Caveolin 1 knockdown inhibits the proliferation, migration and invasion of human breast cancer BT474 cells

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Received October 10, 2013; Accepted February 13, 2014

DOI: 10.3892/mmr.2014.2018

Abstract. Previous studies have demonstrated that caveolin 1 acts as a tumor suppressor in breast cancer, however, few studies have demonstrated that caveolin 1 also serves as a tumor promoter in breast cancer. In the present study, caveolin 1 small interfering RNA was used to knock down caveolin 1 expression in order to investigate the association between caveolin 1 and the proliferation and metastatic abilities of human breast cancer BT474 cells. The results revealed that cell proliferation, migration and invasion were attenuated by caveolin 1 knockdown in BT474 cells. Furthermore, caveolin 1 knockdown in BT474 cells arrested cells in the G0/G1 phase and decreased the number of cells in the S phase. In addition, caveolin 1 knockdown decreased the activation of the extra-cellular signal-regulated kinase 1/2 pathway and inhibited the expression of cell cycle-associated proteins (cyclin D1, c-Fos and β-catenin), whilst the expression of E-cadherin was increased. Furthermore, the protein expression of matrix metalloproteinase-2, -9 and -1 was also inhibited by caveolin 1 knockdown. In combination, these results demonstrated that caveolin 1 knockdown had a tumor suppressing effect on BT474 cells.

Introduction

Breast cancer is one of most common types of malignancy that occurs in females around the world. In the United States, in 2013, ~232,340 females were diagnosed with breast cancer and ~39,620 breast cancer-associated mortalities were estimated (1). In addition, breast cancer is the most common cause of cancer-associated mortality in females in China. Accurate prognosis and effective treatments against breast cancer require a more in depth understanding of the cellular and molecular mechanisms involved in breast cancer development and progression.

Caveolin 1, a 21-24 kDa membrane protein, is a major structural component of caveolae, which are identifiable plasma membrane invaginations. It has been suggested that caveolin 1 functions as a scaffold protein for signal transduction, transformation, endocytosis, cholesterol homeostasis, the cell cycle, cell migration and invasion (2-4).

Emerging evidence has demonstrated that caveolin 1 serves as a tumor suppressor protein and knockdown of caveolin 1 activates anchorage-independent growth of transformed cells (5). However, caveolin 1 has also been demonstrated to have a tumor promoting role in prostate cancer, renal cancer and esophageal squamous cell carcinoma (6-8), suggesting that whether caveolin 1 acts as tumor suppressor or facilitator depends upon specific tumor types.

The potential function of caveolin 1 in the development and progression of breast cancer remains unclear. In human breast cancer MCF-7 cells, the overexpression of caveolin 1 is associated with the suppression of cell growth and inhibition of migration and invasion (9). However, there is evidence that caveolin 1 also acts as a tumor promoter in breast cancer. It has been reported that the depletion of caveolin 1 decreased migration, polarization and focal adhesion in MDA-MB-231 cancer cells (basal-like phenotype) (10). This is consistent with another study, which demonstrated that caveolin 1 is highly associated with the breast cancer basal-like phenotype (11). In the present study, human breast cancer BT474 cells were used to analyze the role of caveolin 1 in BT474 cells. It was hypothesized that caveolin 1 may serve as a tumor promoter in BT474 cells, leading to tumor growth, migration and invasion.

Materials and methods

Cell lines and reagents. The human breast cancer BT474 cell line was purchased from American Type Cell Culture (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, South Logan, UT, USA) and 1% penicillin/streptomycin (Beyotime Biotech, Nanjing, China) in the presence of 5% CO\textsubscript{2} and at 37°C. Antibodies [anti-caveolin 1, anti-p-extra-cellular signal-regulated kinase 1/2 (ERK1/2) and anti-ERK1/2] were purchased from Cell Signaling Technology, Inc. (Beverly,
MA, USA). Anti-matrix metalloproteinase 1 (MMP-1), anti-MMP-2 and E-cadherin were purchased from Epitomics, Inc. (Burlingame, CA, USA). Anti-MMP-9, anti-cyclin D1 and anti-GAPDH were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-c-Fos and anti-β-catenin were purchased from Abcam (Cambridge, MA, USA).

Small interfering RNA (siRNA). The target siRNA against human caveolin 1 (si-h-Cav-1) was designed and constructed by Guangzhou Ribio Biotech Co., Ltd (Guangzhou, China). The sequence was as follows: si-h-Cav-1, forward 5'-GCA UCAACUUCCGAGAAGAdTdT-3' and reverse 3'-dTdTTCGUA GUUGAACCUCUUUCU-5'.

Prior to transfection the medium was replaced with penicillin/streptomycin-free RPMI-1640 complete medium. BT474 cells were then transfected with si-h-Cav-1 or negative control siRNA (siCon) using Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA).

