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Original Article

Caveolin-1 regulates proliferation and metastasis of human breast cancer cells by activating Her-2

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Abstract: Caveolin-1 is an important gene in breast cancer. Based on the analysis of cellular and molecular levels. Further discussions on the regulation of phosphorylation of Her-2 Caveolin-1 (Cav-1) and roles in the Akt/ERK pathway have been made. We selected 50 cases of invasive breast cancer tissues and used immunohistochemical techniques to assess cav-1, and to understand its relationship with EGFR, Her-2, Ki-67 expression as well as with other conventional clinico-pathological parameters. Ectopic expression of cav-1 in human breast cancer cell lines was performed to investigate changes in cellular proliferation, migration. The role of cav-1 in p-Her-2 expression and signaling was evaluated by Western blot. The expression of Cav-1 is associated with Cav-1 and Her-2/p-Her-2 and malignance in the breast tissue. The expression of Cav-1 is associated with lymph node metastasis and clinical stage. Cav-1 expression at higher levels of human breast cancer cell proliferation, migration and invasion ability has changed, with the increase of EGF stimulation. Cav-1 had no obvious effect on total expression of Her-2, but can regulate the phosphorylation level of Her-2, under the EGF stimulation; it can decline activation levels of Akt/ERK pathway. Cav-1 may promote the growth of breast cancer cells via enhancing EGF-induced Her-2 activation (phosphorylation) and downstream signal transduction.

Keywords: Breast cancer, Her-2, signal pathway, caveolin-1, tumor suppressor

Introduction

Breast cancer is the most common cancer in women. The 5-years relative survival rate in patients with local breast cancer is 98%. However, the rate is only about 27% in patients with distant metastases [1]. Development of the normal breast and breast carcinogenesis involves a complex interplay of growth factors, steroids, activation of oncogenes and inactivation of tumor suppressor genes. The ErbB family of transmembrane receptor tyrosine kinases [ErbB1 or epidermal growth factor receptor (EGFR), ErbB2 or Her-2/c-neu, ErbB3, and ErbB4] and ligands has been consistently implicated in mammary gland tumor genesis in both humans and rodents. The Her-2 proto-oncogene encodes a tyrosine kinase receptor that is amplified and overexpressed in human breast tumors. Caveolae are flask-shaped invaginations of the plasma membrane that were first observed by electron microscopy in the1950. Many signal molecules are localized in caveolae including Src family kinases, H-ras, protein kinase C, epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor, and endothelial nitric oxide synthase; Cav-1 can interact with these signal molecules via its scaffolding domain and plays an important role in signal transduction, membrane traffic [2]. The exact role of cav1 in tumor regulation is unclear. Many studies suggest that cav1 is a tumor suppressor gene. Down-regulation of cav1 expression was observed in breast, lung, colon, and ovarian cancers. In contrast, other studies have reported that cav1 expression was up-regulated in human cancers, including prostate cancer and esophageal squamous cell carcinoma [3, 4]. Sunaga et al. [5] reported that cav1 acted like a tumor suppressor gene in small cell lung cancer, whereas in non small cell lung cancer, it seems required for cell survival

and growth. These results suggest that the roles of cav1 vary depending upon the type of tumor.

Extra-cellular signal-regulated kinase 1/2 (ERK1/2) pathway is well implicated in cell transformation, invasion, and metastatic processes [6, 7]. Williams et al. [8] found that tumors lacking cav1 expression promoted ERK1/2 hyperactivation in mammary tumorigenesis.

Recent studies have shown reduced caveolin-1 protein levels in a number of human cancers including lung cancer, colonic cancer, ovarian cancer, sarcoma and even breast cancer, suggesting a negatively regula-tory role for caveolin-1 in tumour development [9-12]. In this study, we investigated the expression pattern of caveolin-1 in invasive ductal carcinoma by immuno-histochemistry and also its value as a tumour suppressor in breast carcinogenesis. We also evaluated caveolin-1 status and its relationship to EGFR, Her-2, Ki-67 expression as well as to other conventional clinico-pathological parameters.

Studies shown that a loss of caveolin-1 (Cav-1) in the stromal compartment is a novel biomarker for predicting poor clinical outcome in all of the most common subtypes of human breast cancer, including the more lethal triple negative subtype [13, 14]. A loss of stromal Cav-1 predicts early tumor recurrence, lymph-node metastasis, tamoxifen-resistance, and poor survival.

Subjects and methods

Patients and specimens

Fifty female patients diagnosed with breast invasive carcinoma at the Affiliated Hospital of Hebei University between 2006 and 2008 were included in this study. 20 cases of adjacent noncancerous tissue were considered as the negative control group, and all of specimens were reviewed by double-blind study. All of the primary patients were female ranging from 28 to 76 years old (mean, 52.3 years old) without chemotherapy, radiotherapy, or hormone therapy before surgical resection. In accordance with Eison and Eiiis semi-quantitative, levels in 50 cases of histological grade I II III are respectively 26, 18 and 6; TNM stage as NCCN 20l0 criteria defined 13 cases of I, 19 cases of II, 15 cases of III, 3 cases of IV. 29 cases with lymph node metastasis; 21 cases with no lymph node metastasis. ER +40 cases; ER-10 cases. PR +35 cases; PR -15 cases. Her-2 +24 cases; Her-2 -26 cases. 20 cases of tumor diameter within 2 cm and 30 cases more than 2 cm. Ki-67 +23 cases; Ki-67 -27 cases. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Hebei Medical University. Written informed consent was obtained from all participants.

