Original Article

Wnt/β-catenin pathway is required for epithelial to mesenchymal transition in CXCL12 over expressed breast cancer cells

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Abstract: CXCL12 is positively associated with the metastasis and prognosis of various human malignancies. Cancer-associated fibroblasts (CAFs), the main cells secreting CXCL12, are capable of inducing epithelial to mesenchymal transition (EMT) of breast cancer cells. However, it has not been completely understood whether CXCL12 is involved in EMT of breast cancer cells and the underlying mechanisms. The present study aimed to investigate the effects of CXCL12 on the EMT and cancer stem cell (CSC)-like phenotypes formation by transfecting pEGFP-N1-CXCL12 plasmid into MCF-7 cells. Real time-PCR and Western blot analysis demonstrated the successful over expression of CXCL12 in MCF-7 cells. Cell counting kit-8 assay, wound healing assay and Transwell invasion analysis confirmed that over expression of CXCL12 significantly promoted the proliferation, migration and invasion in MCF-7 cells (P<0.05). In addition, ALDH activity was dramatically enhanced compared with parental (P<0.001), accompanied by the notably elevated mRNA and protein levels of OCT-4, Nanog, and SOX2 in CXCL12 overexpressed-MCF-7 cells (P<0.001). Furthermore, we observed the down regulation of E-cadherin and up regulation of vimentin, N-cadherin, and α-SMA in CXCL12 overexpressed-MCF-7 cells (P<0.01). Meanwhile, western blot and immunofluorescence assay showed that over expression of CXCL12 activated Wnt/β-catenin pathway to induce EMT of MCF-7 cells, as evidenced by the increased expression of E-cadherin after silencing β-catenin by siRNA interference (P<0.001). Collectively, our findings suggested that over expression of CXCL12 could trigger EMT by activating Wnt/β-catenin pathway and induce CSC-like phenotypes formation to promote the proliferation and metastasis in MCF-7. Hence, CXCL12 may become a promising candidate for breast cancer therapy.

Keywords: CXCL12, breast cancer, epithelial to mesenchymal transition, cancer stem cell-like phenotypes, Wnt/β-catenin pathway

Introduction

Breast cancer, a frequent hormone-dependent tumor among women, accounts for 23% in all female malignancies with 3.1% increasing rate annually [1-3]. Orthotopic breast cancer is non-lethal, but once the cancer cells undergo epithelial-to-mesenchymal transition (EMT), the loose connections between cells will result in the intravasation of primary tumor cells and the formation of metastases, which is fatal to the patients. On the other hand, EMT is helpful for the cancer stem cell (CSC)-like phenotypes formation of cancer cells to resist DNA damage which makes the cancer cells unresponsive to chemotherapeutic and apoptotic drugs [4]. Therefore, searching for the key genes involved in the progression of EMT is crucial for developing effective approaches for breast cancer therapy.

CXCL12 (stromal cell-derived factor-1, SDF-1), belongs to the CXC subfamily, is one of the most evolutionary conserved chemokines [5]. Clinical researches revealed that the expression of CXCL12 was up regulated in various carcinoma including breast carcinoma, ovarian carcinoma, and papillary thyroid carcinoma [6-8], which was notably correlated with the prognosis [9]. Besides, the organs which exhibit an enhanced
secretion of CXCL12, such as lung, liver, bone marrow, kidney, and brain, are the most favorable metastatic sites of breast cancer [10-12]. In vitro studies confirmed that CXCL12 bound to CXCR4 or CXCR7 on the surface of breast cancer cells to activate intracellular pathways mediating tumor initiation, promotion, progression and metastasis [13]. In addition, both Soon et al. and Yu et al. have reported that breast cancer-associated fibroblasts (CAFs) could induce EMT in breast cancer cells [14, 15]. CAFs are one of a major source of CXCL12 production [4]. However, the contribution of CXCL12 to the progression of EMT in breast carcinoma and the detailed mechanisms has not been well understood.

In our study, we successfully established the over expressing CXCL12 MCF-7 cell model to evaluate whether CXCL12 could affect the proliferation, migration, invasion, EMT and the acquisition of a CSC-like phenotype in breast cancer cells and explore the related pathway. Our result identified that over expression of CXCL12 induced EMT by Wnt/β-catenin pathway, and conferred CSC-like phenotype on MCF-7 cells, thus enhanced the proliferation, migration, and invasion of breast cancer cells.

Materials and methods

Cell and mammosphere culture

MCF-7 cell line was obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with streptomycin/penicillin (100 U/mL) and 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) at 37°C in a humidified atmosphere containing 5% CO2.

