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Original Article

Down-regulation of FOS-like antigen 1 enhances drug sensitivity in breast cancer

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Abstract: Objective: Multidrug resistance (MDR) to chemotherapeutic drugs is an important reason for clinical chemotherapy failure. So far, the relationship between FOS-like antigen1 (FOSL1) and chemotherapy sensitivity of breast cancer remains unclear. This study investigates the relationship between FOSL1 and chemotherapy sensitivity of breast cancer and its molecular mechanism. Methods: Doxorubicin-resistant MCF-7/ADR breast cancer cells were transfected with NC (control) or FOSL1 siRNA and assayed for cell viability and relative colony number by MTT assay and colony formation, respectively. The expression level of FOSL1 was detected by immunohistochemistry (IHC). The relationship between FOSL1 and chemotherapy sensitivity was analyzed by a one-way of variance analysis and Pearson's chi-square test among a total of 50 patients with stage II and III breast cancer before and after they received epirubicin-based neoadjuvant chemotherapy (NCT) between 2012 and 2017. Results: The expression of FOSL1 was increased in breast cancer tissues compared with normal breast tissues ($P<0.05$), and the expression of FOSL1 was decreased after NCT treatment compared with breast cancer tissues (or before NCT). This lower expression of FOSL1 was correlated with chemotherapy resistance or chemotherapy sensitivity ($P<0.05$). Moreover, the expression level of FOSL1 was markedly lower in NCT-sensitive patients than that of NCT-resistant patients ($P<0.05$). Conclusion: Down-regulation of FOSL1 potentiated chemotherapy sensitivity of breast cancer, and its lower expression attenuated chemotherapeutic drug resistance in human breast cancer cells. FOSL1 might be a drug target for predicting chemotherapy effect in breast cancer.

Keywords: FOSL1, doxorubicin, chemotherapy sensitivity, chemotherapy resistance, breast cancer

Introduction

Breast cancer ranks as the most common malignancy among female malignant tumors around the world [1]. So far, progress has been made in improving the curative effect of breast cancer by the use of comprehensive treatment measures including epirubicin-based neoadjuvant chemotherapy (NCT) treatment that is widely applied in breast cancer patients, in order to reduce tumor volume, reduce clinical stage, afford the opportunity of surgery or breast preservation, and improve prognosis [2-4]. Studies have shown that the epirubicin-based NCT of breast cancer induced pathologic complete response, and the prognosis was better [5, 6]. However, multidrug resistance (MDR) is the main cause of tumor chemotherapy failure. Hence, it is of great significance to search for new therapeutic targets to improve the therapeutic effect of breast cancer and to explore its underlying mechanism.

Doxorubicin is a cytotoxic anthracycline antibiotic which is also an anti-cancer chemotherapy agent, including breast cancer treatment [7, 8]. Epirubicin is a semisynthetic L-arabino derivative of doxorubicin which has an antineoplastic effect by inhibiting Topoisomerase [9]. Epirubicin and doxorubicin are common chemotherapy drugs in the treatment of breast cancer which both inhibit topoisomerase II, thus stop DNA replication and interfere with the synthesis of protein and RNA [10]. In recent years, epirubicin-based NCT treatment has been widely used for the treatment of breast carcinoma because it has few side effects [11, 12].

FOS-like antigen1 (FOSL1) is an important member of the FOS family, which is frequently

overexpressed in multiple types of human cancers including breast cancer. Many studies have shown that FOSL1 is associated with tumor invasion, metastasis and chemotherapy resistance of breast cancer, lung cancer, et al. [13, 14]. However, the relationship between FOSL1 and chemotherapy resistance of breast cancer remains unclear. Hence, the purpose of this study was to investigate the expression of FOSL1 in epirubicin-based chemotherapy of breast cancer, as well as the correlation between it and chemotherapy resistance to further improve the treatment of breast cancer.

