

miRNA-542-3p downregulation promotes trastuzumab resistance in breast cancer cells via AKT activation

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Abstract. Trastuzumab (Herceptin) has been widely used in breast cancer treatment. However, the majority of cancers that initially respond to trastuzumab begin to progress again within 1 year. Despite the high resistance rate, the molecular mechanisms underlying this disease are not well understood. In the present study, microRNA (miRNA-542-3p) modulated trastuzumab resistance in SKBR3 and MCF7/Her2 breast cancer cell lines. Trastuzumab induced miRNA-542-3p expression in SKBR3 and MCF7/Her2 cells. Furthermore, knockdown of miRNA-542-3p in the two cell lines resulted in decreased drug sensitivity to trastuzumab and cell apoptosis. The blockage of G1/S checkpoint by trastuzumab was rescued as well. miRNA-542-3p knockdown also activated the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, while LY294002 reversed the effect of miRNA-542-3p knockdown. In summary, the results suggested that miRNA-542-3p downregulation may contribute to the trastuzumab resistance in breast cancer via, at least in part, the PI3K-akt pathway. Our findings provide new molecular mechanisms in trastuzumab resistance.

Introduction

Breast cancer is the first most commonly diagnosed invasive malignant disease and the second leading cause of cancer mortality in women. In 2014, the estimated new cases and deaths due to breast cancer in the United States were 232,670 and 40,000, respectively. The incidence of breast cancer accounts for up to 29% of female malignant diseases, while the mortality accounts for up to 15% (1). Breast cancer is

therefore considered a major public health issue worldwide. The proto-oncogene *HER-2* encodes a transmembrane tyrosine kinase receptor with extensive homology to the epidermal growth factor receptor. Overexpression of HER2 has been shown in 20-30% of patients with breast cancer and is associated with metastasis and poor prognosis (2). HER2-positive tumors constitute a group of breast cancers with specific biological features and therapeutic options (3).

The *HER2* gene targeted therapy has been applied in the clinical treatment of breast cancer. Trastuzumab (Herceptin), a humanized monoclonal anti-ERBB2 antibody, is known to significantly improve clinical outcome for early and advanced HER2-positive breast cancer (4). Trastuzumab inhibits HER2 dimerization and growth factor signaling cascades downstream of HER2, including the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway and RAS/RAF/MEK/MAP kinase (MAPK) pathway. In addition, it has been demonstrated that Fc portion of trastuzumab participates in antibody-dependent cellular cytotoxicity (ADCC) function (5-7). Despite its initial efficacy, acquired resistance to trastuzumab develops in the majority of patients with metastatic breast cancer, and a large subset never responds, demonstrating primary resistance (5,8,9).

MicroRNAs (miRNAs) are small non-coding, single-stranded RNAs that regulate crucial biological processes by inhibiting gene expression at a post-transcriptional level. The abnormal expression of miRNAs was observed in a various types of human cancer. miRNAs may function as tumor suppressors or oncogenes, depending on whether they specifically target oncogenes or tumor-suppressor genes (10,11). Recent findings have demonstrated that miRNA-542-3p is associated with tumor progression via c-Src-related oncogenic pathways (12). Furthermore, miRNA-542-3p induces growth arrest and inhibits tumor angiogenesis by targeting angiopoietin-2 (13,14).

In the present study, we detected the function of miRNA-542-3p in breast cancer. The result showed that miRNA-542-3p expression was induced by trastuzumab in SKBR3 and MCF7/Her2 cell lines. Knockdown of miRNA-542-3p impaired trastuzumab-mediated apoptosis and G1/S checkpoint blockage. Furthermore, miRNA-542-3p depletion activated the PI3K-AKT pathway and LY294002 reversed the effect of miRNA-542-3p knockdown. Collectively, our results suggested that miRNA-542-3p is an important regulator of the

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PI3K-AKT pathway and downregulation of miRNA-542-3p contributes to trastuzumab response and resistance.

Materials and methods

Cell culture, antisense miRNA and transfection. SKBR3 and MCF7/HER2 breast cancer cell lines were obtained from the Type Culture Collection of Chinese Academy of Sciences and were maintained in DMEM with 10% FBS in a 5% CO₂-humidified, 95% air incubator. Antisense miRNA-542-3p was purchased from Ambion (Austin, TX, USA). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA).