For mammosphere culture, MCF-7 cells were planted into ultralow attachment plates (Corning, NY, USA) at a density of 1×10^5 cells/mL and cultured in serum free DMEM/F12 (1:1) medium supplemented with 20 ng/mL epidermal growth factor (EGF, PeproTech, St. Louis, MO, USA), 10 ng/mL basic fibroblast growth factor (b-FGF, PeproTech), ITS (insulin, transferrin and selenium, Sigma-Aldrich, St. Louis, MO, USA), and B27 (GIBCO). 2 mL fresh mammosphere media was added every 2 to 3 days without decanting old media. Mammospheres were collected every seven days for ALDH activity assay.

Plasmid and transfection

The plasmid pEGFP-N1-CXCL12 harboring CXCL12-coding sequences was constructed by Wanleibio Co., Ltd. (Shenyang, China). When cells reached to 80%-90% confluence, the pEGFP-N1-CXCL12 and the empty vector (pEGFP-N1) were transfected into MCF-7 cells respectively using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) strictly according to the manufacturer's directions. The non-transfected control cells were experimented in parallel. The stably transfected cells were screened from complete DMEM containing 400 μg/mL G418 (Invitrogen) after 24 h of transfection, and the expression of CXCL12 was detected at 7 d-14 d of transfection.

Cell counting Kit-8 (CCK-8) assay

Cells were inoculated in 96-well plates (2×10^3 cells per well) with five replicates for each sample and cultured at 37°C in an atmosphere of 5% CO2 for 24 h, 48 h, 72 h, and 96 h respectively, followed by incubation with CCK-8 (Beyotime, Haimen, China) for 1 h. Thereafter, optical density (OD) values were read at 450 nm with a microplate reader (BIOTEK, Vermont, USA).

Wound healing assay

Cells were planted in 6-well plates and cultured to 80%-90% confluence. Subsequently, the artificial wounds were created on the confluent cell monolayer using 200 μL pipette tips, and the detached cells were washed twice with FBS free culture medium. Then the cells were grown in FBS free DMEM medium, and migrating cells were imaged under an inverted microscope and calculated at 12 h and 24 h of culturing.

Transwell invasion assay

The 24-well transwell chambers (Corning, Tewksbury, MA, USA) were pre-coated with matrigel (BD Biosciences, San Jose, CA, USA). Cells were resuspended in complete DMEM and plated in the upper chamber of the transwell at a density of 2×10^4 per well. 800 μl DMEM plus 30% FBS was added into the lower chamber. Cells were cultured in the transwell system for 24 h, then the non-migrated cells at the upper-surface of the membrane were removed with cotton swabs. The invading cells which migrated to the undersurface of the
membrane were fixed in 4% paraformaldehyde for 20 min before stained with crystal violet for 5 min. The number of invaded cells was counted in five randomly selected fields in a blinded manner under an inverted microscope.

**ALDH activity analysis**

The activity of ALDH was measured by Aldehyde Dehydrogenase Activity Colorimetric Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). In brief, mammosphere cell suspension (200 μl ALDH binding buffer per 1×10⁶ cells) was centrifuged at 13000 g for 10 min. Thereafter, 50 μl supernatant was incubated with 2 μl ALDH substrate, 43 μl ALDH binding buffer, and 5 μl acetaldehyde for indicated times in the dark. The absorbance was determined by a microplate reader at 450 nm, and ALDH activity was calculated according to the manufacturer’s instruction.

**Immunofluorescence assay**

Cells were grown on coverslips to 80% confluence and fixed in 4% formaldehyde for 15 min followed by permeabilized in 0.1% Triton X-100 (Amresco, Cochran Road Solon, USA) for 30 min. After washed with PBS for three times, the coverslips were blocked with goat serum (Solarbio, Beijing, China) for 15 min at room temperate. Thereafter, cells were incubated with relevant primary antibodies against β-catenin and E-cadherin (both 1:200 diluted, BOSTER, Wuhan, China) at 4°C overnight and subsequently stained with Cy3-labeled goat anti-rabbit IgG secondary antibody at a dilution of 1:200 (Beyotime, Haimen, China) at room temperature. Unbound antibodies in both steps were removed with PBS. The coverslips were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and rinsed with PBS before being mounted inversely onto slides with anti-fluorescent mounting media (Solarbio). Protein fluorescences were observed under a laser scanning confocal microscope.

**β-catenin-siRNA interference**

siRNA for β-catenin and control siRNA were obtained from GenePharma Co., Ltd (Shanghai, China). Sequences were as follows: β-catenin-siRNA: 5'-CAGGACGUCUUAAGUAAUA-3’; control siRNA: 5'-UUUCUGAGCGUGUCGCU-3’. 75 pmol β-catenin-siRNA and control siRNA were transfected into CXCL12 over expressing MCF-7 cells respectively using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) completely following the manufacturer’s instructions. Cells were collected at 24 h after transfection for western blot.