Materials and methods

Clinical information and tissue specimens

A total of 50, 30-53 year old female patients with breast cancer before and after neoadjuvant chemotherapy at the First Affiliated Hospital of Anhui Medical University and the Second People's Hospital of Hefei were enrolled in this study from 2012 to 2017. The tissues included breast cancer tissues and adjacent normal tissues. Patients with primary breast cancer who had previously received surgery, chemotherapy and endocrine or local radiotherapy or with distant metastasis and contraindicated chemotherapy, or who had acute infection, human immunodeficiency virus infection or other diseases were excluded. Neoadjuvant chemotherapy drugs including an anthracycline chemotherapy such as epirubicin (EPI) (0.10 g/m^2, intravenous) plus paclitaxel (TAX) (0.17 mg/m^2, intravenous) was administered in treatment of breast cancer and each time the chemotherapy was administered on day 1, 21 days apart, and lasted for 4 cycles.

The histologic grade of cancerous tissues was based on the World Health Organization classification of tumors [15], and the pathologic tumor stage was determined according to the 6th edition of the tumor-node-metastasis classification of the International Union Against Cancer [16]. All tissues obtained during surgery were fixed with paraformaldehyde, embedded in paraffin, and cut into 4 μm sections. The schema used in this study was approved by the Institutional Review Boards of the First Affiliated Hospital of Anhui Medical University and the Second People's Hospital of Hefei, and written informed consent was obtained from all patients.

Immunohistochemistry (IHC)

Immunohistochemistry analysis [17] of FOSL1 protein expression was performed using a two-step immunohistochemical staining kit (Shanghai Changdao Biotech Co., Ltd., Shanghai, China) according to the manufacturer's protocol with anti-FOS-like antigen 1 (FOSL1) antibodies (cat. no. ab232745). The tissues from advanced breast cancer patients before and after neoadjuvant chemotherapy were fixed with 4% paraformaldehyde, dehydrated and embedded in paraffin. Then for immunoperoxidase microscopy, the paraffin-embedded breast cancer samples were cut into sections (4 μm thick) for immunostaining of FOSL1. In a nutshell, after dewaxing and rehydration, a microwave pretreatment in 0.01 M sodium citrate buffer (pH 6.0) for 15 min was done to expose antigens present in the breast cancer tissues. To block endogenous peroxidase activity, the tissue slides were immersed in 3% hydrogen peroxide for 10 min. The tissue sections were then incubated with primary antibody overnight at 4°C after washing with phosphate-buffered saline (PBS). After rinsing in PBS, slides were incubated with secondary antibody for 1 h. The sections were later washed with PBS and incubated with DAB solution (Shanghai Changdao Biotech Co., Ltd) for 3 min at room temperature. All slides were restained with hematoxylin for 1-3 min at room temperature. Known positive samples were used as the positive controls, and for the negative controls, PBS was used as the primary antibody. The microscopic examination was performed by using a Leica DM2000 light microscope (Leica, Heidelberg, Germany).

Scoring of stained sections and evaluation of chemotherapy efficacy

The expression of FOSL1 in breast tissues was evaluated by two senior pathologists using a double-blind method. The staining sections were graded according to the staining intensity and quantity, and the method from Pinto's article was used for reference [18]. In brief, swatches where ≥10% of breast tissue cells were stained with any intensity were viewed as positive for expression of FOSL1, nevertheless, swatches where <10% of breast tissue cells were stained with any intensity were viewed as negative for the expression of FOSL1 [19].

According to the pathologic evaluation criteria, no residual tumor cells was considered complete remission; no more than 10% residual tumor cells was judged to be mostly remission; no more than 50% residual tumor cells was judged to be partially remission; more than 50% residual tumor cells was assessed as mild remission and no obvious necrosis or stromal response in the tumor tissue was judged as no remission. Complete remission, mostly remission and partial remission were judged to be chemotherapy sensitive, while mild remission and no remission were judged as chemotherapy resistance [20].

