MiR-346 promotes the biological function of breast cancer cells by targeting SRCIN1 and reduces chemosensitivity to docetaxel

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A B S T R A C T
MicroRNAs (miRNAs) are a class of highly conserved small noncoding RNAs that play pivotal roles at the post-trancriptional level in the biological function of various cancers, including breast cancer. In our study, miR-346 mimir, inhibitor, negative control or si-SRCIN1 were transfected into MCF-7 and MCF-7/Doc cells, respectively. Quantitative real time PCR (qRT-PCR) was used to measure miR-346 and SRCIN1 mRNA expressions and western blot was used to detect the expression of SRCIN1 in protein level. CCK-8 and colony formation were employed to verify cell viability and proliferation. Flow cytometry showed the apoptosis. Transwell was performed to detect migration and invasion. The luciferase reporter assay data showed the target correlation of miR-346 and SRCIN1. Firstly, we found that the expression of miR-346 was higher in breast cancer tissues than in their paired corresponding non-cancerous tissues and there was significant inverse correlation between miR-346 and SRCIN1. Overexpression of miR-346 promoted cell proliferation, colony formation, migration and invasion, and reduced apoptosis, sensitivity to Docetaxel (Doc). SRCIN1 was identified as a direct target of miR-346, whose silencing promoted cell proliferation and the IC50 of Doc. Moreover, SRCIN1 silencing reduced the effect of miR-346 down-expression. Taken together, miR-346 may function as an oncogenic miRNA and mediate chemosensitivity to docetaxel through targeting SRCIN1 in breast cancer, targeted modulation of miR-346 expression may became a potential strategy for the treatment.

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1. Introduction

Breast cancer is the most common malignancy, accounting for nearly one-third of all cancers, and is the second leading cause of cancer death after lung cancer in women (DeSantis et al., 2014). Although the significant treatment modalities have developed and mortality rate has a significant reduction, the mechanisms of carcinogenesis and metastasis still remained unknown (Liu et al., 2016; Spanheimer et al., 2013). Docetaxel (Doc), a vital chemotherapeutic agent, is an indispensable treatment of the standard treatment in breast cancer. Furthermore, Docetaxel resistance is a major reason for the failure of breast cancer treatment, and the mechanisms of drug-resistance also still remained puzzled. Recently, it was discovered that miRNAs play a key role in breast cancer biological processes, including carcinogenesis, metastasis and drug-resistance. For example, miR-139-5p inhibited the biological function and enhanced the Docetaxel resistance by targeting Notch1 in breast cancer (Zhang et al., 2015a). Wang et al. found that miR-222 was associated with Adr-resistance in breast cancer through suppression of MDR1 (Wang et al., 2014). Zhang et al. showed that miR-411-5p inhibited breast cancer cell proliferation and metastasis by targeting GRB2 (Zhang et al., 2016).

MicroRNAs (miRNAs), known as short, highly conserved small non-coding RNA molecules, down-regulate gene expression by binding to their 3′-untranslation regions (3′UTRs) at the post-transcriptional level and played significant roles in various biological processes (Krol et al., 2010). Previous studies had shown that miR-346 promoted the migration and invasion of HeLa cells by regulating Argonaute 2.
night, then the number of colonies was counted. However, the functional role of miR-346 in breast cancer was still unknown. In this study, we primarily disclosure that change the expression of miR-346 in breast cancer cell affects their resistance to Doc through inhibiting SRCIN1.

2. Materials and methods

2.1. Cell culture and clinical tissues

Human breast cancer cell line MCF-7 and human breast epithelial cell line HBL-100 were purchased from ATCC (Rockville, MD, USA). By gradually increasing the concentration of docetaxel, we successfully established the drug resistant subline, MCF-7/Doc, in our laboratory. All cell lines were cultured with RPMI-1640 (KEYGEN, Nanjing, China) containing 10% fetal bovine serum (Applied Biosystems, Gibco, USA) in a humidified atmosphere containing 5% CO2 at 37 °C. Breast cancer tissues and paired corresponding non-cancerous tissues were derived from patients (n = 24) diagnosed at the Jiangsu Cancer Hospital, Affiliated Hospital of Nanjing Medical University, from 2012 to 2015. All of the patients were given written informed consent and the study was approved by the Ethics Committee of the Jiangsu Cancer Hospital.