Chemicals and antibodies. Trastuzumab was obtained from the Tianjin Medical University Cancer Institute and Hospital. LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-cyclin D1, anti-p27^{kip}, anti-AKT, anti-p-AKT, anti-GSK-3 β , anti-p-GSK-3 β , anti-FOXO1a, anti-p-ERK and anti- β -actin antibody were purchased from Cell Signaling Technologies Inc. (Danvers, MA, USA).

Apoptosis and BrdU incorporation. Apoptotic rate and proliferation rate were measured by a PE Annexin V Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA) and a Cell Proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany), respectively. The measurements were performed following the manufacturer's instructions.

Cell cycle analysis. Cells were collected and fixed in 75% ethanol at 4°C overnight. After washing with PBS, the cells were stained with PI/RNase staining buffer (BD Pharmingen) for 10 min at room temperature. The DNA content of cells was measured by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Proportions of cells in G1, S, and G2/M phases were analyzed using ModFit Software (Verity Software House Inc., Topsham, ME, USA).

RNA extraction and RT-qPCR. RNA was extracted using the TRIzol RNA isolation kit (Invitrogen Life Technologies). miRNAs were reverse-transcribed to generate cDNA using stem-loop reverse transcriptase (RT) primers. miRNA expression was calculated relative to the expression of RNU48 (P/N: 4373383, for human) (Applied Biosystems, Foster City, CA, USA). miRNA-specific primers for miRNA-542-3p were obtained from Applied Biosystems (P/N: 4378101).

MTT assay. Cells (8x10³ cells/well) were placed in 96-well plates. At 24 h following treatment, the cells were continually cultured for 24–72 h. At 24, 48 and 72 h, 10 μ l of 0.5 μ g/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) was added to each well. The cells were incubated at 37°C for another 2 h, the medium was removed and the precipitated formazan was dissolved in 100 μ l of DMSO. Following agitation for 20 min, the absorbance was detected at 570 nm on a μ Quant Universal Microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

Western blot analysis. Cell lysates were separated on 8% SDS denatured polyacrylamide gel electrophoresis (PAGE)

gels, transferred to nitrocellulose membranes and blocked in phosphate-buffered saline/Tween-20 containing 5% non-fat milk. The membranes were incubated with antibodies overnight at 4°C. The membranes were then incubated with the HRP-labeled corresponding IgG for 1 h. The protein expression level was assessed by enhanced chemiluminescence and the membranes were exposed to film (Fujifilm, Tokyo, Japan). We also performed western blot analysis to detect the expression of cyclin D1 and p27^{kip}.

Statistical analysis. Experimental results are presented as mean \pm standard deviation (SD). Statistically significant differences between groups were indicated using a two-tailed unpaired Student's t-test. $P < 0.05$ was considered significant.

Results

Trastuzumab induces miRNA-542-3p expression in breast cancer cells. To investigate the potential role of miRNA-542-3p in trastuzumab resistance, we treated the breast SKBR3 cancer cell line with trastuzumab at concentrations of 1, 5 and 10 μ g/ml or vehicle. After 24 h, miRNA-542-3p expression was analyzed by RT-qPCR. The miRNA-542-3p expression was upregulated after trastuzumab treatment and correlated with trastuzumab concentration (Fig. 1A). Furthermore, when we treated the MCF7/HER2 cell line, a MCF7 breast cancer cell line over-expressed with HER2, with trastuzumab, similar results were obtained (Fig. 1B). The results suggested that miRNA-542-3p may be induced by trastuzumab in breast cancer cells and may be important in trastuzumab-mediated antitumor effects.

miRNA-542-3p suppression restores proliferation rate of trastuzumab-treated cells. To investigate the role of miRNA-542-3p in trastuzumab treatment, we suppressed miRNA-542-3p expression using miRNA-542-3p antisense oligonucleotides (ASO-miR). ASO-NC was used as the control. SKBR3 and MCF7/HER2 cells were transfected with ASO-miR and ASO-NC. Then miRNA-542-3p expression was analyzed using RT-qPCR. As shown in Fig. 2A, miRNA-542-3p expression was inhibited efficiently. After miRNA-542-3p knockdown, trastuzumab was added. Compared with the control, miRNA-542-3p depletion rescued trastuzumab-induced proliferation suppression (Fig. 2B). To confirm these findings, we performed BrdU incorporation assay. miRNA-542-3p depletion increased BrdU incorporation rate of SKBR3 and MCF7/HER2 cells following trastuzumab treatment (Fig. 2C). Taken together, these results indicated that miRNA-542-3p may participate in trastuzumab-induced tumor growth suppression and downregulation of miRNA-542-3p may be a cause of trastuzumab resistance.

