell Cycle Detection Using Flow Cytometric Analysis

Apoptotic rate of MCF-7/Adr or MCF-7 cells was measured by using an Annexin V-FITC/PI Labeled Apoptosis Detection Kit (Thermo Fisher, Waltham, MA, USA). A total of $2x10^6$ cells was suspended in 1000 μL binding buffer, labeled using 10 μL annexin V-FITC and 10 μL PI according to the manufacturer's instructions. The samples were immediately analyzed after being maintained in the dark for 10 min. FITC (-) and PI (-) cells were assigned as live cells and FITC (+) but PI (-) as early apoptotic cells.

For cell cycle analysis, PI Staining Cell Cycle Detection Kit (Thermo Fisher, Waltham, MA,

USA) was recruited. The harvested cells after treatment were centrifuged and the supernatant was removed. Next, a total of $1x10^6$ cells were immersed in cooling ethyl alcohol at 20°C for 48 h. Cell cycle analysis was detected after cells incubated in 50 μg/mL propidium iodide (PI) solution in the dark for 30 min. The percentage of cells in different phases were measured and calculated.

These two assays were performed with a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Every experiment was repeated at least three times.

Dual-luciferase Assay

Dual-Luciferase assay (Promega, Madison, WI, USA) was enlisted to measure the luciferase activity. The wild type or mutant miR-574 binding site SMAD4 3'-UTR cDNA fragment after being amplified was cloned into pGL3 luciferase vector (Promega, Madison, WI, USA), relatively. A549 cells were transfected with miR-574 mimics together with the conducted pGL3/WT or pGL3/mutant vector. These cells were cultured for 24 h, and the activity of luciferase was determined and recorded using luminometer machine (Promega, Madison, WI, USA).

Western Blotting Assay

The proteins of MCF-7/Adr and MCF-7 cells were extracted using radioimmunoprecipitation assay (RIPA) reagent (Beyotime, Shanghai, China). The protein concentrations were measured by bicinchoninic acid (BCA) quantitative detection reagent kit (Beyotime, Shanghai, China). Then, the protein was separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) after being degenerated and shifted to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes loaded with proteins were immersed into block non-specific protein interactions, and next soaked in 5% fat-free milk with the primary antibody against SMAD4 or glyceraldheyde 3-phosphate dehydrogenase (GAPDH) at 4°C overnight. The membranes were then incubated with secondary antibody marked by horseradish peroxide (HRP) for 1 h after being washed with tris-buffered saline-tween (TBST) buffer for three times at room temperature. The membranes were next detected using enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA) following the instructions. All these antibodies were purchased from Cell Signaling Technology company (Danvers, MA, USA).

Statistical Analysis

Statistic package for social science 18.0 (SPSS Inc., Chicago, IL, USA) software was used to do the statistical analysis and all results were showed as mean ± SD. Comparison between groups was done using One-way ANOVA test followed by LSD (Least Significant Difference) post-hoc test. p-values < 0.05 were considered to have significant difference.

Results

miR-574 was Overexpressed in MCF-7/Adr Dox-resistant Cell Lines Compared with MCF-7 Cell Line

To demonstrate the relationship between miR-574 and Dox-resistance, we first obtained MCF-7/Adr and MCF-7 cell lines. The IC50 of MCF-7/Adr cells were significantly higher than the parallel MCF-7 ones (Figure 1A). Next, we detected the miR-574 expression in the two cell lines and found that the miR-574 level in Dox-resistant MCF-7/Adr cells was remarkably higher than that in parallel normal MCF-7 cell line (Figure 1B). These results indicated that miR-574 might play an important role in breast cancer cell doxorubicin resistance.