Cells transfection and grouping

Human breast cancer cell line MCF-7/ADR cells were obtained from KeyGENE (Nanjing, China). Doxorubicin was purchased from Beyotime (Shanghai, China). The cells were cultured in RPMI-1640 medium containing with 10% fetal bovine serum (Gibco, USA), 1% penicillin sulfate (KeyGENE, China), and 1% streptomycin sulfate (KeyGENE, China), and then incubated at 37°C, 5% CO_2. FOSL1 was down-regulated in MCF-7/ADR cells using FOSL1 RNA interference (siFOSL1). Cells were seeded in six-well plates at 1.5×10^5/ml one day before the transfection and cultured to a density of 65% within 24 hours, and the mixture of Lipofectamine 3000 and siFOSL1 was then added to the transfected cells. The transfection efficiency of siFOSL1 was detected by qRT-PCR after 24 hours. Detailed grouping information was as follows: siFOSL1 group was transfected with siFOSL1; the negative control (siNC) group was transfected with siNC-FOSL1.

MTT assay

To measure the cell mitosis, siNC and siFOSL1 transfected into MCF-7/ADR cells were incubated with MTT buffer (0.5 mg/ml, Thermo Fisher, USA) on a 96-well plate at 37°C. After 72 hours, the absorbance at 570 nm was detected using a spectrophotometer (Bio Rad, USA). This experiment was repeated three times. Meanwhile, we detected the sensitivity of siNC transfected MCF-7/ADR cells and siFOSL1 transfected MCF-7/ADR cells to doxorubicin. Cells were seeded into 96-well plates and cocultured with 2 μM doxorubicin. The proliferation of breast cancer cells was measured by using 0.5 mg/ml MTT buffer at 72 h. This experiment was repeated three times.

Soft agar assay for colony formation assay

For evaluating cellular transformation in vitro, the soft agar colony forming assay was performed. Cells were seeded in 6-well plates at 0.5×10^3/ml and cultured in DMEM medium containing 10% FBS and 3% agarose. After 12 days, colonies that appeared were fixed with pre-cold methanol and stained with 2% Giemsa solution. The experiment was repeated three times.

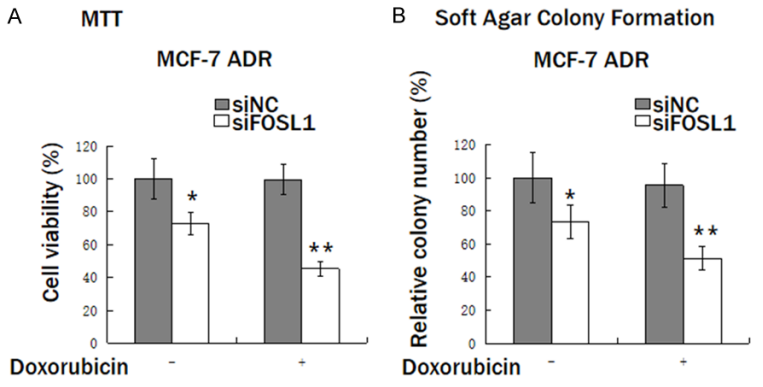
Statistical analysis

All statistical analyses were performed with SPSS 18.0 software. The measurement data were presented as mean ± standard deviation (SD). A one-way analysis of variance followed by Bonferroni or Tamhane post hoc tests was used for MTT assay and cell colony formation assay. Pearson's chi-square test was used to analyze the correlation between chemotherapy efficacy and FOSL1 expression. A value of $P<0.05$ was considered significant.

Results

Down-regulation of FOSL1 can weaken doxorubicin resistance in MCF-7/ADR cells and suppress cell growth and proliferation