miRNA-542-3p suppression rescues trastuzumab-mediated cell cycle arrest. Cells treated with trastuzumab undergo arrest during the G1 phase of the cell cycle, with a concomitant reduction in proliferation (9). To investigate whether miRNA-542-3p restored breast cancer cells proliferation by rescuing cell cycle arrest, we determined alteration of cell cycle profile. SKBR3 and MCF7/HER2 cells exhibited G1/S checkpoint arrest following treatment with trastuzumab. However, when miRNA-542-3p was depleted, trastuzumab-induced G1 arrest was rescued (Fig. 3A and B).

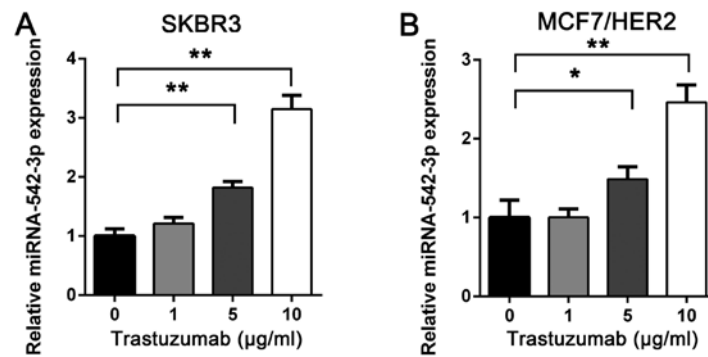


Figure 1. Trastuzumab induces miRNA-542-3p expression in (A) breast cancer cells SKBR3 and (B) MCF7/HER2 cells were treated with different concentrations of trastuzumab (72 h) as indicated ($\mu\text{g/ml}$). The miRNA-542-3p expression was analyzed by RT-qPCR. Columns, mean of six determinations; bars, SD. * $P < 0.05$, ** $P < 0.01$.