2.2. Cell transfection

MCF-7 and MCF-7/Doc cells were plated in 6-well plates (2.5 × 10^5 cell/well) for 24 h, then transfected with human hsa-miR-346 mimic, hsa-miR-346 inhibitor, hsa-miR-346 negative control, small inhibitory RNA (siRNA) against human-SRCIN1 (si-SRCIN1) and NC-siRNA (RIBOBIO, Guangzhou, China) using Lipofectamine (Applied Biosystems, Lifetechnologies, USA). 12 h–48 h after transfected with miRNA or siRNA, cells were harvested for the next experiment.

2.3. RNA isolation and real-time quantitative PCR

We used Trizol reagent (Applied Biosystems, Invitrogen, USA) to extract total RNA from tissues and cells. The concentration and quality of total RNA were detected by spectrophotometric and electrophoretic assay. First-strand cDNA was synthesized using the SuperScript III first-strand synthesis system (Applied Biosystems, Invitrogen, USA). Real-time PCR was carried out in the ABI 7300 real-time PCR machine (Applied Biosystems, USA) using the SYBR Select Master Mix (Applied Biosystems, Lifetechnologies, USA). After normalization with reference to expression of U6 or β-actin, the relative expression levels of miR-346 and SRCIN1 were calculated by the 2^-ΔΔCt method.

2.4. Cell proliferation and colony formation assay

Cell proliferation was detected by the Cell Counting Kit-8 (CCK-8). 24 h after the transfection with miRNA, MCF-7 Cells (2.0 × 10^3) were plated into 96-well plates and cultured for 1, 2, 3 or 4 days, in addition, MCF-7 Cells (5.0 × 10^3) and MCF-7/Doc (5.0 × 10^3) were plated into 96-well plates and incubated for 48 h with various concentrations of DOC, then discarded the old culture media, 10 μl CCK-8 (Dojindo, Japan) in 100 μl culture medium was added to each well and incubated for a further 1 h at 37 °C. The absorbance was measured at a wavelength of 450 nm. For the colony formation assay, 200 cells per well were seeded into a six-well plate after transfection, 200 cells per well were seeded into a six-well plate. Approximately 10 days later, the colonies were fixed in 4% methanol 30 min and stained with 1% crystal violet overnight, then the number of colonies was counted.

2.5. Cell apoptosis assay

MCF-7 and MCF-7/Doc cells were incubated with 10 ng/ml Doc or 100 ng/ml Doc for 48 h respectively after transfected for 48 h. Then, we used trypsin to digest cells, rinsed cell pellets twice with ice-cold PBS and collected cells by centrifugation at 1000 rpm for 5 min. Apoptosis was determined by dual staining with Annexin V-FITC apoptosis Detection Kit I (KEYGEN, Nanjing, China) according to the manufacturer’s protocol.

2.6. Cell migration and invasion assay

The cell migration and invasion ability was tested by 6.5 mm diameter tissue culture inserts with an 8.0 μm pore size Transwell (Corning, USA), placed into the wells of 24-well culture plates, with or without Matrigel Basement Membrane Matrix (Corning, USA). After transfection, cells were cultured and starved with serum-free RPMI 1640 in 6-well plates for 24 h. Then, transfected cells (2 × 10^4) in 200 ml serum-free RPMI 1640 were placed into the upper chamber, at the same time, 500 ml RPMI 1640 with 20% FBS was added to the lower chamber. After incubated in a humidified atmosphere for 24 h, cells that migrated or invaded through the Transwell membrane were fixed with 4% methanol for 30 min, washed 3 times with distilled water and stained with 1% crystal violet overnight at room temperature. Then, gently scraped off the cells remained on the upper surface of the Transwell membrane with a cotton swab and four random fields were counted under a microscope (100 magnification).

2.7. MiRNA target gene identification

MiRWalk2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2), including miRWalk, miRanda, RNA22 and Targetscan databases, was used to predict the target genes of miRNA.

2.8. Dual luciferase activity assay

To detect the binding specificity, we constructed the wild-type and mutant seed region of SRCIN1 3′UTR, which contained the putative target site for the mature miR-346, and cloned them into the pGL3-control vector. MCF-7 cells were co-transfected with miR-346 mimic, inhibitor or negative control, pGL3 -SRCIN1 3′UTR-WT vector, pGL3-SRCIN1 3′UTR-MT vector and phRL-SV40 control vector (Promega, USA) using Lipofectamine (Applied Biosystems, Lifetechnologies, USA) in 24-well plates. After transfected for 24 h, the relative luciferase activity was detected by the Dual-Luciferase Reporter Assay System (Promega, USA) and normalized with renilla luciferase activity using SpectraMax i3 (Molecular Devices, USA).