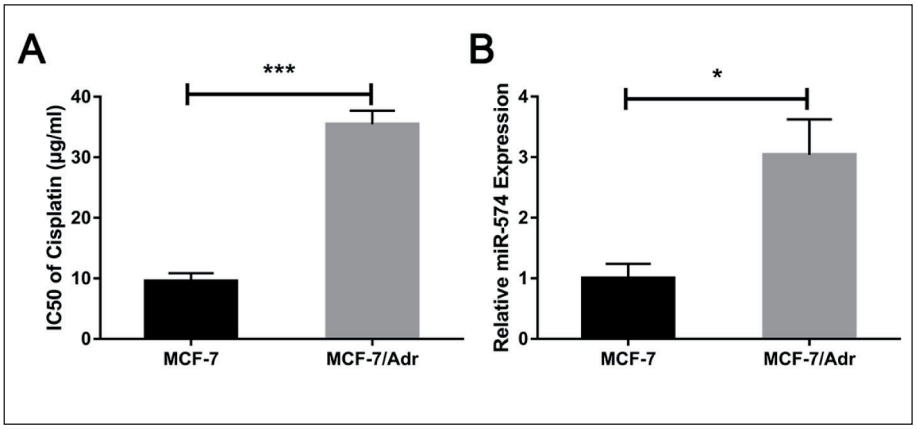

Figure 1. miR-574 was overexpressed in MCF-7/Adr Dox-resistant cell lines compared with MCF-7 cell line. A- Analysis of IC50 of miR-377 in MCF-7/Adr Dox-resistant cell lines compared with MCF-7 cell line. B- Analysis of miR-574 expression level in MCF-7/Adr cells compared with MCF-7 cells. miR-574 was detected by qRT-PCR and U6 was used as an internal control. Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