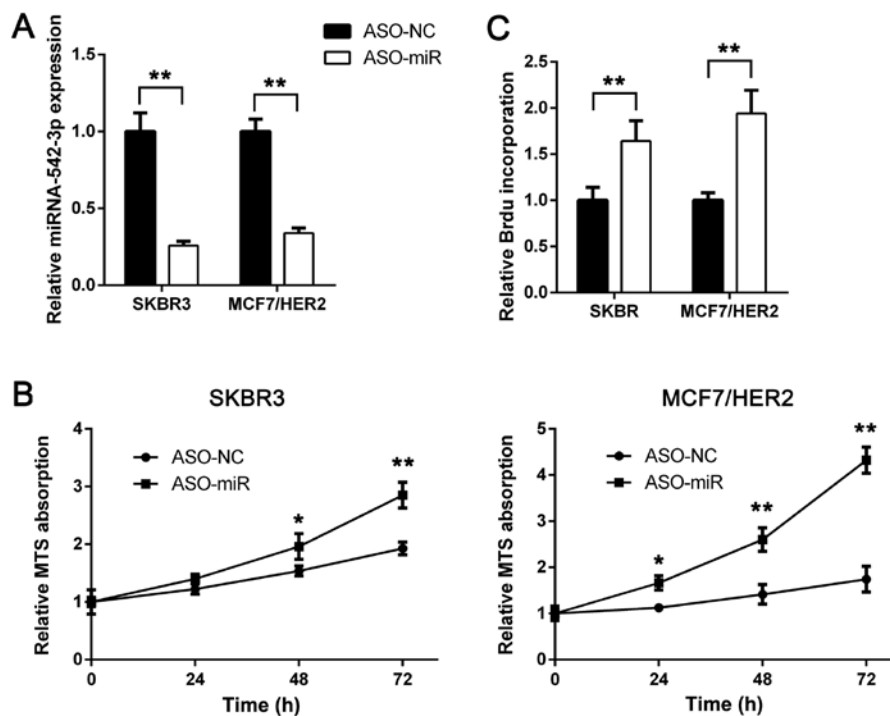


Figure 2. (A) miRNA-542-3p suppression restores the proliferation rate of trastuzumab-treated cells. SKBR3 and MCF7/HER2 cells were transfected with miRNA-542-3p antisense oligonucleotides (ASO-miR) or negative control antisense oligonucleotides (ASO-NC). After 48 h, miRNA-542-3p expression was analyzed by RT-qPCR. Columns, mean of six determinations; bars, SD. (B) SKBR3 and MCF7/HER2 cells were transfected with miRNA-542-3p ASO-miR or ASO-NC. After 48 h, cells were treated with trastuzumab (10 $\mu\text{g/ml}$) and proliferation curve was measured by MTT assay. (C) Cell proliferation rate was also determined by BrdU incorporation assay. Columns, mean of six determinations; bars, SD. * $P < 0.05$, ** $P < 0.01$.

We also performed western blot analysis to detect the expression of cyclin D1 and p27^{kip}. Trastuzumab may reduce the expression of cyclin D1 and upregulate cyclin-dependent kinase (cdk) inhibitor p27^{kip} (9). As expected, trastuzumab down-regulated cyclin D1 expression and induced p27^{kip} expression in SKBR3 and MCF7/HER2 cells. However, when miRNA-542-3p was suppressed, the expression of cyclin D1 and p27^{kip} was restored (Fig. 3C). Taken together, these results indicated that miRNA-542-3p is important in trastuzumab-induced G1 arrest.

miRNA-542-3p suppression impairs trastuzumab enhancement on taxol-induced apoptosis. Trastuzumab pretreatment increases taxol-induced apoptosis (15,16). We further investigated whether miRNA-542-3p participates in breast

cancer cell survival. SKBR3 and MCF7/HER2 cells were or were not treated with taxol plus trastuzumab. The two cell lines were more vulnerable to taxol when pretreated with trastuzumab compared with treatment with taxol alone. When miRNA-542-3p expression was suppressed, SKBR3 and MCF7/HER2 cells exhibited resistance to trastuzumab-induced apoptosis enhancement (Fig. 4). Taken together, these results indicated that miRNA-542-3p depletion promotes breast cancer cell survival.

miRNA-542-3p suppression activates AKT pathway. HER-2 activates multiple cell signaling pathways, including the PI3K and MAPK cascades. Trastuzumab reduces signaling from these pathways, promoting cell cycle arrest and apoptosis (6).

