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Knockdown of CD24 inhibits proliferation, invasion and sensitizes breast cancer MCF-7 cells to tamoxifen *in vitro*

Z.-L. MA[1], Y.-P. CHEN[2], J.-L. SONG[2], Y.-Q. WANG[2]

[1]Department of Breast Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[2]Department of Laboratory, Women and Children's Hospital of Qingdao, Qingdao, Shandong, China

ZhongLiang Ma and Yanping Chen contributed equally to this work

Abstract. – OBJECTIVE: CD24 is overexpressed in breast cancer, and patients with high CD24 expression was resistant to tamoxifen treatment. Furthermore, treatment with CD24 antibody to inhibit CD24 expression could induce apoptosis and inhibits migration in breast cancer cells *in vitro*. In this study, we investigated the anti-tumor efficacy of CD24 knockdown using siRNA targeting CD24 on proliferation, invasion and sensitivity to tamoxifen (TAM) of breast cancer MCF-7 cells *in vitro*.

MATERIALS AND METHODS: CD24 siRNA vector (CD24-siRNA) and empty plasmid vector (EP) were transiently transfected into the breast cancer MCF-7 cells and the knockdown efficacy was assessed by Western blot analysis. The effects of CD24 knockdown on cell viability, apoptosis and sensitivity to TAM in MCF-7 cells were determined using methyl thiazolyl blue tetrazolium bromide (MTT), ELISA and terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assays. The effects of CD24 knockdown on cell invasion and migration were determined using chemoinvasion assay and wound scratch assay, respectively.

RESULTS: Transfection of CD24-siRNA effectively down-regulated CD24 expression in MCF-7 cells *in vitro*. CD24 suppressed showed significantly decreased proliferation, invasion and increased apoptosis as well as increased sensitivity to TAM *in vitro* in MCF-7 cells.

CONCLUSIONS: Knockdown of CD24 expression by CD24-siRNA significantly inhibited invasion and cell viability, and induced apoptosis and increased sensitivity of MCF-7 cells to TAM, indicating that knockdown of CD24 by siRNA might be a potential therapeutic approach against human breast cancer.

Key Words:
Breast cancer, Tamoxifen, CD24, Invasion, Apoptosis.

Introduction

CD24 is a glycosylphosphatidylinositol-anchored membrane protein; its heterogeneous molecular weight rangs from 30 to 70 kDa[1]. The protein is related to the membrane via glycosylphosphatidylinositol (GPI)-anchor and is mainly located in membrane lipid raft domains[2]. It has recently found that CD24 is overexpressed in many human cancers, such as pancreatic cancer[3], intrahepatic cholangiocarcinoma[4], osteosarcoma[5], gastric cancer[6], extrahepatic bile duct cancer[7]. In intrahepatic cholangiocarcinoma, patients with CD24 positive tumors had significant shorter survival time, and CD24 expression and tumor stage were independent prognostic factors[4]. In laryngeal squamous cell carcinoma (LSCC), overexpression of CD24 in LSCC is associated with invasiveness, metastatic potential and high tumor proliferation status[8]. CD24 overexpression also has a poor prognosis in human gliomas[9], nonmelanoma skin cancer[10], bladder cancer[11] and hepatocellular carcinoma[12]. In breast cancer, overexpression of CD24 was also associated with poor prognosis[13-17].

CD24 expression has been demonstrated to regulate tumour cell proliferation, migration and invasion in osteosarcoma, ovarian cancer and gastric cancer AGS cells[18-20]. Knockdown of CD24 also enhances chemosensitivity in gastric cancer AGS cells[20].In MCF-7 breast cancer cells, treatment with CD24 antibody could induce apoptosis and inhibits migration[21]. Schabath et al[22] has showed that CD24 was a regulator of CXCR4 function that could be relevant for breast cancer growth and metastasis. CD24 overexpression increases cell proliferation and adhesion in a

Corresponding Author: Yanping Chen, MD; e-mail: chenyanpingqd@sina.com

rat carcinoma cell line through activation of integrins[23].

Tamoxifen (TAM) is the most frequently used drug for the treatment of breast cancer. Surowiak et al[24] has found CD24 was the potential marker of breast cancers which are resistant to tamoxifen treatment. We, therefore, suggested that knockdown of CD24 might sensitize breast cancer cells to tamoxifen treatment.