Immunohistochemical (IHC)

A manual immunohistochemical procedure was performed, using 4-μm-thick tissue sections, then HE staining and immunohistochemical. The antibodies used in this study were anti-cav-1, anti-Her-2, anti-p-Her-2 and anti-Ki-67. PBS was used as the negative control group. SP immunohistochemical staining was used; methods of operation were according to reagent instructions. DAB after the color with hematoxylin, dehydrated and transparent neutral gum after cementing. Each section was randomly selected five high number > 400 each horizons 200 cells, according to the color strength and positive cell percentage of marking and grading. By semi-quantitative determination of Caveolin 1 coloring, dyeing strength score ranged from 0 to 3 (0 = no, 1 = weak, 2 = medium, 3 = strong), and dyeing cell density ratio from 0 to 100. Results were determined by cell density of dyeing strength multiplying the percentage. In order to facilitate statistical analysis, the results of immune dyeing using dichotomy were divided into low expression group and high expression group. According to the colour intensity, Her-2 and p-Her-2 were divided into four grades, only 3 + as positive. Ki-67 positive cells percentages were calculated in every 500 tumor cells, and then the average positive rate in five views were calculated respectively.

Cell culture

The human breast cancer cell line MDA-MB-453, MCF-7, MDA-MB-436 and MDA-MB-231 (Columbia University in the United States gifes) was cultured in minimal essential medium (MEM) (life technologies Gibco, grand Island, NY, USA) with 10% (v/v) fetal bovine serum (FBS) (thermo Scientific HyClone, Waltham, Ma, USA) and penicillin (100 units/ml), streptomycin (100 μg/ml) (north China Pharmaceutical

Co., Shijiazhuang, Hebei, China) in a humidified atmosphere with 5% CO_2 at 37°C.

Western blot was used to examine Cav-1 and Her-2 levels

Serum cells were placed immediately on ice, washed twice in cold PBS and lysed with cold lysis buffer as described previously. After protein concentration was determined using the BCA protein determination kit (Pierce, Rockford, Il, USA), the same volume protein samples were transferred to the polyvinylidene fluoride membrane (Millipore, Billerica, Ma, USA) on 10% SDS-page electrophoresis. After TBST liquid containing 5% skimmed milk was closed, each membrane respectively incubated with rabbit anti-Cav-1 (1:3000) (Santa Cruz, Dallas, Texas, USA), rabbit anti-Her-2 antibodies (1:1000) (Danvers, Ma, USA), rat anti-β-actin (1:5000) (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA). PVDF membrane were washed three times after the homologous horseradish peroxidase (1:5000) (Beyotime, Haimen, Jiangsu, China) incubated twice, and then ECL (Beyotime, Haimen, Jiangsu, China) color and exposed in dark room. The stripes width and grey value were read by ImageJ software (national Institutes of Health, Bethesda, MD, USA). The data was analyzed by statistical Microsoft. This experiment was repeated three times.

Cell transfection

The cells of exponential growth were transfected in experiments. By QIAGEN plasmid extraction kit, pcDNA3.1-Cav-1 plasmid was extracted and pcDNA3.1 plasmid was considered as the negative control group. And MDA-MB-453 cells were transfected. All specific steps refered kit instructions. Experimental groups: transfection Cav-1 genome (Cav), transfection empty carrier group (pcDNA3.1) and blank control group (MDA-MB-453). The cells were screened after 48 hours with 1640 medium without serum starvation 4 h, and finally washed once with cold PBS, and were immediately plunged into liquid nitrogen. Western blot was used to detect expression of Cav-1 of each group as the above method. RT-PCR method was used to detect expression of m-RNA expression of three kinds of cells. With the three kibds, the total RNA was extracted respectively, through the reverse transcription box of cDNA synthesis. β-actin was regarded internal reference, Cav upstream primer: 5'-GTCGCTCTCAGGAACAGCAG-3' and downstream primers: 5'-AGGGGACGGCCCTTTAAT-3'. GAPDH upstream primer: 5'-GAAGGTGAAGGTCGGAGTC-3' and downstream primers: 5'-GAAGATGGTGATGGGATTTC-3'. Tested expression level of Cav-1 mRNA.

MTT colorimetric assa

Cav-1, pcDNA3.1 cells were cultured in 96 well plates, 8000/cell, 100 μl/cell. Each kind of cells set up four groups: 0 nm, 4 nm, 20 nm, 100 nm EGF. After 1640 culture medium containing different concentration of EGF 48 h cultivation, it was to MTT (5 mg/ml) (Sigma-Aldrich, Saint Louis, MO, USA) 20 μl/cell. Continued to cultivate 4 h. Abandoned culture medium, then added 150 μl DMSO in every cell to dissolve the formazan abundantly for 10 min oscillation. 490 nm wavelength OD value of each cell was detected by enzyme-labeled meter. Then, the results were recorded and analyzed, this experiment was repeated three times.