Western blot analysis. The cells were seeded in six-well plates and were allowed to grow to 60-80% confluence. The concentration of the total protein was determined using the bicinchoninic acid protein assay kit (Beyotime Biotech). SDS-PAGE (Beyotime Biotech) was used to separate the total protein, prior to transfer onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk in 0.1% Tris-buffered saline with Tween 20 for 1 h at 37˚C, prior to being incubated with primary antibody overnight at 4˚C followed by three washes in 0.1% TBST for 5 min. The membranes were then incubated in horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5,000; Boster Biological Technology, Co., Ltd., Wuhan, Hubei, China) for 1 h at 37˚C, following washing in 0.1% TBST for 5 min three times. An enhanced chemiluminescence Substrate Reagent kit (Thermo Fisher Scientific, Waltham, MA, USA) was added and the band intensity of the blot was quantified using a gel imager (Bio-Rad, Hercules, CA, USA). GAPDH was used as an internal standard.

Immunofluorescence analysis. The cells were seeded in six-well plates at 50-60% confluence, prior to being treated for 48 h with si-h-Cav-1 or siCon, respectively. The cells were washed with cold phosphate-buffered saline (PBS) twice and then fixed in 4% paraformaldehyde at room temperature for 10 min. The cells were subsequently washed twice in PBS and the slides were blocked with 1% bovine serum albumin (BSA; Amresco, Solon, OH, USA) in 0.1% PBS with Tween 20 with 0.3 M glycine for 30 min. The slides were then incubated with anti-caveolin 1 antibody for 2 h at room temperature. Fluorescein isothiocyanate-conjugated goat-anti-rabbit immunoglobulin G was used to detect the primary antibody for 1 h at room temperature in the dark and DAPI (0.5 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) was used to label nuclei for 3 min at room temperature in the dark. Image capture and processing were performed using an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Cell proliferation assay and chemotherapy sensitivity assay. To assess cell proliferation and chemotherapy sensitivity to doxorubicin (Dox; Sigma-Aldrich), the Cell Counting kit-8 (CCK-8) assay (Dojindo Lab., Kumamoto, Japan) was used in accordance with the manufacturer's instructions. The cells were seeded in 96-well plates at a density of 5x10⁴ cells/well. The culture medium was removed and 100 µl diluted CCK-8 (1:9, diluted in RPMI-1640 medium) was added to each well, and the cells were incubated at 37˚C for 1.5 h. The optical density was then detected at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific).

Colonial formation assay. For the colony formation assay, following transfection with si-h-Cav-1 or siCon, the cells were seeded in six plates (3x10²/well). The cells were cultured for 9 days prior to being stained with crystal violet, and then images of the cells were captured and analyzed for colony formation.

Transwell migration and invasion assays. The upper transwell chamber (8 µm pore size; Corning Inc., Union City, CA, USA), coated (Sigma-Aldrich; invasion assay) or not coated with ECM gel (migration assay), was covered by 5x10⁴ cells in 200 µl medium containing 0.1% BSA. The lower chamber was then filled with 200 µl RPMI-1640 medium containing 30% FBS (HyClone Laboratories). The cells were then cultured at 37˚C and 5% CO₂ for 22 h, prior to being fixed with 70% ethanol and stained with 0.1% crystal violet. The number of cells were counted in multiple random fields using the Olympus IX71 fluorescence microscope (Olympus).

Flow cytometric analysis. For the cell cycle assay, cells (1-2x10⁴) were collected and washed twice with PBS and centrifuged at 1,500 x g for 5 min. The cells were then resuspended and fixed in 70% ethanol in PBS overnight at 20˚C. Fixed cells were washed twice with PBS and centrifuged at 1,500 x g for 5 min prior to being resuspended in 500 µl propidium iodide (50 µg/ml; BioSharp, Seoul, South Korea) with RNase A (50 µg/ml; Amresco). The cells were then incubated at 4˚C for 30 min in the dark and subsequently analyzed using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis. Statistical analysis of all the data were performed using the SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA). The results are presented as the mean ± standard deviation. Student's t-test was used to evaluate significant differences and P<0.05 was considered to indicate a statistically significant difference.

Results

Stable knockdown of caveolin 1 in human breast cancer BT474 cells. In order to investigate the role of caveolin 1 in human breast cancer, BT474 cells were transfected with si-h-Cav-1 to knockdown caveolin 1 expression or siCon as a control. The successful knockdown of caveolin 1 was confirmed using western blotting (Fig. 1A) and immunofluorescence analysis (Fig. 1B).

Effect of caveolin 1 knockdown on cell growth, migration and invasion in BT474 cells. To examine whether caveolin 1 knockdown affects the cell growth of BT474 cells, BT474
Cells were transfected with si-h-Cav-1 or siCon in 96-well plates. Cell proliferation was evaluated using the CCK-8 kit at different time points (24, 48, 72 and 96 h). The results demonstrated that cell growth significantly decreased 72 and 96 h after caveolin 1 knockdown in BT474 cells compared with cells transfected with siCon (Fig. 2A). Transwell assays were performed to detect the effect of caveolin 1 knockdown on the migration and invasion of BT474 cells. The results demonstrated that caveolin 1 knockdown in BT474 cells attenuated their metastatic ability (Fig. 2B).