In the present study we first knockdown of CD24 expression in human breast cancer cell line MCF-7 by RNA interference (RNAi) to determine its effect on the proliferation and invasive ability as well as the sensitivity of MCF-7 cells to TAM *in vitro*.

Materials and Methods

Cell Culture and Transfection

Human breast cancer cell line MCF-7 was purchased from ATCC, Shanghai, China. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 8% fetal calf serum (FCS) under 5% CO_2. CD24 siRNA (h) (sc-29978) and control siRNA was gifted by Dr. Jiao, the Affiliated Hospital of Qingdao University[20]. MCF-7 cells were transiently transfected with CD24 siRNA and control siRNA respectively using lipofectamine 2000 (Invitrogen, Shanghai, China) for 48 hs according to the manufacturer's instructions. Then the MCF-7 cells were verified by Western blot analysis.

Tamoxifen Treatment

MCF-7 cells or MCF-7 cells transfected with CD24 siRNA and control siRNA for 48 hs, then exposed to tamoxifen (0.01-1 μM) for 72 h, CD24 and estrogen receptor (ER) were assessed by western blot analysis.

Western Blot Analysis

Total protein from cells at different time points under different condition was extracted using RIPA lysis buffer containing 50 μg/ml phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined by Bicinchoninic Acid (BCA) protein assay kit (Boster Biology Co., Wuhan, China). The protein samples were denatured at 100°C for 10 min and then preserved at -20°C for later use. The protein samples were separated by 7.5% SDS-polyacrylamide gels and transblotted onto Nitrocellulose blotting membrane (0.22 μm). Membranes were blocked with

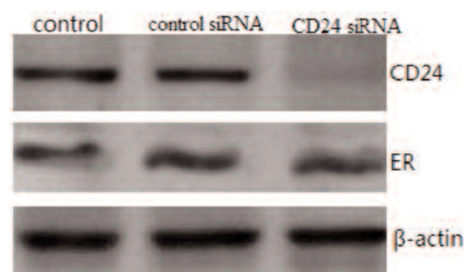

Figure 1. The effect of siRNA transfection on CD24 and ER expression by western blot assay. β-actin served as a loading control.

5% skim milk for 1 h at room temperature, and probed with primary antibodies (rabbit anti-CD24, Anti-ER and mouse anti-β-actin) overnight at 4°C. After incubation with the appropriate anti-rabbit, anti-goat or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000, Boster, China) for 1.5 hs at room temperature, immunoreactive bands were visualized by the chemiluminescence dissolvent (Thermo Scientific, Waltham, MA, USA, USA) and exposed to the X-ray film (Kodak, Rochester, MA, USA). The determination of grayscale value was processed by Image J. All experiments were repeated by three times over multiple days.

MTT Assay

The cell viability was detected by the MTT assay. Briefly, MCF-7 cells transfected with CD24 siRNA or control siRNA for 96 hs (1×10^4 cells/well of 96-well plate), or transfected with CD24 siRNA or control siRNA for 48 hs, then exposed to tamoxifen (0.1 μM) for 72 h and thereafter 25 ml of MTT solution (5 mg/ml in PBS) was added. After 2 h of incubation, 100 ml extraction buffer (20% SDS in 50% dimethylformamide) was added. After an overnight incubation at 37°C, absorbance was red at 570 nm.

ELISA Detection

The cell death detection ELISA kit was used for assaying apoptosis according to the manufacturer's instruction. Briefly, 4×10^3 cells (transfected with CD24 siRNA and control siRNA for 24 hs) per well were plated onto a 96-well microtiter plate in culture medium. Cells were exposed to different concentrations of tamoxifen for 72 h. After treatment, the samples was lysed and then overlaid and incubated in microtiter plate modules coated with antihistone antibody. Then, samples were cultured with anti-DNA peroxidase fol-

lowed by color development with ABTS substrate. The optical densities of the samples were detected using Ultra Multifunctional Microplate Reader (Tecam, Durham, NC, USA) at 405 nm.

TUNEL detection

Terminal deoxynucleotide transferase labeled free DNA 3' ends with fluorescein-conjugated dUTP was used by the In Situ Cell Death Detection kit (Roche Diagnostics, Guangzhou, China). Cytospin preparations were fixed and labeled according to the manufacturer's instruction. Four independent ×100 fields containing a minimum of 300 cells on each of two replicate slides were determined for nuclear labeling by fluorescence microscopy for each treatment or condition.