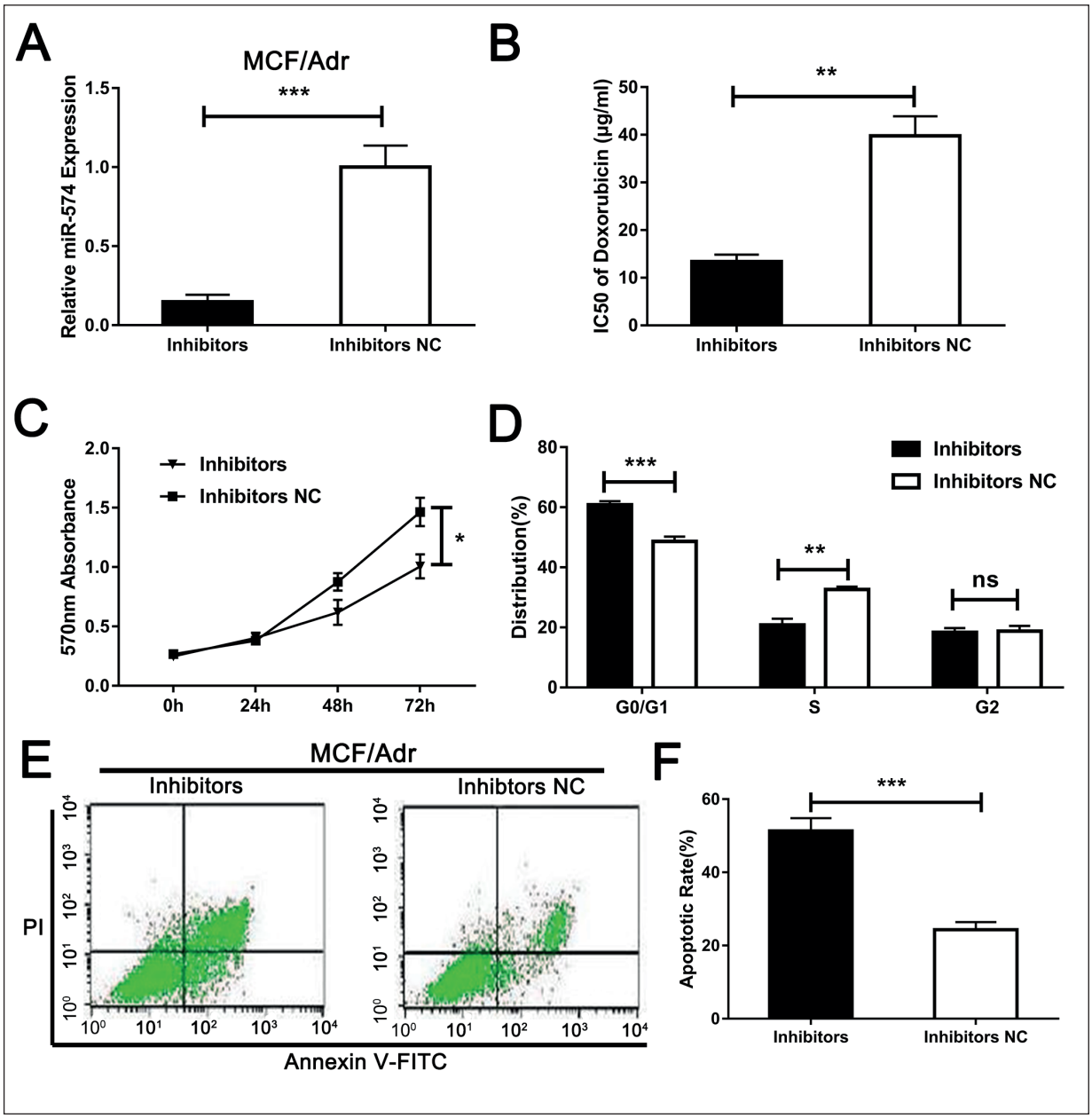

Figure 2. Down-regulation of miR-574 reversed doxorubicin resistance of MCF-7/Adr cells. A- Expression of miR-574 in miR-574 inhibitors treated MCF-7/Adr cells, B- IC 50 of miR-574 inhibitors treated MCF-7/Adr compared with inhibitors NC group. C- MTT assay was performed to determine proliferation of MCF-7/Adr cells treating with miR-574 inhibitors compared to negative control. D- Cell cycle distribution of miR-574 inhibitors transfected MCF-7/Adr cells compared with negative control after treated with 2 μL doxorubicin. E, F- Cell apoptotic rate of miR-574 inhibitors transfected MCF-7/Adr cells compared with negative control after treated with 2 μL doxorubicin. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Knockdown of miR-574 Reversed Doxorubicin Resistance of MCF-7/Adr Cells and Inhibited Cell Growth

To further analyze the function of miR-574 in MCF-7/Adr cells, we knockdown the miR-574 level by using miR-574 inhibitors and the efficiency was shown in Figure 2A. The IC 50 of miR-574 inhibitors treated cells was remarkably decreased than the negative control group (Figure 2B). MTT assay displayed that the proliferation ability of MCF-7/Adr cells was significantly reduced by miR-574 down-regulation (Figure 2C). The flow

cytometric analysis demonstrated that the percentage of cell circle distribution in G0/G1 phase in miR-574 inhibitors group was increased but the percentage of S phase was decreased comparing with negative control group (Figure 2D). In addition, the cell apoptotic rate after 2 μg/mL doxorubicin treatment of miR-574 knockdown MCF-7/Adr cells was higher than the inhibitors NC group (Figure 2E-F). These data partially suggested that miR-574 knockdown could re-sensitize MCF-7/Adr cells to doxorubicin, reduce cell growth, promote cell apoptosis, and arrest cell in G0/G1 phase.

Up-regulation of miR-574 Enhanced Doxorubicin Resistance and Promoted Cell Proliferation of MCF-7 cells.

To confirm the results of miR-574 knockdown, we over-expressed miR-574 in MCF-7 cells using miR-574 mimics (Figure 3A). The IC 50 of MCF-7 cells to doxorubicin were markedly improved by mir-574 up-regulation (Figure 3B). Cell proliferation of miR-574 mimics treated MCF-7 cells increased significantly than the control group (Figure 3C). The cell circle analysis showed that miR-574 over-expression decreased cell distribution in G0/G1 phase but increased in S phase comparing with mimics NC group (Figure 3D). The apoptotic rate after 2 μg/ml doxorubicin treatment of MCF-7 cells was reduced after miR-574 mimics transfection (Figure 3E-F). These results verified the data of miR-574 down-regulation in MCF/Adr cells and indicated miR-574 promoted drug resistance of breast cancer cells.

SMAD4 mediated the Dox-resistant effect of miR-574 in breast cancer cells

To further investigate the underlying mechanism of miR-574, we next searched several database (TargetScan, Pitar, miRwalk) and found SMAD4 as a potential target of miR-574. The binding site of miR-574 in SMAD4 3'-UTR was showed in Figure 4A. Next, we conducted a dual-luciferase assay to identify our assumption. Figure 4B showed the activity of luciferase in wild type SMAD4 3'-UTR group was decreased but no difference in mutant SMAD4 3'-UTR group. Western-blot assay confirmed that the SMAD4 protein level was significantly increased in miR-574 down-regulated MCF/Adr cells than in negative control group but decreased in miR-574 up-regulated MCF-7 cells (Figure 4C-D). These data suggested that SMAD4 was a target for miR-475 in breast cancer cell and miR-574 enhanced doxorubicin resistance of MCF-7 cells.