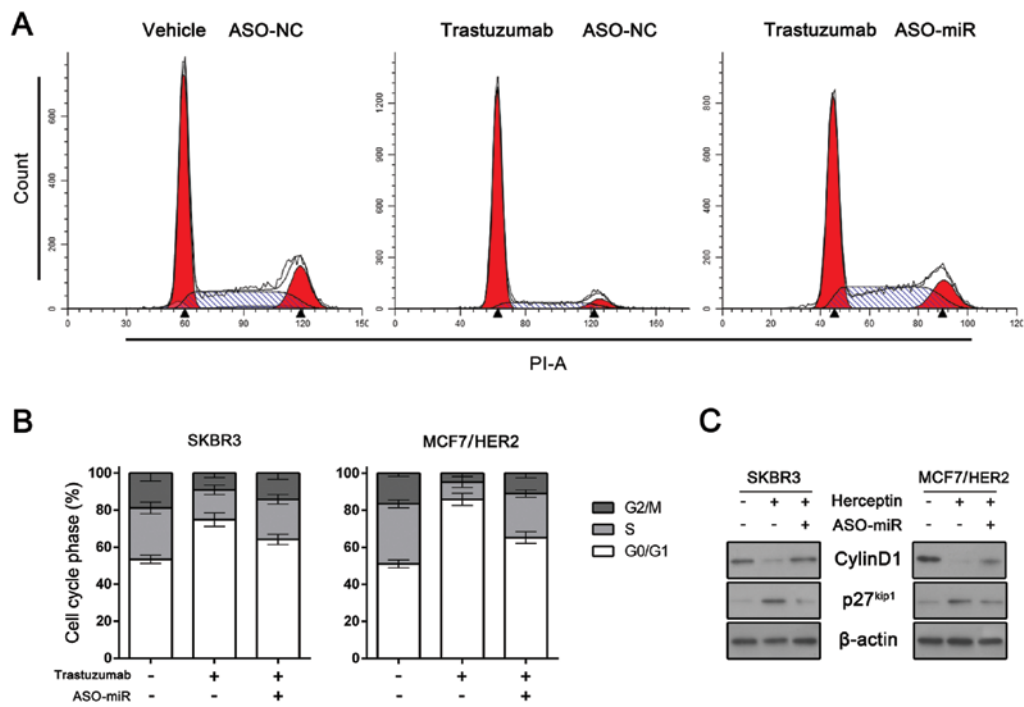


Figure 3. miRNA-542-3p suppression rescues trastuzumab-mediated cell cycle arrest SKBR3 and MCF7/HER2 cells were transfected with miRNA-542-3p antisense oligonucleotides (ASO-miR) or negative control antisense oligonucleotides (ASO-NC). After 48 h, the cells were treated with trastuzumab (10 μ g/ml). After another 24 h, cell cycle analysis was performed. (A) Representative cell cycle distribution of SKBR3 cells in cell cycle analysis. (B) Cell cycle distribution of SKBR3 and MCF7/HER2 cells. Columns, mean of 6 determinations; bars, SD. (C) SKBR3 and MCF7/HER2 cells were transfected with miRNA-542-3p ASO-miR or ASO-NC. After 48 h, cyclin D1 and p27^{kip} expression was measured by western blot assay.

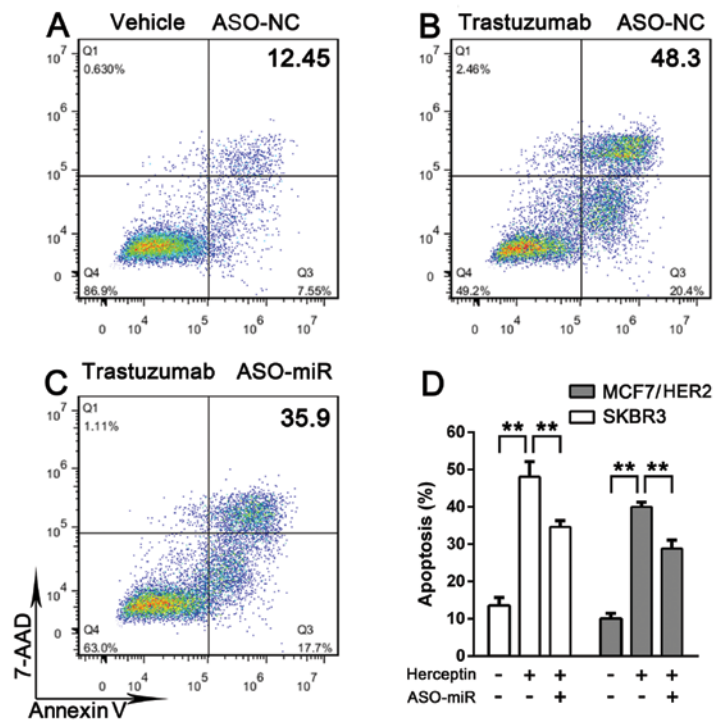


Figure 4. miRNA-542-3p suppression impairs trastuzumab enhancement on taxol-induced apoptosis SKBR3 and MCF7/HER2 cells were transfected with miRNA-542-3p antisense oligonucleotides (ASO-miR) or negative control antisense oligonucleotides (ASO-NC). After 48 h, cells were or were not treated with taxol (10 nM) in the presence of trastuzumab (10 μ g/ml). After another 24 h, the apoptotic rate was analyzed. (A-C) Representative apoptosis analysis of SKBR3 cells. (D) Apoptotic rate of SKBR3 and MCF7/HER2 cells. Columns, mean of six determinations; bars, SD. * P <0.05, ** P <0.01.

Since miRNA-542-3p knockdown may reduce the tumor suppressive effect of trastuzumab, we hypothesized that miRNA-542-3p may regulate the PI3K or MAPK pathway. AKT is the core transducer and regulator of PI3K pathway (17).

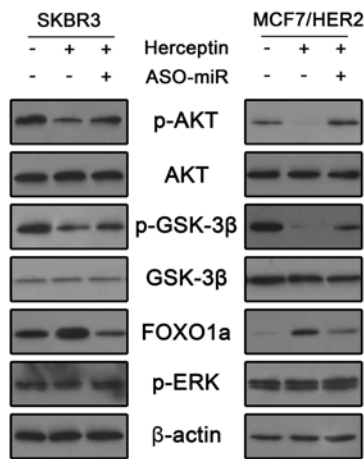


Figure 5. miRNA-542-3p suppression activates AKT pathway. SKBR3 and MCF7/HER2 cells were transfected with miRNA-542-3p antisense oligonucleotides (ASO-miR) or negative control antisense oligonucleotides (ASO-NC). After 48 h, the cells were or were not treated with trastuzumab (10 μ g/ml). After another 24 h, western blot analysis was performed to determine the expression of the indicated proteins.