Wound healing assay was used to examine the migration ability in stably transfected cells

Cav-1, pcDNA3.1 cells were cultured in 24 well plates, 1 ml per plate (about 2×10^6/cell). Each cell inoculated 6 holes, which would be cultivated for 24 h and then hungered for 4 h. A straight line was drawn at the bottom of the plate and the culture medium was washed and replaced. Each kind of cells was respectively divided into two groups: without EGF (EGF; Miltenyi Biotec, auburn, Ca, USA) group and 20 nm EGF group, both for 3 plates. They were observed under 10 × inverted microscope and photographed at 0 h, 24 h, 48 h, 72 h, 96 h, and the culture medium was changed every 24 h. Measuring software was used to analyse as the following formula, Migration rate = (scratches initial width-scratches the current width)/2/scratch the initial width × 100%.

Invasion assay

Serum-starved cells (1×10^6 cells in 200 μl of serum-free MEM) were placed in the top chamber of transwell invasion chambers (8.0 μm polycarbonate membrane inserts; Corning, Lowell, Ma, USA). The lower chamber was filled with 600 μl MeM containing 10% FBS. After a 24-h incubation period in a humidified atmosphere containing 5% CO_2 at 37°C, noninvasive cells were removed from the upper surface of the transwell membrane with a cotton swab,

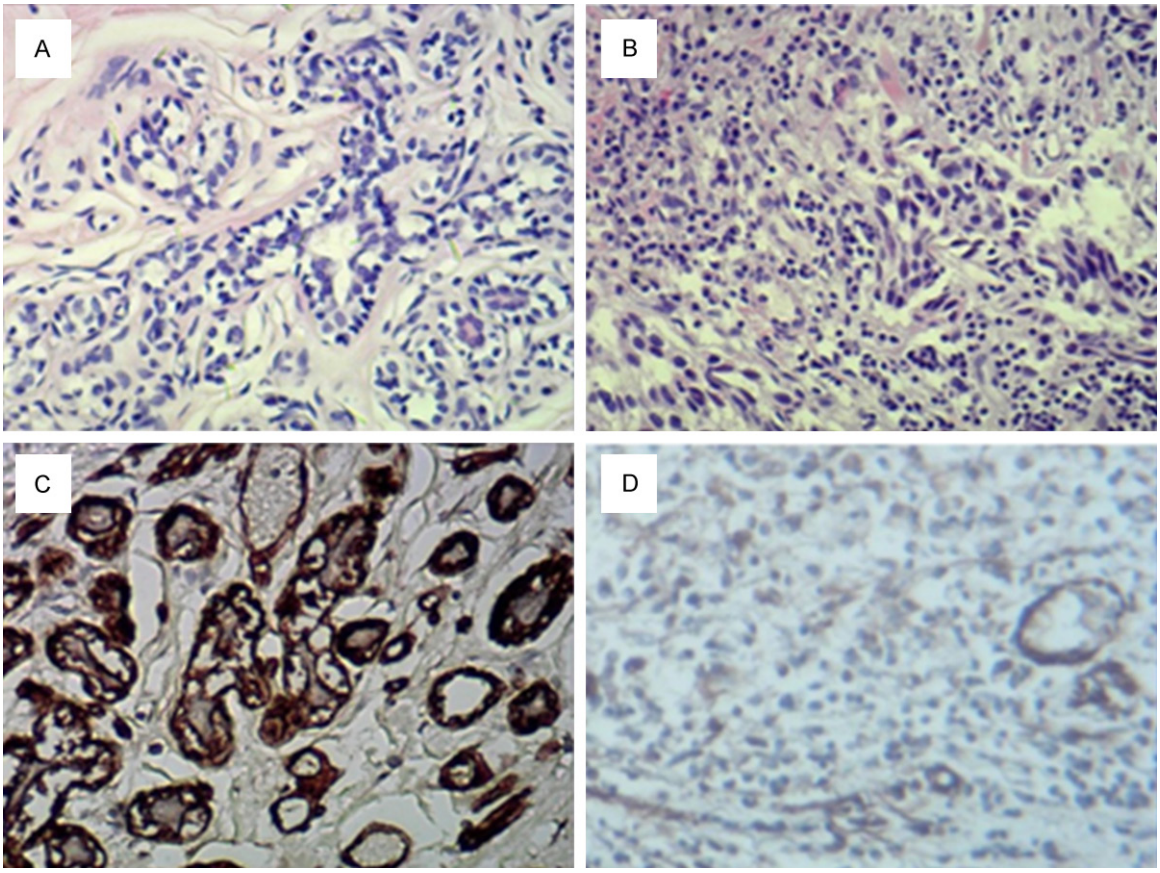

Figure 1. Immunohistochemical analysis of Caveolin-1 expression in normal, human breast tissue and breast cancer tissue. Hematoxylin and eosin staining in normal breast tissue (A) and in breast cancer tissue (B). Immunohistochemical analysis of Caveolin-1 expression in normal breast tissue (C) and in breast cancer tissue (magnification, × 400).