miR-574 Expression Levels Were up-Regulated in blood Samples from Patients with Advanced Breast Cancer Receiving Doxbased Chemotherapy

Doxorubicin was applied to treat advanced breast cancer patients usually. So, we collected 30 paired blood samples from patients before and after getting Doxbased chemotherapy to measure the miR-574 expression level. The analysis verified the levels of miR-574 expression were increased in the blood samples of patients following chemotherapy based on doxorubicin compared to the samples before the treatment (Figure 5A). These data indicated miR-574 might act as an important influencing factor in therapeutic resistance of breast cancer.

Discussion

Although chemotherapy for breast cancer developed rapidly, advanced breast cancer remains to be a huge challenge for cancer therapies due to drug resistance[2,4,16]. Therefore, it is urgent to identify the underlying mechanism of doxorubicin resistance in breast cancer, which could improve the prognosis of treatment. miRNAs have been proved to be involved in different processes of doxorubicin resistance in several cancers. Zou et al[17] reported miR-495 could sensitize MDR cancer cells to doxorubicin and taxol via repressing MDR1 level. Zhao et al[18] found miR-137 up-regulated by HDAC8 inhibition increasing the doxorubicin sensitivity of neuroblastoma cells. In addition, Duan et al[19] proved that miR-15b modulated multidrug resistance *in vitro* and *in vivo* in human osteosarcoma. Moreover, in breast cancer, miR-181b regulating Bim level and miR-133a regulating uncoupling protein 2 expression could promote chemoresistance in breast cancer cells[20, 21].

Some researches[14,15] pointed out that miR-574 could regulate tumorigenesis and progression in prostate cancer, and high-express in formalin-fixed paraffin-embedded drug resistant breast tissues. We confirmed that miR-574 expression level was significantly higher in Dox-resistant MCF-7/Adr cells compared with normal MCF-7 cells. Next, up-regulation of miR-574 significantly promoted MCF-7 cell proliferation while down-regulation of miR-574 decreased cell growth in MCF-7/Adr cells. Furthermore, cell apoptotic rate treated with doxorubicin was significantly lower in miR-574 over-expressing MCF-7 cells compared with control group; however, higher in miR-574 knockdown MCF/Adr