2.9. Western blot

Total protein from cells was extracted by RIPA buffer (Beyotime, China) and Equalized by NuPAGE LDS Sample Buffer (4×) (Applied Biosystems, Lifetechnologies, USA) and NuPAGE Reducing Agent (10×) (Applied Biosystems, Lifetechnologies, USA). Protein samples were separated by 5% MOPS SDS Running Buffer (20×) (Applied Biosystems, Lifetechnologies, USA) with NuPAGE Bis-Tris Mini Gels (Applied Biosystems, Lifetechnologies, USA) before transferred to PVDF membranes (Sigma, Germany). After washed with TBST and blocked with western blocking buffer (Beyotime, China), transfected membranes were incubated with primary antibodies against human SRCIN1 (1:1000, Cell Signaling Technology, America) or β-actin (1:1000, Proteintech, America) overnight at 4 °C, then washed with TBST again and incubated with the Goat anti-Rabbit IgG peroxidase-conjugated secondary antibody (1:2000, Fmncas, Nanjing China). The protein band was detected by Chemiluminescence with BeyoECL Pus (Beyotime, China) using ChemiDoc XRS + Imaging System (Bio-Rad, America).
2.10. Statistical analysis

Experiments were shown from three independent experiments in triplicate and representative of all the data. Using SPSS 19.0 by t-test or one-way ANOVA and Spearman rank test for Statistical analysis. The data are expressed as the mean ± SD, and \( P < 0.05 \) was considered significant.

3. Results

3.1. miR-346 was up-regulated in breast cancer tissues and cell lines

We first determined the expression patterns of miR-346 in breast cancer tissues, and miR-346 expression was significantly higher in breast cancer tissues than in their paired corresponding non-cancerous tissues.
(Fig. 1A). Then, we examined the expression level of miR-346 in breast cancer cell lines (MCF-7 and MCF-7/Doc) and the breast epithelial cells HBL-100. miR-346 expression was highest in MCF-7/Doc cells, followed by the MCF-7 cells, and finally the HBL-100 cells (Fig. 1B).

3.2. miR-346 promoted cell proliferation, colony formation and inhibited apoptosis

After MCF-7 cells were transfected with miR-346 mimic, miR-346 inhibitor, or miR-346 negative control for 48 h, respectively. qRT-PCR was used to determine the expression level of miR-346 (Fig. 2A). Then a CCK-8 assay was performed, miR-346 mimic showed promoted proliferation, in contrast, miR-346 inhibitor had the opposite effect on cell proliferation (Fig. 2B). Moreover, miR-346 mimic cells exhibited more colonies compared with controls and miR-346 inhibitor had the opposite effect (Fig. 2C). Then we detected the rate of cellular apoptosis by flow cytometry analysis to determine the connection between miR-346 and apoptosis, compared with the negative controls, miR-346 mimic inhibited apoptosis and miR-346 inhibitor had the opposite effect (Fig. 2D).

3.3. miR-346 promoted cell migration and invasion

We next focused on whether miR-346 could mediate the metastasis and invasive ability of breast cancer cells. After transfection and starvation, we seeded cells into Transwell chamber and incubated for 24 h. We found that miR-346 mimic promoted the MCF-7 cell migration compared to the negative control, moreover, upregulation of miR-346 promoted the MCF-7 cell invasion (Fig. 3A and B).

3.4. miR-346 reduced chemosensitivity to docetaxel of breast cancer cells

As shown in Fig. 1B, the expression level of miR-346 significantly upregulated in MCF-7/Doc, compared with its parental MCF-7 cell line. To further determined the effect of miR-346 expression in chemosensitivity to docetaxel, miR-346 mimic, miR-346 inhibitor or miR-346 negative control were also successfully transfected into MCF-7/Doc cells (Fig. 4A). Detected by cck-8 assay, miR-346 mimic reduced chemosensitivity, the half maximal inhibitory concentration (IC50) of Doc in MCF-7 and MCF-7/Doc cells, and miR-346 inhibitor had the opposite effect (Fig. 4B, C and D). Compared with the negative controls, miR-346 mimic reduced docetaxel-induced apoptosis of MCF-7, furthermore miR-346 inhibitor promoted docetaxel-induced apoptosis of MCF-7/Doc (Fig. 4E and F).