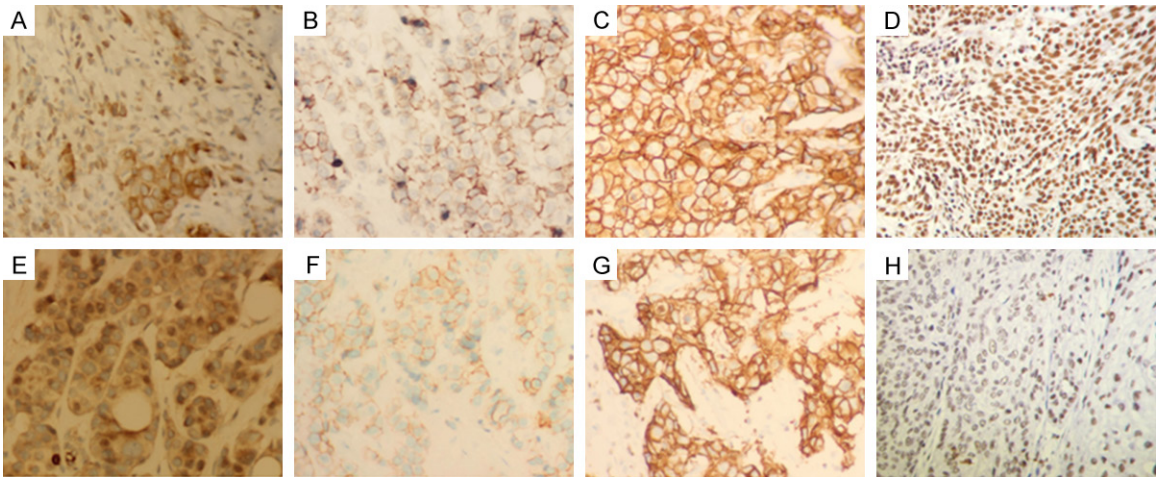

Figure 2. Immunohistochemical staining of breast cancer tissue. The group of Cav-1 weak expression (A) shows expression of p-Her-2 (B), Her-2 (C) and Ki-67 (D). However, the other group shows an adverse result, the group of Cav-1 strong expression (E) shows expression of p-Her-2 (F), Her-2 (G) and Ki-67 (H) (magnification, × 400).

and invasive cells on the lower membrane surface were fixed with Precooled 95% ethanol, stained with H&E, photographed and counted through a microscope at 200 × magnification in five fields. These experiments were repeated twice.

Table 1. Relationship between the expressions of Cav-1 in breast cancer tissues and the clinical characteristics

Characteristic	N	Cav-1		*P*
		Low Expression	High Expression	
Age (year)				
< 50	16	10	6	0.125
≥ 50	34	28	6	
Tumor size (cm)				
≤ 2	20	13	7	0.137
> 2	30	25	5	
Lymphnode				
Positive	29	25	4	0.047
Negative	21	13	8	
Grade				
I	26	19	7	0.848
II	18	14	4	
III	6	5	1	
ER				
Positive	40	29	11	0.246
Negative	10	9	1	
PR				
Positive	35	26	9	0.665
Negative	15	12	3	
Her-2				
Positive	24	21	3	0.067
Negative	26	17	9	
Ki-67				
Positive	23	20	3	0.094
Negative	27	18	9	
Menopaus				
Pre	13	8	5	0.156
Post	37	30	7	
TNM				
I+II	29	19	10	0.041
III+IV	21	19	2	

The expression of Cav-1, 4G10, Her-2, p-Akt, Akt, p-ERK, ERK and β-actin were assessed by western blot

The three kinds of cells which had been culture were to go through the Western blot that was used to detect the expression of each protein as methods mentioned above. A dilution ratio of resistance was as follows rabbit polyclonal antibody Cav-1 1:3000, rabbit anti Her-2 1:1000; polyclonal antibody of the latter, mouse fight 4 G10 (Millipore Temecula, Ca, USA) monoclonal antibody 1:2500, rabbit polyclonal antibody against p-ERK 1:3000 (Danvers, Ma, USA), rabbit polyclonal antibody against ERK 1:800, rabbit anti p-Akt (Danvers, Ma, USA) polyclonal antibody 1:000, rabbit polyclonal antibody against Akt 1:000, rat anti beta actin monoclonal antibody 1:5000. The experiment was repeated twice.

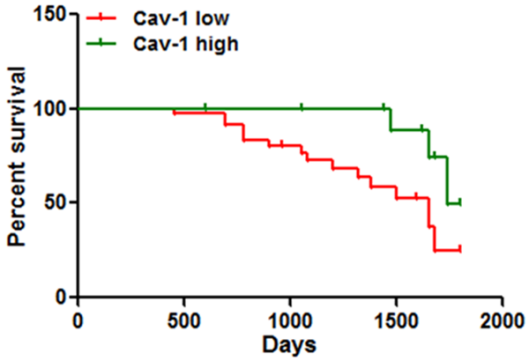

Figure 3. Kaplan-Meier survival analysis of patients with breast cancer. A significant difference was found between the overall survival of patients with breast cancer that have low and high Cav-1 levels ($P = 0.032$).