Cell Invasive Assays

48 hs after CD24 siRNA or control siRNA transfection, MCF-7 cells were collected, resuspended in culture medium, and incubated in a chemoinvasion chamber kit containing polycarbonate filter coated with Matrigel (BD Biosciences, Bedford, MA, USA) for 5 h. The invasive capability of cells was measured as per Vendor's protocol. Cells were counted under a microscope in five predetermined fields at a magnification of ×200.

Wound-Healing Assay

Cells were cultured in standard conditions. Until 90-100% confluence, the migration potency was detected using scratch wound healing assay as reported previously[25]. Briefly, the scratched plates were photographed at the center of the wells. The scratched cells were stained under standard conditions for 24 hs. Plates were washed once again, and then the plates were photographed at the same sites of the wells using the same magnification. The cells migrating into the scratched area from the wound edge per picture were counted. The impact of CD24 siRNA on cell migration potency was determined by comparing the mean of migration width with control siRNA.

Statistical Analysis

Statistical analyses were carried out by using Windows (version 11.0). Data presented are means of the three or more independent experiments ± *SE*. Statistically significant differences were determined by X^2 and Student's *t*-test. *p* values <0.05 were considered significant.

Results

Effect of siRNA on CD24 and ER Expression in MCF-7 Cells

As showed by western blot analysis, MCF-7 cells transfected with CD24 siRNA displayed a significant reduction in the expression levels of CD24 protein (Figure. 1). Control siRNA did not exhibit any effect on protein levels of CD24 and ER (Figure. 1). These data confirmed the suppression effect of siRNA. Furthermore, CD24 did not have any effect on ER expression.

Effect of TAM on CD24 and ER Expression in MCF-7 Cells

MCF-7 cells were treated with TAM (0.01-1 μM) for 72 h, CD24 and ER was assessed by western blot analysis. As showed in Figure 2, treatment with TAM (0.01-1 μM) did not have any effect on CD24 and ER expression.

Effects of CD24 Inhibition on MCF-7 Cell Growth and Apoptosis

To study the anti-proliferative effect of CD24 inhibition, CD24 inhibited MCF-7 cells and control cells were digested and counted at 96 h after siRNA transfection. Compared with control cells, significant growth inhibition was showed in CD24-inhibited MCF-7 cells. As shown in Figure 3A, the number of CD24-inhibited MCF-7 cells was less than 55% of control at 96 h after CD24 siRNA transfection.

As inhibition of cell growth could be the consequence either of apoptosis, ELISA and TUNEL assay was then performed. There was no significant change of apoptosis in control siRNA transfected MCF-7 cells by ELISA (Figure 3B) and TUNEL (Figure 3C) detection. However, apoptotic cells were significantly increased in CD24 siRNA transfected MCF-7 cells by ELISA (Figure 3B) and TUNEL (Figure 3C) detection.

CD24 Sliencing Potentiates TAM Induced Cytotoxicity

We studied the combination effect of CD24 siRNA with TAM on apoptosis induction. MCF-7 cells were transfected with CD24 siRNA or control siRNA for 48 hs, then treated with TAM for 72 h after siRNA transfection before ELISA and TUNEL assay. As shown in Figure 3E and F, dramatic increase of apoptosis showed in MCF-7 cells treated with TAM in combination with

CD24 siRNA compared with cells treated with TAM in combination with control siRNA. Furthermore, MCF-7 cells treated with TAM in combination with CD24 siRNA showed a marked growth inhibition relative to control siRNA transfectants as determined by MTT assay (Figure 3D). These results indicate that CD24 inhibition resulted in MCF-7 cell growth suppression and increased apoptosis, and further, enhanced cell sensitivity to TAM.

CD24 Sliencing Suppresses cell Migration and Invasion

CD24-inhibited MCF-7 cells and the control MCF-7 cells were subjected to scratch wounding. A significant wound closure inhibition was observed in the CD24 inhibited cells (Figure 4A), although the control MCF-7 cells migrate rapidly into the wound area to result in almost complete wound closure within 24 h (Figure 4A).