Effect of caveolin 1 knockdown in BT474 cells on colony formation, the cell cycle and Dox-induced cell death. To further confirm the impact of caveolin 1 knockdown on cell growth, the cell cycle was analyzed using flow cytometry. The results demonstrated that the number of BT474 cells in G0/G1 phase increased, whilst the number of cells in the S phase decreased following caveolin 1 knockdown (Fig. 3A). The efficiency of cell colony formation was analyzed and it was found to decrease in caveolin 1 knockdown BT474 cells (Fig. 3B). The cells were treated with Dox for 12 h, prior to being transfected with si-h-Cav-1 or siCon for 36 h. BT474 cells in the si-h-Cav-1 group demonstrated higher sensitivity to the Dox treatment compared with cells in the siCon group (Fig. 3C).

Effect of caveolin 1 knockdown in BT474 cells on the expression of proteins involved in the cell cycle, migration and invasion. BT474 cells were further investigated from a mechanistic perspective. Caveolin 1 knockdown reduced the activation of the ERK1/2 pathway (Fig. 4A) and decreased the expression of proteins involved in the cell cycle, including cyclin D1, c-Fos and β-catenin (Fig. 4B). Caveolin 1 knockdown in BT474 cells also led to the upregulation of E-cadherin (Fig. 4B). Furthermore, the protein expression of the MMP family (MMP-2, MMP-9 and MMP-1) was also investigated and it was found that MMP expression decreased with caveolin 1 knockdown (Fig. 4C).

Discussion

Caveolin 1 has been demonstrated to have a suppressing and promoting role in pancreatic cancer, lung cancer, esophageal squamous cell carcinoma, renal cell carcinoma, prostate cancer and melanoma (6-8,12-14). Previous studies have found that patients with a high caveolin 1 expression have more progressive diseases, and caveolin 1 has been demonstrated to have tumor promoting and pro-survival functions in more advanced disease stages (15,16). By contrast, several studies have also revealed that caveolin 1 functions as a tumor suppressor in breast cancer, which has been confirmed in breast cancer MCF7 cells and several animal models (17,18). However, there is also evidence that caveolin 1 serves as a tumor promoter in breast cancer (19). In the present study, it was demonstrated that caveolin 1 had a tumor promoting role in BT474 cells. Knockdown of caveolin 1 resulted in the suppression of cell proliferation, migration and invasion of BT474 cells.
It has been demonstrated that caveolin 1 is able to negatively regulate cell proliferation. Knockdown of caveolin 1 resulted in a decrease in the number of cells in the G0/G1 phase population and an increase in the number of cells in the S phase population, through driving the expression of cyclin D1, an essential factor in the G1/S transition and in tumor formation (20-22).
However, caveolin 1 knockdown had a different effect on cell growth and the cell cycle in BT474 cells, resulting in a significant reduction in cell growth (Fig. 2A) associated with decreased cyclin D1 expression, and increased G0/G1 phase population and reduced S phase population. Furthermore, c-Fos, as well as β-catenin, has previously been demonstrated to function as a nuclear transcription factor (23,24). In addition, in the present study, their expression was found to be decreased by caveolin 1 knockdown in BT474 cells (Fig. 4B).

Not only has caveolin 1 been identified as a tumorigenic activity-associated gene, but it has also been suggested that it is involved in multiple-drug resistance to chemotherapy in numerous types of carcinoma (25,26). In the present study, it was demonstrated that caveolin 1 knockdown increased Dox-induced cell death in BT474 cells and therefore sensitized those cells to Dox treatment (Fig. 3C).

E-cadherin has previously been demonstrated to be important in tumor metastasis through modulating the process of cell-cell adhesion, establishment of cell polarity and cytoskeletal rearrangement (27). Downregulation of caveolin 1 has been demonstrated to be associated with a reduction in E-cadherin expression and, therefore, cell motility, as well as enhancing the metastatic ability of tumor cells (28). However, in contrast to previous studies, the inhibition of caveolin 1 in the present study resulted in an induction in the protein level of E-cadherin in BT474 cells (Fig. 4B).

In conclusion, the results from the present study indicate the potential capacity of caveolin 1 as a tumor promoter in BT474 cells. The role of caveolin 1 in cancer progression has been demonstrated to be controversial and complex. The present study provides novel insights into the function of caveolin 1 in breast cancer. It was demonstrated that caveolin 1 has a tumor promoting role in BT474 cells and the results suggest that caveolin 1 may be used as a metastatic marker in carcinomas. However, this requires further investigation before it may be used as a practical diagnostic and prognostic marker.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant no. 81001171) and the Key Technologies R&D Program of Hubei Province (grant no. 2007AA302B07).
References