To test the effect of FOSL1 on doxorubicin resistance of MCF-7/ADR cells, we performed siRNA knockdown experiments. Indeed, knockdown of FOSL1 by siRNA significantly decreased doxorubicin resistance of MCF-7/ADR cells in the MTT assay, which indicated that the proliferation capacity of MCF-7/ADR cells was markedly decreased by FOSL1 down-regulation compared with the negative control group (siNC) (**Figure 1A**, top panel). We next tested the relationship between FOSL1 and doxorubicin resistance and respectively, siNC or siFOSL1 was transfected into MCF-7/ADR cells culturing with doxorubicin. Then, after culturing with doxorubicin, knockdown of FOSL1 by siRNA significantly reduced proliferation capacity compared with the siNC group (**Figure 1A**, bottom panel). Consistently, soft agar assay for colony formation assay suggested that the relative colony number of siFOSL1 treated MCF-7/ADR cells was markedly decreased compared with siNC group (**Figure 1B**, top panel), and after culturing with doxorubicin, the



	siNC	siFOSL1
Vehicle / Doxorubicin (Fold decrease)	1.00 (NS)	1.60 (**)

	siNC	siFOSL1
Vehicle / Doxorubicin (Fold decrease)	1.05 (NS)	1.43 (**)

Figure 1. Down-regulation of FOSL1 weakened doxorubicin resistance of MCF-7/ADR cells and suppressed cell growth and proliferation. A. MTT assay was performed to test cell proliferation after siFOSL1 was transfected into MCF-7/ADR cells compared to negative control before and after co-culturing with doxorubicin. B. Soft agar colony forming assay was used to evaluate the relative colony number of siFOSL1 or siNC transfected into MCF-7/ADR cells separately before and after co-culturing with doxorubicin. *P<0.05 compared with siNC group or siNC group treated by doxorubicin. **P<0.01 compared with siNC group or siNC group treated by doxorubicin.

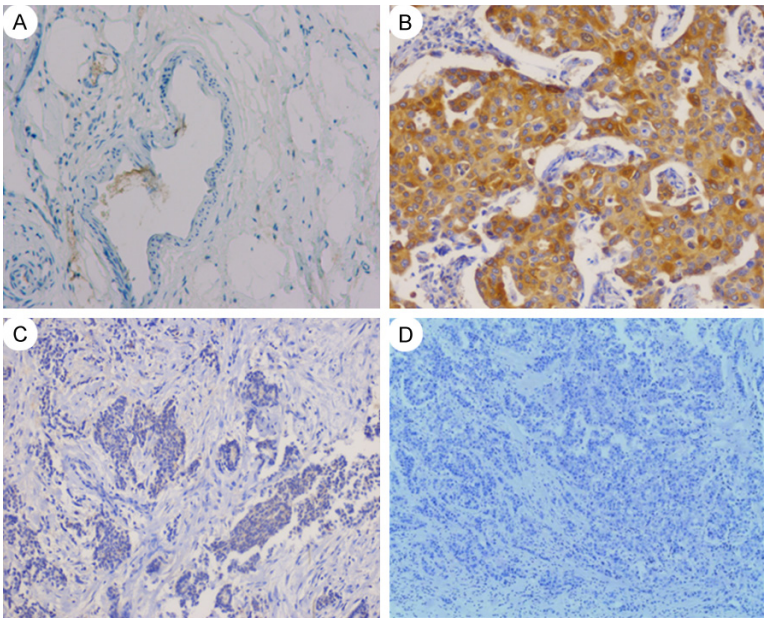


Figure 2. Immumohistochemical staining was performed to test the level of FOSL1 in breast cancer tissues before and after neoadjuvant chemotherapy (×100). A. Expression of FOSL1 in normal breast tissues was low. B. Expression of FOSL1 in breast cancer tissues was high compared to normal breast cancer tissues. C. Expression of FOSL1 was lower after neoadjuvant chemotherapy of breast cancer compared with before neoadjuvant chemotherapy (or breast cancer tissues). D. There was no FOSL1 expression in the negative control group (PBS instead of primary antibody).

relative colony number of siFOSL1 treated into MCF-7/ADR cells was significantly reduced comparing with the siNC group (**Figure 1B**, bottom panel). These results also gave evidence of that knockdown of FOSL1 by siRNA inhibited cell growth and proliferation and further reversed doxorubicin resistance of MCF-7/ADR cells.