Statistical analysis

For all experiments, raw data was analyzed and graphed using GraphPad Prism 5 (GraphPad Software, la Jolla, Ca, USA), and appropriate statistical tests were chosen according to the data analyzed using GraphPad Prism 5 or SPSS version 16.0 (SPSS Software, Armonk, NY, USA). Unless otherwise stated, all data is reported as mean ± SEM; and indicate $P < 0.05$ and $P < 0.01$, respectively.

Results

The correlation of Cav-1 and Her-2/p-Her-2 between breast cancer tissue and clinicopathology

Our research results indicate that the expression of Cav-1 is associated with Cav-1 and Her-2/p-Her-2 and malignance in the breast tissue. Immunohistochemical results showed that the positive expression of Cav-1 is mainly distributed in the cytoplasm of mammary epithelial cells. The degree of positive expression in normal breast tissue is higher, but lower in invasive breast cancer cells (**Figure 1**), and its expression is reduced with the increasing malignant

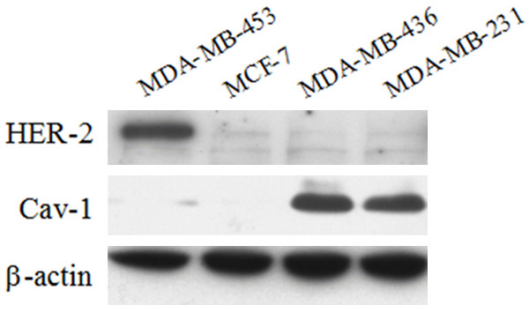

Figure 4. Western blot was used to detected expression of Her-2 and Cav-1 about MDA-MB-453, MCF-7, MDA-MB-436 and MDA-MB-231 cells.

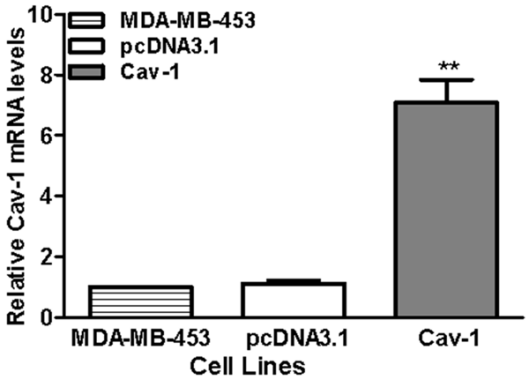

Figure 5. Total RNA was prepared from MDA-MB-453 cells either stably transfected with pcDNA3.1 or Caveolin-1pcDNA plasmid, and quantitative real time PCR was used to analyze the expression of the Caveolin-1 gene and normalized to the expression levels of the GAPDH gene. The levels of Caveolin-1 mRNA relative to Control are showed.

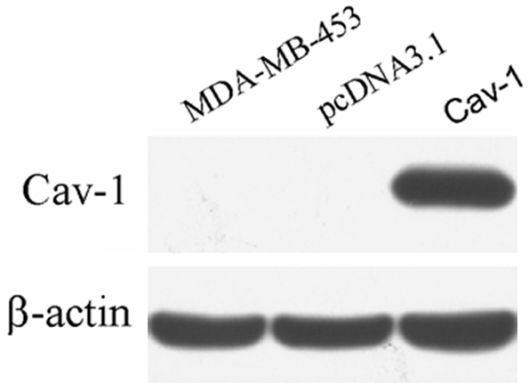

Figure 6. The differential expression of Caveolin-1 in MDA-MB-453 cell lines after stably transfected with pcDNA3.1-Cav-1 plasmid detected by Western-blot.

degree, which is statistically significant. The result of Cav-1 in low expression of breast cancer is similar to epithelium in lung cancer, colon

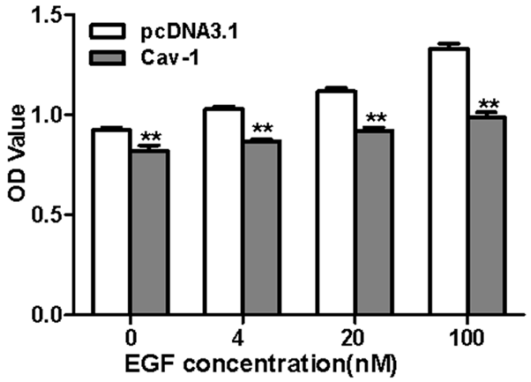

Figure 7. The proliferation conditions of MDA-MB-453 cells stably transfected with pcDNA3.1 or Caveolin-1pcDNA plasmid detected by MTT after EGF stimulating for 48 h. **P < 0.01, vs. PcDNA3.1 group (corresponding EGF concentration).

cancer, ovarian cancer and stromal sarcoma. The expression of Her-2/p-Her-2 was mainly distributed in the mammary gland epithelial cell membrane. The expression level of Cav-1 in invasive breast cancer has nothing to do with Her-2, and is negatively correlated with p-Her-2 expression ($P < 0.05$). As a tumor suppressor, this preliminary revealed that Cav-1 can affect activation of Her-2. Cav-1 influences the proliferation through regulating Her-2 (**Figure 2**).