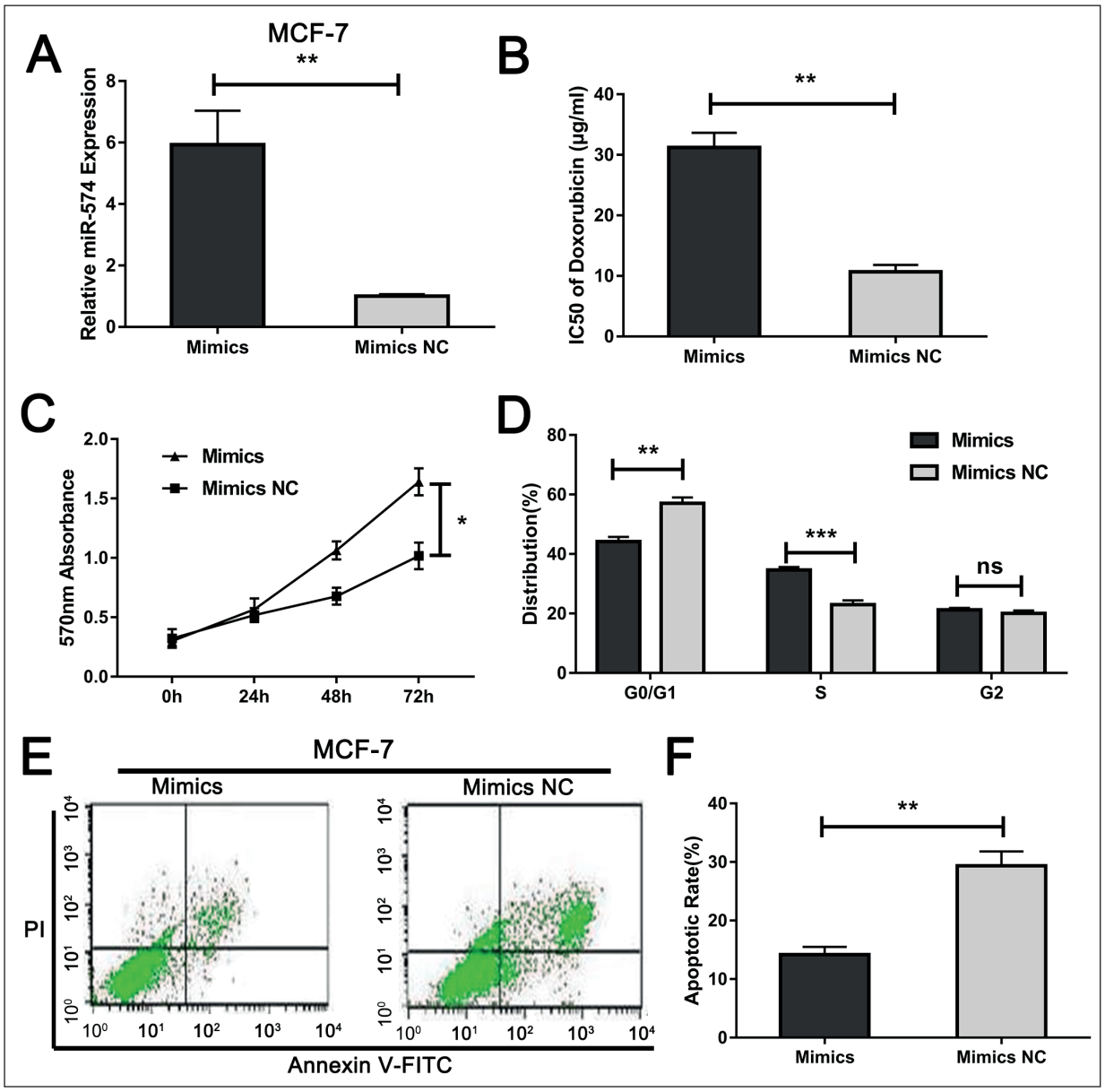

Figure 3. Up-regulation of miR-574 reversed doxorubicin resistance of MCF-7 cells. A- Expression of miR-574 in miR-574 mimics treated MCF-7 cells. B- IC 50 of miR-574 mimics treated MCF-7 compared with mimics NC group. C- MTT assay was performed to determine proliferation of MCF-7 cells treating with miR-574 mimics compared to NC group. D- Cell cycle distribution of miR-574 mimcs treated MCF-7 cells compared with NC group after cultured with 2 μL doxorubicin. E, F- Cell apoptotic rate of miR-574 mimcs treated MCF-7 cells compared with NC group after treated with 2 μLL doxorubicin. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

cells comparing with negative control group. Additionally, cell circle distribution was regulated. The percentage of miR-574 mimics treated MCF-7 cells in G1 phase, increased, whereas the percentage of miR-574 inhibitors transfected MCF-7/Adr cells in G1 phase, decreased after doxorubicin treatment compared with each control group. All these results ectopic miR-574 expression could influence doxorubicin sensitivity in breast cancer cells.

SMAD4, as a tumor suppressor, is a mediator of the transforming growth factor (TGF) β signaling pathway[22,23]. SMAD4 could regulate multidrug resistance in several cancers. In colorectal cancer, loss of SMAD4 induced 5-fluorouracil resistance via Akt pathway and SMAD3/4 effected chemotherapeutic drug sensitivity; in human osteosarcoma, SMAD4 mediated drug resistance by miR-202 via inhibiting apopto-