Expression of FOSL1 was down-regulated in breast cancer samples from patients with breast cancer receiving neoadjuvant chemotherapy

We detected the FOSL1 expression in breast cancer tissues collected from patients before and after receiving neoadjuvant chemotherapy and in adjacent normal tissues (**Figure 2**). We divided the expression level of FOSL1 into high or low by the staining intensity and quantity scores. Immunohistochemistry confirmed a high labeling of FOSL1 expression in breast cancer tissue (**Figure 2A**) compared with normal breast tissue (**Figure 2B**), whereas after chemotherapy, the expression of FOSL1 was down-regulated (**Figure 2C**). As shown in **Table 1**, the expression of FOSL1 was high in 34 patients (68%) and the expression level of FOSL1 was low in 16 patients (32%) before chemotherapy; however, the expression of FOSL1 was high in 17 patients (34%) and the expression of FOSL1 was low in 33 patients (66%) after chemotherapy. These results verified that the expression of FOSL1 was decreased in the tumor samples after neoadjuvant chemotherapy compared to samples before treatment (P<0.05).

Table 1. Expression of FOSL1 in breast cancer tissues before and after neoadjuvant chemotherapy

Group	n	FOSL1 expression		x^2	*P*-value
		High expression n (%)	Low expression n (%)		
Pre-chemotherapy	50	34 (68.0)	16 (32.0)	11.565	0.001
Post-chemotherapy	50	17 (34.0)	33 (66.0)		

Table 2. Relationship between FOSL1 and chemotherapy resistance or chemotherapy sensitivity

Group	n	FOSL1 expression		x^2	*P*-value
		High expression n (%)	Low expression n (%)		
Chemotherapy sensitive	32	7 (41.2)	25 (75.0)	5.824	0.016
Chemotherapy resistant	18	10 (58.8)	8 (25.0)		

Table 3. Differences in FOSL1 expression before and after neoadjuvant chemotherapy, between chemoresistant and chemosensitive groups

Group	n	Changes in FOSL1 expression before and after neoadjuvant chemotherapy		x^2	*P*-value
		Decreased n (%)	Unchanged/Increased n (%)		
Chemotherapy sensitive	32	20 (86.9)	12 (44.0)	9.742	0.002
Chemotherapy resistant	18	3 (13.1)	15 (56.0)		

Expression level of FOSL1 in breast cancer tissue from patients who were sensitive to chemotherapy was lower than in those who were resistant to chemotherapy

We can divided into two groups based on clinical response to neoadjuvant chemotherapy in a total of 50 patients. One group was chemotherapy-resistant and other was chemotherapy-sensitive. We further tested the expression of FOSL1 in the two groups to understand its role in chemotherapy resistance. As seen in **Table 2**, the FOSL1 expression was high in 7 patients (41.2%) and FOSL1 was low in 25 patients (75.0%) in the chemotherapy-sensitive group; whereas, the expression of FOSL1 was high in 10 patients (58.8%) and FOSL1 was low in 8 patients (25.0%) in the chemotherapy-resistant group. These results suggested that the expression of FOSL1 in breast cancer of chemotherapy-sensitive group was lower than in the chemotherapy-resistant group ($P<0.05$). We subsequently tested that the change of the expression level of FOSL1 before and after neoadjuvant chemotherapy in two groups: chemotherapy-resistant group and chemotherapy-sensitive group. As shown in **Table 3**, in the chemotherapy-sensitive group, the level of FOSL1 was decreased in 20 patients (86.9%) post-chemotherapy compared to pre-chemotherapy and the level of FOSL1 was unchanged or increased in 12 patients (44.0%). However, in the chemotherapy-resistant group, the level of FOSL1 was decreased in 3 patients (13.1%) post-chemotherapy compared to pre-chemotherapy and the expression level of FOSL1 was unchanged or increased in 15 patients (56.0%). These results demonstrated that after neoadjuvant chemotherapy, the expression level of FOSL1 was markedly increased in the chemotherapy-resistant group, but, the expression of FOSL1 was decreased dramatically in the chemotherapy-sensitive group ($P<0.05$).