Since the wound healing model is an integrated process of cell migration and proliferation, we then performed the chemoinvasion assay to further determine the cell migration ability. Chemoinvasion assay was also performed at 48 h after siRNA transfection. Compared with control cells, these CD24 inhibited cells displayed a marked decrease in number of invasive cells (Fig. 4B). Taken together, inhibition of CD24 would decrease cell migration and invasion.

Discussion

CD24 is a very novel gene, and overexpressed in various human malignancies, and was shown to increase tumor invasion[26]. Most studies so far focuses on its role in cancer progression. However, its expression is not limited to tumor cells and was shown to be expressed in many cell types, including breast cancer cells[13].

Gene silencing by RNA interference has be as a powerful method that is useful for the investigation of functional genomics. In the present study, we successfully transfected siRNA targeting CD24 gene into human breast cancer cell line MCF-7. CD24 expression was effectively suppressed by CD24 siRNA transfection. Previously, it has been found that CD24 sliencing reduced tumour cell proliferation in the models of colorectal and pancreatic cancers[27]. So CD24 represents a promising novel therapeutic target to inhibit tumour cell growth.

We, then, examined the effect of CD24 inhibition on the proliferation and apoptosis of MCF-7 cells. The proliferation potential of MCF-7/siRNA cells was inhibited compared with that of the MCF-7 / control siRNA cells. Furthermore, apoptotic cells were significantly increased in the MCF-7/siRNA cells compared with that of the MCF-7 / control siRNA cells.

CD24 overexpression could enhance cancer cells invading through vessel walls by increasing

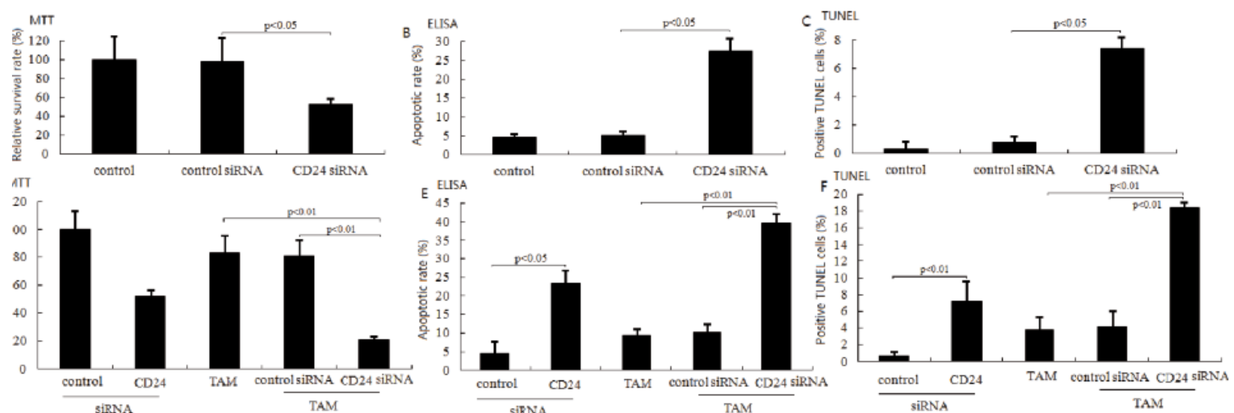

Figure 3. Effects of CD24 siRNA alone or in combination with TAM on cell growth and apoptosis in MCF-7 cells. CD24-inhibited MCF-7 cells and the control MCF-7 cells were seeded in 24-well plates at a density of 1 × 104 viable cells per well and were cultured in growth chamber for 96 hs. A, Survival rate of MCF-7 cells exposed to CD24 siRNA alone for 96 h was detected by MTT. B, Apoptosis rate of MCF-7 cells exposed to CD24 siRNA alone for 96 h was detected by ELISA; C, Apoptosis rate of MCF-7 cells exposed to CD24 siRNA alone for 96 h was detected by TUNEL. D, Survival rate of MCF-7 cells exposed to CD24 siRNA alone for 48 hs and were then exposed to TAM for an additional 72 h for MTT assay. E, Apoptosis rate of MCF-7 cells exposed to CD24 siRNA alone for 48 hs and were then exposed to TAM for an additional 72 h for ELISA assay. F, Apoptosis rate of MCF-7 cells exposed to CD24 siRNA alone for 48 hs and were then exposed to TAM for an additional 72 h for TUNEL assay. Data shown (mean ± SD) here is from three independent tests.