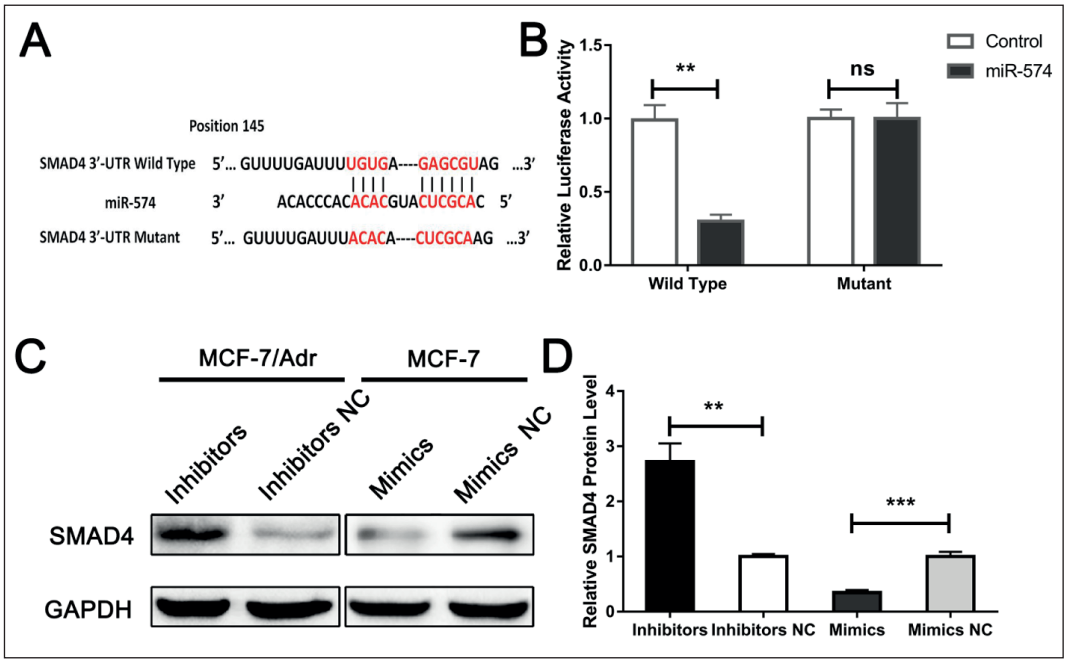

Figure 4. SMAD4 was a direct target of miR-574. A- The predicted binding sites of miR-574 in the 3'-UTR of SMAD4. B- Dual-luciferase reporter assay was used to determine the binding site. A549 cells treated by mimics or control were transfected with pGL3 construct containing the WT or mutant SMAD4 3'-UTR site. C- Levels of SMAD4 and GAPDH protein measured by Western-blot in miR-574 knockdown MCF-7/Adr cells and miR-574 overexpressing MCF-7 cells. D- The relative protein level of SMAD4 and GAPDH. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ns: no significant.

sis and in hepatoma cells, SMAD4 regulated by miR-130a-3p adjusted gemcitabine resistance[24-27]. In breast cancer, SMAD4 co-worked with TGF-β pathway inhibited chemotherapy action against breast cancer[24,28-30]. In our study, SMAD4 was found to be a direct target of miR-574, and miR-574 over-expression significantly inhibited SMAD4 expression level. Based on the results of previous studies and our experiments, we suggested that miR-574 might regulate cell proliferation, apoptosis, cell cycle distribution and drug resistance of breast cancer cells via repressing SMAD4. However, the mechanism of miR-574 functions rendered as a network structure; further studies are still needed to elucidate the pathological function of miR-574.

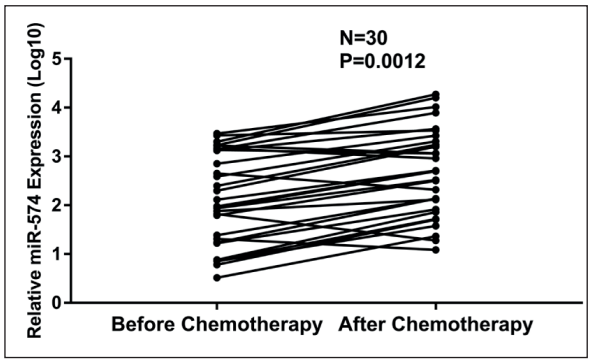

Figure 5. miR-574 expression levels were up-regulated in blood samples from patients with advanced breast cancer receiving Dox based chemotherapy.

Conclusions

For the first time we demonstrated that miR-574 expression increased in doxorubicin-resistant MCF-7/Adr cells compared with MCF-7 cells. Additionally, miR-574 level in blood samples of patients with advanced breast cancer after chemotherapy was up-regulated. Furthermore, experimental assays confirmed up-regulation of miR-574 promoted the doxorubicin resistance of breast cancer cells via repressing SMAD4. All these results suggested miR-574 could act as novel target for improving the curative effect of chemotherapy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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