Western blot analysis showed that although total AKT expression was not affected, miRNA-542-3p suppression upregulated phospho-AKT, an active form of the protein (Fig. 5). GSK-3 β is a downstream effector of AKT that is involved in cell cycle regulation. Compared with transfected ASO-NC, SKBR3 and MCF7/HER2 cells, ASO-miR expressed a more active form than GSK-3 β , and phospho-GSK-3 β (Fig. 5).

FOXO transcription factors are major substrates of AKT kinase and have been suggested as a tumor suppressor (18). FOXO1a has been known to participate in trastuzumab resistance through p27^{kip} and cyclin D1 regulation (19). Since miRNA-542-3p suppression regulates p27^{kip} and cyclin D1, we determined whether it also regulates FOXO1a. Western blot analysis showed that trastuzumab increased FOXO1a expression in SKBR3 and MCF7/HER2 cells. However, after miRNA-542-3p knockdown, FOXO1a was obviously down-regulated (Fig. 5).

We also examined whether miRNA-542-3p affects ERK pathway activation. Although trastuzumab inhibited ERK phosphorylation in the SKBR3 and MCF7/HER2 cell lines, miRNA-542-3p knockdown had no impact on ERK phosphorylation (Fig. 5). Taken together, these results showed that miRNA-542-3p is an essential PI3K-AKT pathway regulator in breast cancer.

PI3K-AKT pathway activation is required for miRNA-542-3p suppression-induced trastuzumab resistance. Since trastuzumab mediated PI3K-AKT, inhibition is important in its antitumor effect (6,9). We hypothesized that miRNA-542-3p suppression-induced trastuzumab resistance is PI3K-AKT pathway-dependent. To determine this possibility, we treated miRNA-542-3p knockdown cells with PI3K inhibitor, LY294002. Phospho-AKT downregulation was confirmed by western blot analysis (Fig. 6A). Cell proliferation was examined using BrdU incorporation assay. As expected, PI3K inhibition suppressed miRNA-542-3p-mediated BrdU incorporation in SKBR3 and MCF7/HER2 cells (Fig. 6D). Furthermore, the cell cycle arrest was examined. Compared with miRNA-542-3p knockdown

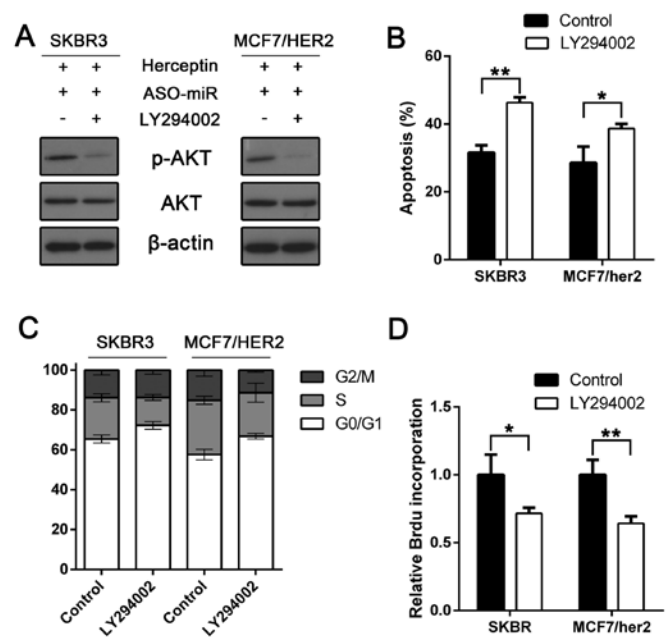


Figure 6. PI3K-AKT pathway activation is required for miRNA-542-3p suppression-induced trastuzumab resistance. SKBR3 and MCF7/HER2 cells were transfected with miRNA-542-3p antisense oligonucleotides (ASO-miR). After 48 h, the cells were or were not treated with trastuzumab (10 μ g/ml) in the presence of LY294002 (10 μ M). After another 24 h, (A) AKT expression, (B) apoptosis rate, (C) cell cycle phase and (D) BrdU incorporation rate were analyzed. Columns, mean of six determinations; bars, SD. * P <0.05, ** P <0.01. PI3K, phosphatidylinositol 3-kinase.