From the relationship between the expressions of Cav-1 and clinical pathologic factors of breast cancer in the table (**Table 1**), it can be seen that the expression of Cav-1 is associated with lymph node metastasis and clinical stage, that is statistically significant. The reducing expression of Cav-1 influences the 5-year survival rate of patients. It indicates that Cav-1 plays an important role in breast cancer metastasis and infiltration. In patients with Cav-1 positive expression, the 5-year survival rate was higher than that negative expression. Kapian-Meier method is adopted to improve the survival analysis, and the results show that the low Cav-1 expression indicates shorter overall survival and poor prognosis (**Figure 3**). The expression level of Cav-1 has nothing to do with age, tumor diameter, ER/PR, Ki-67, or whether patient is in menopause.

Cav-1 reduces the proliferation, migration and invasion in human breast cancer cell lines

Western blot was used to detected expression of Her-2 and Cav-1 between MDA-MB-453, MCF-7, MDA-MB-436 and MDA-MB-231-1 cells

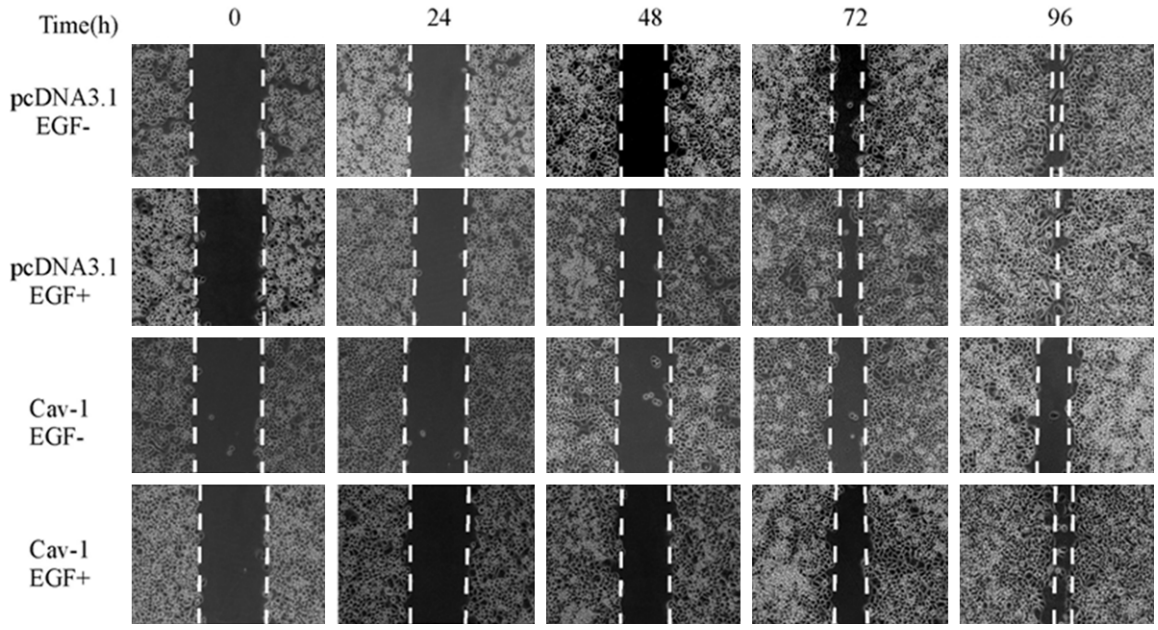

Figure 8. Migration rates in MDA-MB-453 cells stably transfected with pcDNA3.1 or Caveolin-1pcDNA plasmid observed by Wound healing assay.

(**Figure 4**). It shows that the expression of Cav-1 is lower and Her-2 is higher in MDA-MB-453 cells. It also can be validated by building stable transfected pcDNA3.1-Cav-1 plasmid, Western blot and RT-PCR (**Figures 5**, **6**). Western blot showed that expression level of Cav-1 is obviously more in Cav-1. Caveolin plasmid transfection group than in empty plasmid transfection group, which is a significant difference and suggests that successful transfection.

The role of Cav-1 in basal and EGF-induced cell growth and viability was then evaluated using the MTT assay (**Figure 7**). Cav-1 cells were accompanied by significant reduction in basal and EGF-stimulated growth as compared with pcDNA3.1 cells. As Cav-1 expression level increased, the proliferation ability of human breast cancer cell significantly reduced, as the same as EGF stimulation.

From the wound healing assay with 20 nm EGF after 96 hours stimulation in breast cancer cells (**Figure 8**), it can be observed the migration ability of Cav-1 cells is significantly lower than pcDNA3.1 at each time point when it is without EGF stimulation. After 20 nm EGF stimulation, the mobility of pcDNA3.1 and Cav-1 cells were higher than without EGF stimulation group at each point in time that. The expression level of Cav-1 is negativly correlated with migration ability of breast cancer cell. The ability of migration can speed up after EGF stimulation. Increasing the expression of Cav-1 can inhibit breast cancer cell migration.