3.5. SRCIN1 was inversed with the expression of miR-346 in breast cancer, as a direct target

To further explore the mechanism of miR-139-5p in breast cancer cells, miRWalk2.0 was used to identify potential target genes. The predicted interaction between miR-346 and the target sites in the SRCIN1
3'UTR was illustrated in Fig. 5A. The SRCIN1 expression levels were significantly down-regulated in breast cancer compared to the adjacent normal tissues (Fig. 5B), also in breast cancer cells (Fig. 5C). Moreover, by linear regression analysis, we found that there was a significant inverse correlation between miR-346 and SRCIN1 (Fig. 5D). Additionally, in breast cancer cells, miR-346 mimic significantly reduced the expression of SRCIN1 mRNA and protein, and miR-346 inhibitor had the opposite effect (Fig. 5E and F). To further investigate whether miR-346 directly and negatively regulates SRCIN1 expression, we constructed luciferase reporter plasmid that contained wild-type (WT) and mutant (MT) miR-346 target sequences of the SRCIN1-3'UTR. After co-transfection experiments in breast cancer cells, dual-luciferase reporter assays showed that miR-346 mimic inhibited the luciferase activity of the WT reporter plasmid, but not the MT reporter plasmid (Fig. 5G).

3.6. SRCIN1 knock-down reduced the effect of miR-346 down-expression in breast cancer cell

After si-SRCIN1 and miR-346 inhibitor were successfully transfected into MCF-7 and MCF-7/Doc cells, miR-346 inhibitor didn’t significantly promoted the expression of SRCIN1 mRNA and protein in co-transfected MCF-7 and MCF-7/Doc cells (Fig. 6A and B), si-SRCIN1 significantly promoted breast cancer cell proliferation, miR-346 inhibitor had the opposite effect, however, in the cells co-transfected with miR-346 inhibitor and the si-SRCIN1, miR-346 inhibitor didn’t have significantly effect in cell proliferation (Fig. 6C). Similarly, si-SRCIN1 significantly promoted the IC50 of Doc of breast cancer cells, but, si-SRCIN1 didn’t obviously promoted the IC50 of Doc in co-transfected MCF-7 and MCF-7/Doc cells compared with cells transfected with si-SRCIN1 only (Fig. 6D).

**Fig. 4.** miR-346 reduced chemosensitivity to docetaxel in breast cancer cells. (A) The transfection efficiency of miR-346 was detected by qRT-PCR. (B) and (C) The percent survival of MCF-7 and MCF-7/Doc with different concentrations of Doc was detected by cck-8 assay. (D) The connection between miR-346 and IC50 of Doc in MCF-7 and MCF-7/Doc cells. (E) Flow cytometry analysis was used to detect the connection between miR-346 and cell Doc-induced apoptosis in MCF-7 and MCF-7/Doc cells. (F) The Doc-induced apoptosis rate of Flow cytometry analysis. *P < 0.05; **P < 0.01; *** P < 0.001.
Fig. 5. SRCIN1 was inversely correlated with the expression of miR-346 and inversely correlated with the expression in breast cancer, as a direct target. (A) Predicted SRCIN1 3′-UTR binding site for miR-346. (B) The relative expression of SRCIN1 mRNA in breast cancer tissues compared with their paired corresponding non-cancerous tissues was detected by qRT-PCR. (C) The relative expression of SRCIN1 mRNA in breast cancer cells was detected by qRT-PCR. (D) The inverse correlation between miR-346 and SRCIN1 in breast cancer tissues was calculated by linear regression analysis (Spearman rank test, \(r = -0.5696; P = 0.0037\), two-tailed). (E) The relative expression of SRCIN1 mRNA in transfected MCF-7 cells was detected by qRT-PCR. (F) The protein levels of SRCIN1 in transfected MCF-7 cells were detected by Western blot. (G) The relative luciferase activity of the reporter gene in transfected MCF-7 cells. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 6. SRCIN1 knock-down reduced the effect of miR-346 down-expression in breast cancer cell. (A) The relative expression of SRCIN1 mRNA in transfected MCF-7 and MCF-7/Doc cells was detected by qRT-PCR. (B) The protein levels of SRCIN1 in transfected cells were detected by Western blot. (C) The cell proliferation of MCF-7 cells transfected with siRNA and inhibitor was detected by CCK-8 assay. (D) The IC50 of Doc in transfected MCF-7 and MCF-7/Doc cells. *P < 0.05; **P < 0.01; ***P < 0.001.
4. Discussion

To date, release 21 of the miRBase database contains 28,645 entries representing hairpin precursor miRNAs in 223 species, 2588 mature miRNA sequences have been identified in humans (http://www.mirbase.org/). Importantly, miRNAs played a crucial regulatory role in the control of maintaining the balance of gene regulating networks, and their regulatory role was essential, affecting various biological phenomena including signaling activation, proliferation, differentiation, apoptosis, angiogenesis, as well as invasion and metastasis (Chen et al., 2013). In addition, alterations of miRNAs were involved in many diseases such as neurological disorders, various types of cancer, cardiovascular diseases, and autoimmune diseases (Zhang et al., 2015b; Seok et al., 2016). It is worth mentioning that previous studies suggested that miR-346 played an important role in several pathological processes including Graves’ disease (Chen et al., 2015), rheumatoid arthritis (Semaan et al., 2011), inflammatory response (Alsaleh et al., 2009; Bartoszewski et al., 2011), and metabolic processes (Tsai et al., 2009).