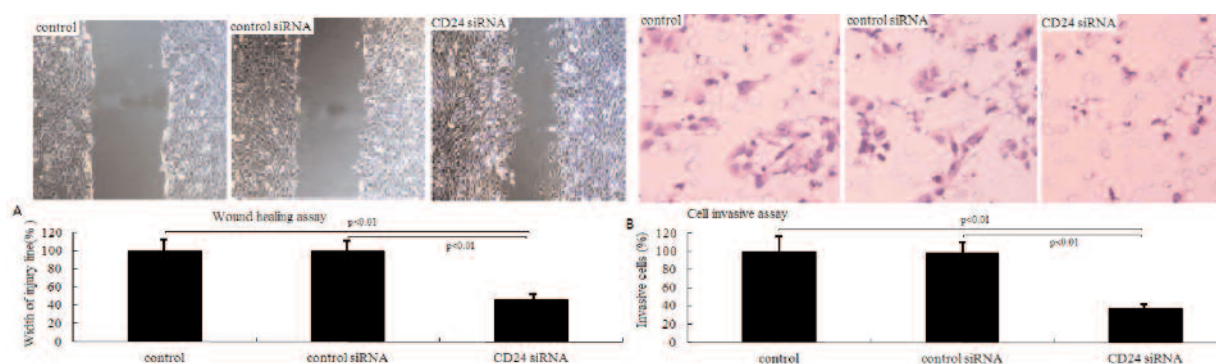

Figure 4. CD24 sliencing by specific siRNA results in defects of cell migration and invasion in MCF-7 cells. A, Wound healing assay for CD24 siRNA transfected cells. Cells were incubated at 48 h after siRNA transfection and seeded into 24-well plate with number adjust to equal in each group. Scratch was conducted on the confluent monolayer the next day and cells were cultured in growth chamber for 24 h. Images of the migrating cells were collected under optical microscopy. B, chemoinvasion assay of MCF-7 cells was performed at 48 h after siRNA transfection. Migrated cells were counted under the optical microscope. The experiments were done three times independently.

their adherence to platelets and endothelial cells under physiologic conditions[28]. It has showed that overexpression of CD24 is associated with invasion of urothelial carcinoma[11] and with migration and invasion in gliomas[29]. We, therefore, suggested that CD24 has the effect to promote invasion of cancer cells. So, CD24 represents a promising novel therapeutic target to inhibit invasion and metastasis of cancers. We showed in the present study that CD24 siRNA inhibited the invasiveness of MCF-7 cells *in vitro*. Our findings further proved that CD24 is involved in migration in MCF-7 cells, and indicated that tumor progression can be inhibited by CD24 silencing.

Numerous clinical trials have found that over 40% ER-positive cases no remission can be got with tamoxifen therapy. In approximately 10% ER-negative cases tamoxifen treatment results in remission[30]. The potential of accurate determination of sensitivity to tamoxifen treatment would permit subjecting the resistant cases to alternate therapeutic modalities and, thus, would improve the effectiveness of breast cancer therapy. In the present work, we found that CD24 inhibition resulted in MCF-7 cell growth suppression and increased apoptosis, and further, enhanced cell sensitivity to TAM.

At present, tamoxifen is the most frequently applied drug for the treatment of breast cancer. It acts mainly through blocking the function of estrogen receptor alpha (ER). Although CD24 inhibition enhanced cell sensitivity to TAM, it remained possible that responses to stress may affect CD24 and ER expression in the MCF-7 cells. Therefore, we wished to evaluate whether TAM treatment could affect CD24 and ER expression in the MCF-7 cells. MCF-7 cells were treated with TAM (0.01-1 μM) for 72 h, CD24 and ER was assessed by western blot analysis. The result showed that treatment with TAM (0.01-1 μM) did not have any effect on CD24 and ER expression. Thus, CD24 did correlate with sensitivity to TAM. Silencing of CD24 was effective alone and in combination with TAM in CD24 positive breast cancer cells.

Conclusions

This report showed that the CD24 silencing by siRNA inhibited proliferation and invasion, and promoted apoptosis of human breast cancer cell line MCF-7 *in vitro* and increased its sensitivity to the anti-tumor drug TAM. Our results suggested that CD24 might be a promising target for breast cancer treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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