Another critical step in cancer metastasis is the detachment of tumor cells from the primary sites and invasion of the surrounding tissues. Transwell was used to detect that increased expression level of Cav-1 can change invasion ability of human breast cancer cells (**Figure 9**). The number of Cav-1 penetrated through cell membrane cell (16 + 2) is 2 times lower than the control group pcDNA3.1. It shows that increased expression level of Cav-1 can reduce the invasion ability of human breast cancer cells.

Cav-1 influences activation of Her-2 receptor in human breast cancer cells

Western blot is used to detect Her-2/phosphorylation Her-2[(4G10 clone to detect active, phosphorylated form of Her-2 (p-Her-2)], ERK/phosphorylation of ERK and Akt/phosphorylated Akt protein expression levels between Cav-1 and pcDNA3.1 cells after EGF stimulation (**Figure 10A**). After Her-2 antibodies and phosphorylated tyrosine resistance (4G10) detection in EGF, the total expression of Her-2 has no obvious changes between two kinds of cells. The expression of Cav-1 and p-Her-2 significantly decreased at all time points compared

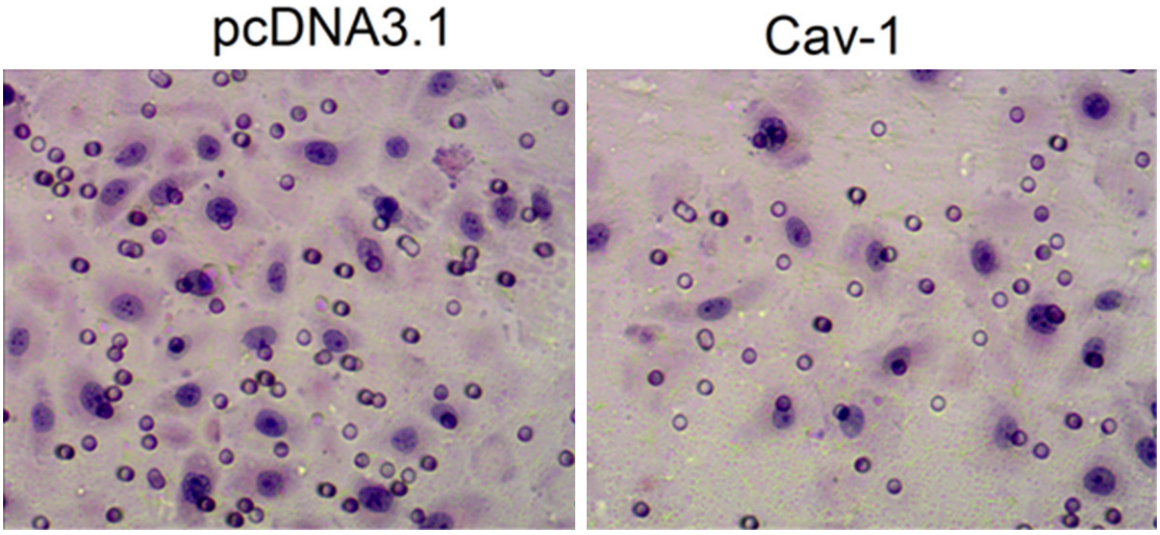

Figure 9. The invasion abilities of MDA-MB-453 cells stably transfected with pcDNA3.1 or Caveolin-1pcDNA plasmid examined by Transwell cell invasion assay. Cells were cultured for 24 h and then fixed by 95% alcohol for 30 min, stained with hematoxylin and eosin, and photoed under 200 × microscope. The number of invasion cells was counted in five fields.