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In vivo, we found that miR-346 expression was significantly higher in breast cancer tissues than in their paired corresponding non-cancerous tissues. In vitro, we also showed that miR-346 expression was higher in MCF-7 and MCF-7/Doc cells than in HBL-100 cells, and miR-346 promoted the function of breast cancer cells. These results were the same as listed above and implied that miR-346 acted as an oncopogene in the pathogenesis of breast cancer. However, dependent on the cell and tumor types, miRNAs may exert different functions (Cao et al., 2014). In malignant gliomas, miR-346 was down-regulated and post-transcriptionally up-regulated expressions of glioma-relevant genes, like TERT and SEMA6A (Wolter et al., 2016). There were other kinds of cell lines and cancer that we needed to verify.

SRCIN1, also known as p140 Cas-associated protein (p140CAP), mainly expressed in epithelial-rich tissues like mammary glands, lungs, colon and kidneys, contained two amino acids, two proline-rich regions, and two coiled-coil domains, played a crucial role by inactivating SRC in tumor (Di Stefano et al., 2004; Cabodi et al., 2010; Repetto et al., 2013). Di et al. showed that SRCIN1 overexpression inhibited cell spreading, migration and invasion by regulating Csk and Src kinase activity in breast cancer. Src is an inactive state under physiological conditions, the dephosphorylation of Tyr527 in the carboxy-terminal significantly upregulates the Src activity by changing its conformation. However, SRCIN1 controlled this process tightly by activating Csk kinase, which phosphorylated Tyr527 and prevented Src activity (Di Stefano et al., 2007). Screening of breast cancers showed an inverse correlation between the expression of SRCIN1 and malignancy (Damiano et al., 2010). In our study, we also determined SRCIN1 as a tumor suppressor. Recently, SRCIN1 was found to be regulated by several miRNAs. Cao et al. disclosed that the repression of SRCIN1 in lung cancer by miR-150 triggered the activation of the Src/FAK and Src/Ras/ERK pathway and eventually promoted the proliferation and migration (Cao et al., 2014). The overexpression of miR-211 enhanced NSCLC cell proliferation, colony formation, and invasion by regulating SRCIN1 (Ye et al., 2016). Moreover, miR-346, miR-374a and miR-873 were demonstrated as an oncogene by inhibiting SRCIN1 expression in non-small cell lung cancer, gastric cancer and lung adenocarcinoma respectively (Chen et al., 2016; Xu et al., 2015; Gao et al., 2015). Our data shown that SRCIN1 expression was significantly lower in breast cancer tissues and cells, and there was a significant inverse correlation with miR-346.

According to MiRWalk2.0 data, we predicted SRCIN1 as a target of miR-346. In vitro, we found that miR-346 inhibited the expression of SRCIN1, as a direct target, in the breast cancer cells. Moreover, we showed that SRCIN1 significantly inhibited breast cancer cell proliferation, invasion and migration, and reverse the effect of miR-346. Additionally, chemotherapeutic is an essential part in the standardized and comprehensive treatment of breast cancer, including neoadjuvant chemotherapy and postoperative adjuvant chemotherapy, which can effectively reduce the risk of recurrence and metastasis, and prolong the survival period of patients. As we all know, docetaxel is one of the most common chemotherapy drugs of breast cancer. In this study, we found that miR-346 expression was higher in MCF-7/Doc cells than MCF-7 cells, and miR-346 reduced chemosensitivity to Doc in breast cancer cells. Furthermore, SRCIN1 had the opposite effect and reversed the effect by miR-346 in chemosensitivity to Doc. These data confirmed that SRCIN1 functioned as a tumor suppressor and suggested that miR-346 might influence the downstream pathways by targeting SRCIN1 to affect breast cancer cell functions.

In summary, we firstly confirmed that the expression of miR-346 was higher in breast cancer, promoted cell proliferation, colony formation, invasion and metastasis, inhibited apoptosis and reduced chemosensitivity to docetaxel by targeting SRCIN1, which revealed a critical role and appeared to be a promising therapeutic target. Certainly, there would be further studies for us, such as target signaling pathways, demonstrated in Doc-resistant tissues and tumor formation experiment.

Conflict of interest statement

None.

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References