Discussion

Breast cancer is a common malignant tumor in Chinese women, with a high incidence and mortality, which damages women's physical and mental health [21]. Chemotherapy plays an important role during the treatment process of breast cancer. Neoadjuvant chemotherapy, which is systemic chemotherapy prior to local treatment, such as surgery or radiotherapy, is intended to shrink the mass and kill the invisible metastatic cells early enough to facilitate subsequent surgery or radiotherapy. Moreover, neoadjuvant chemotherapy is usually used in patients with middle or advanced-stage tumors in order to shrink the tumor before surgery or radiotherapy [22]. Chemotherapy drugs are currently based on anthracycline, among which doxirubicin and epirubicin are commonly used in adjuvant chemotherapy of breast cancer.

However, treatment of tumors is prone to multidrug resistance, and broad-spectrum drug resistance is an important cause of chemotherapy failure, as well as one of the main reasons why tumors are difficult to treat and easily relapse [23, 24]. Hence, it is of significance to find effective factors to accurately predict the efficacy of neoadjuvant chemotherapy.

The FOS family is a vital component of activating protein-1 (AP-1) transcription factors, which are involved in promoting carcinogenesis [25]. The FOS family includes c-FOS, FOSB, FOS-associated antigen 1 (FOSL1 or FRA1) and FOS-associated antigen 2 (FOSL2 or FRA2). The FOS proteins and JUN proteins (c-JUN, JUNB and JUND) dimerize to form activated protein 1 (AP-1) and then FOS proteins are phosphorylated by ERK kinase after responding to extracellular stimuli, thus regulating downstream genes [26-28]. It is well documented that FOSL1 is associated with tumor metastasis, invasion, and chemotherapy resistance of tumors including breast cancer. Silencing FOSL1 restrained cell growth and proliferation in MCF-7 cells, and reversed the chemotherapy drug resistance to toramifen [29]. FRA1, the protein encoded by the gene FOSL1, significantly enhanced the chemotherapy resistance of breast cancer cells to adriamycin and cyclophosphamide when it had low expression level, and thereby increased the chemotherapy sensitivity of breast cancer cells [30]. Our early research found that miR-130a suppressed cell migration and invasion by targeting FOSL1, and miR-130a reduced drug resistance of breast cancer [31, 32]. These findings highlighted an important role of FOSL1 in chemotherapy resistance of breast cancer. FOSL1 expression was increased in breast cancer tissues, but it decreased after neoadjuvant chemotherapy of breast cancer. Others have noted similar results in breast cancer and prostate cancer, which shows the crucial role of FOSL1 during tumor chemotherapy [33-35]. Our study also indicated that down-regulation of FOSL1 reversed doxorubicin resistance of MCF-7/ADR cells and inhibited cell growth and proliferation. Furthermore, Pearson's chi-square test analyses revealed that the number of breast cancer patients after epirubicin-based neoadjuvant chemotherapy with high expression level of FOSL1 was significantly reduced compared with before neoadjuvant chemotherapy, and especially the sensitivity of breast cancer patients with low expression level of FOSL1 to chemotherapy drugs was also increased. The efficacy of chemotherapy was enhanced in breast cancer patients with relatively lower FOSL1 expression before and after neoadjuvant chemotherapy. Although evidence was provided for FOSL1 as a chemotherapy evaluation biomarker for breast cancer, the precise underlying molecular mechanisms of FOSL1 involvement in chemotherapeutic resistance remains unclear and needs more study.

In conclusion, down-regulated expression of FOSL1 after neoadjuvant chemotherapy of breast cancer compared with pre-therapy levels, and low expression of FOSL1 reversed the resistance to epirubicin-based chemotherapy. Although confirmation of the present findings will be necessary, for the first time we have indicated an important predictive role of FOSL1 in epirubicin-based chemotherapy in breast cancer patients. Our results indicate FOSL1 may be an effective drug target, and can be used to reduce drug resistance and improve the efficacy of chemotherapy.

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Disclosure of conflict of interest

None.

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