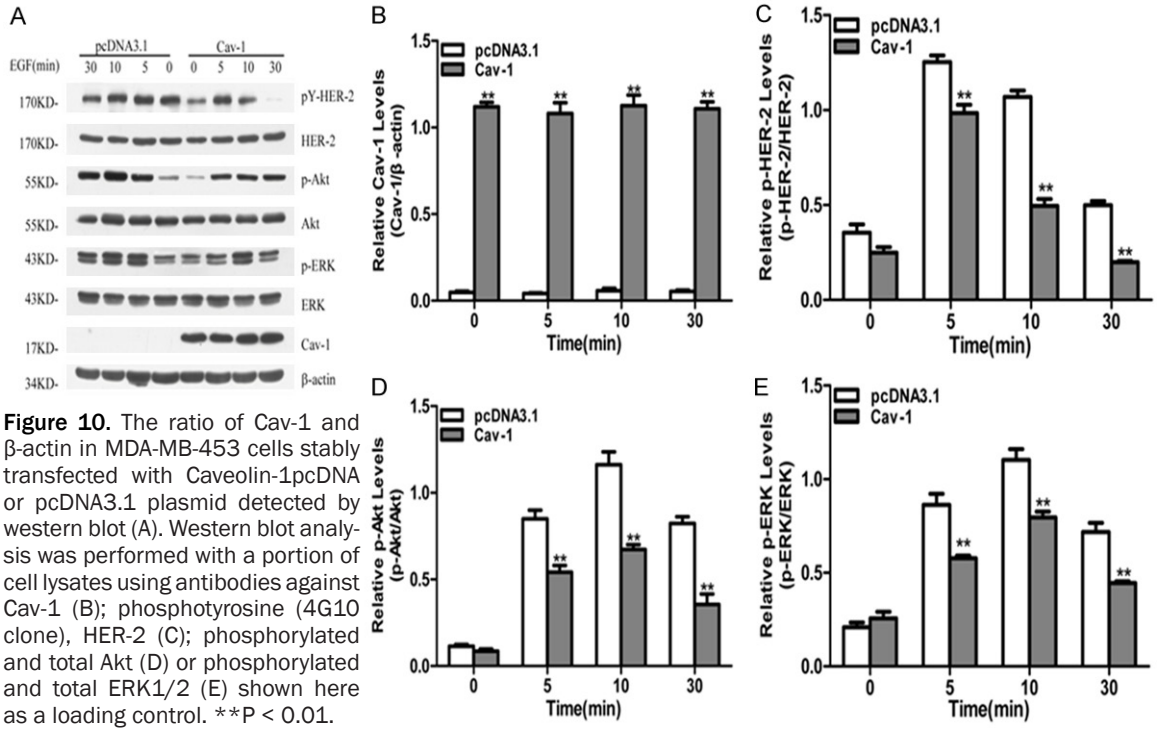

Figure 10. The ratio of Cav-1 and β-actin in MDA-MB-453 cells stably transfected with Caveolin-1pcDNA or pcDNA3.1 plasmid detected by western blot (A). Western blot analysis was performed with a portion of cell lysates using antibodies against Cav-1 (B); phosphotyrosine (4G10 clone), HER-2 (C); phosphorylated and total Akt (D) or phosphorylated and total ERK1/2 (E) shown here as a loading control. **P < 0.01.

with pcDNA3.1 group. This shows that Cav-1 had no obvious effect on total expression of of Her-2, but can regulate the phosphorylation level of Her-2. When the expression of Cav-1 increases, the expression of p-Her-2 decreases, With Akt/ERK antibodies and phosphorylation Akt/ERK under the stimulus EGF the two kinds of cells have no obvious differences in the expression quantity. The expression of p-ERK and p-Akt is less in Cav-1 group than in pcDNA3 group (**Figure 10B-E**). This suggests that under the EGF stimulation, it can decline activation levels of Ras-Raf-MEK-ERK pathway and AKT pathway. Increased expression of Cav-1 can decrease the level of p-Her-2. Cav-1 can regulate proliferation, migration and inva-

sion and a series of biological behaviors of human breast cancer cells by affecting Ras-Raf-MEK-ERK and activation of AKT signal pathway.

Discussion

Caveolae were identified in 1950s by electron microscopy as plasma membrane invagination. Cav-1 was initially identified as Kda membrane protein located at the locus 7a31.1 fragile genomic region which is often deleted in Cancers [15-17]. Cav-1 membrane of Caveolae is enriched in various signaling molecules, receptors, and non receptor tyrosine kinases and lipid which act as intermediateries in cell signaling. Various studies have reported that Cav-1 acted as a tumor suppressor gene in many human cancers. For example, in breast and lung cancers, expression of Cav-1 inhibited cell proliferation and metastasis in vivo and in vitro [9]. However, several recent reports demonstrated that over expression of Cav-1 is associated with poor prognosis in prostate carcinoma and esophageal squamous cell carcinomas suggesting that Cav-1 may play different roles in different tumors. Cav-1 is suppressed in the ovary and breast, colon tumors, and sarcomas, whereas it is up-regulated in kidney, prostate, and stomach tumors [9-12].

In our study, the expression of Cav-1 was down-regulated in Invasive Ductal Carcinoma of breast cancer compared with normal breast epithelial tissue, and showed decreasing trend with increasing tumor genesis and was statically significant.

In contrast, the expression of Her-2/pHer-2 which is mainly distributed in epithelial of cell membrane of mammary gland has reciprocal relation with Cav-1 expression. Though Cav-1 had extinct role in cell proliferation and activated Her-2 for cell proliferation and migration.

Though, Cav-1 expression showed significant role in lymph node metastasis and clinical staging in Breast Cancer. However, our study revealed better survival prognosis with higher expression of Cav-1 gene compared to lower expression showing no correlation between age, tumor diameter, ER/PR, Ki-67 expression level including Pre menopause and post menopause status.

Phosphorylation of Akt/ERK pathway plays important role in cell proliferation, migration and growth of tumor. Thus cell lines transfected with empty vector compare to Cav-1 transfected cells treated with EGF has hypo-posphorylation state which reciprocally effect in tumor genesis and Cav-1 itself work as tumor suppressive gene.

Cav-1 has no obvious effect on total expression of Her-2 but can regulate phosphyrylation level of Her-2.

In conclusion, our data suggest that caveolin-1 over expression enhances Her-2 signaling and confers increased proliferative and migratory properties in breast cancer cells. Her-2 expression has been associated with poor clinical outcome in patients with breast cancer [18].

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Disclosure of conflict of interest

None.

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