alone, the cell cycle arrest in SKBR3 and MCF7/HER2 cells was restored after LY294002 treatment (Fig. 6C). We also assessed LY294002 impact on miRNA-542-3p depletion-mediated apoptosis resistance. In agreement with the above results, LY294002 restored trastuzumab enhancement on taxol-induced apoptosis (Fig. 6B). Taken together, these results indicated that miRNA-542-3p suppression-induced trastuzumab resistance is, at least in part, PI3K-dependent.

Discussion

Trastuzumab, a humanized anti-HER2 monoclonal IgG1 antibody, gained FDA approval in September 1998 for the treatment of HER2-overexpressing breast cancer in adjuvant and metastatic settings. However, the clinical benefit from trastuzumab therapy may be limited due to trastuzumab resistance. The most intensively studied general mechanisms of trastuzumab resistance are: i) obstacles for trastuzumab binding to HER2; ii) upregulation of HER2 downstream signaling pathways; iii) signaling through alternate pathways; and iv) failure to trigger immune-mediated mechanisms to destroy tumor cells (5).

The results describe a new mechanism by which breast cancer acquires trastuzumab resistance. We found that miRNA-542-3p was upregulated in breast cancer cell lines when treated with trastuzumab. Of note, when miRNA-542-3p was silenced breast cancer cells showed resistance to trastuzumab. Trastuzumab-induced proliferation and cell cycle arrest were rescued. The apoptotic rate was also down-regulated when miRNA-542-3p was silenced. Furthermore, miRNA-542-3p silencing activated PI3K-AKT pathway in breast cancer cells. The inhibition of this pathway restored

trastuzumab sensitivity. Taken together, these findings demonstrate that miRNA-542-3p plays an important role in trastuzumab anticancer function.

Recent studies focused on PTEN or PI3K mutation-mediated trastuzumab resistance (20-22). However, few studies have examined the role of miRNA in trastuzumab resistance. In the present study, we found that trastuzumab induced miRNA-542-3p expression. Suppression of miRNA-542-3p in breast cancer cell lines caused trastuzumab resistance. These results confirm the existence of miRNA regulation of trastuzumab resistance.

FOXO transcription factors belong to the forkhead family of transcription factors which are characterized by a distinct forkhead domain. Emerging evidence suggests that FOXO factors play a tumor suppressor role in various types of cancer and are coupled with lifespan extension (18,23). Recent evidence revealed that the FOXO protein family members, FOXO1 and FOXO3 promote autophagy (24,25). Furthermore, it has been suggested that FOXO factors participate in trastuzumab resistance (19,26). In the present study, we confirmed the importance of the role of FOXO1a in trastuzumab antitumor effects. We also found that miRNA-542-3p controlled FOXO1a expression. These results suggest that there may exist a microRNA-dependent mechanism that regulates FOXO1a expression.

The PI3K pathway regulates various cell processes, such as proliferation, growth, apoptosis and cytoskeletal rearrangement (17). There are ample genetic and laboratory studies that suggest the PI3K-AKT pathway is vital to the growth and survival of cancer cells (27). In the present study, we confirmed AKT activation in trastuzumab resistance. We also found that miRNA-542-3p is a negative PI3K-AKT pathway regulator in breast cancer. Furthermore, in the present study, we applied LY294002, an AKT inhibitor, to treat trastuzumab-resistant cancer cells. The results showed marked antitumor effects suggesting targeting of PI3K-AKT pathway. Several therapies target the PI3K-AKT pathway in clinical development for the treatment of cancer. These include dual PI3K-mTOR, PI3K, AKT and mTOR complex catalytic site inhibitors (27,28). However, whether these PI3K-AKT pathway inhibitors also have similar effects remains to be investigated.

In summary, we found a new trastuzumab resistance regulatory mechanism in breast cancer. miRNA-542-3p acts as an AKT-negative regulator to maintain breast cancer cells sensitivity to trastuzumab. However, the exact miRNA-542-3p targets and its application in tumor therapy remain to be investigated.

Acknowledgements

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