**Real time-PCR**

Total RNA of each sample was extracted with an RNA Fast Extraction Kit (BioTeke, Beijing, China) and was reverse-transcribed into cDNA. 1 μl cDNA was amplified with 10 μl SYBR GREEN mastermix (Solarbio, Beijing, China) and 10 μl primers in an Exicycler™ 96 (Bioneer, Daejeon, Korea) with the following cycling profile: initial denaturation at 95°C for 10 min, 40 cycles consisting of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. The following primers were used: CXCL12, 5'-CAGGACGUCUAAUUAAGUAA-3' (Forward) and 5'-UCUGUGUCGUGUCGCU-3' (Reverser); OCT4, 5'-AGCAGTAAGCAGCGACTGCA-3' (Forward) and 5'-CCAGGAGGACCGAGGA-3' (Reverser); Nanog, 5'-GAGGCAACTC-CTTATCC-3' (Forward) and 5'-CCCAATTACACGATAGA-3' (Reverser); SOX2, 5'-CATCACCCCCAGAAAGCC-3' (Forward) and 5'-CCCAATTACACGATAGA-3' (Reverser); β-actin, 5'-TTGTTGCACTCCACGAC-3' (Forward) and 5'-GAAGGTAGTTTCAACGTTT-3' (Reverser). Relative mRNA expressions were calculated by 2^ΔΔCT method. β-actin was used as an internal control.

**Western blot**

Total proteins were extracted by lysing the cells with RIPA lystate (Beyotime, Haimen, China) containing 1% phenylmethanesulfonyl fluoride (PMSF, Beyotime). The concentration of total proteins was quantitated using a bicinchoninic acid (BCA) protein assay kit (Beyotime). 40 μg proteins from each sample were loaded and separated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The blots were then blocked with 5% non-fat milk for 1 h and incubated with the specific primary antibodies as follows: anti-CXCL12, anti-Nanog, anti-SOX2 (all 1:200 diluted, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-OCT4, anti-Vimentin, anti-Wnt1 (all 1:500 diluted, Bioss, Beijing, China), anti-E-Cadherin, anti-N-Cadherin, anti-α-SMA (all 1:400 diluted, Boster, Wuhan, China), and anti-β-catenin (1:400 diluted, Bioss) at 4°C overnight. After washing four times with TBST, the blots were incubated with
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corresponding secondary antibodies (1:5000 diluted, Beyotime) at 37°C for 45 min. An enhanced chemiluminescence (ECL, Qihai Biotec, Shanghai, China) detection method was used to visualize the target bands, and relative band intensities were analyzed by Gel-Pro-Analyzer software (Bethesda, MD, USA). β-actin was served as an internal control.

Statistical analysis

Statistical analysis was carried out by GraphPad Prism 5.0 software. All data are presented as mean ± standard deviation (SD), and the differences between groups were calculated with one-way analysis of variance (ANOVA). Differences were considered to be significant when \( P < 0.05 \).

Results

Stable overexpression of CXCL12 in MCF-7 cells

We constructed the pEGFP-N1-CXCL12 plasmid and transfected the plasmid into MCF-7 cells to study the function of CXCL12. Western blot and RT-PCR were employed to confirm the expression of CXCL12 in positive monoclonal cells. The protein and mRNA levels of MCF-7 cells transfected with CXCL12 were 3.39-fold and (Figure 1A, \( P < 0.001 \)) and 2.81-fold (Figure 1B, \( P < 0.001 \)) higher than those in respective control cells transfected with pEGFP-N1, indicating that CXCL12 had been over expressed in MCF-7 cells.

Over expression of CXCL12 boosts the proliferation, migration and invasion of MCF-7 cells

CCK-8 assay, wound healing and Transwell invasion assay were performed to investigate the role of CXCL12 in the proliferation, migration and invasion of MCF-7 cells. As shown in Figure 2A, compared with parental, the growth capability of CXCL12 over expressed MCF-7 cells had been elevated significantly since 48 h of culture (\( P < 0.05 \)). Besides, the wound healing rates in CXCL12-overexpressed MCF-7 cells were much higher than parental at the point of 12 h and 24 h after wound creation (Figure 2B, \( P < 0.01 \)). At the same time, over expression of CXCL12 remarkably increased the invasive
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Number of MCF-7 cells (54.2±5.5) as compared to parental (27.2±2.95) (Figure 2C, P<0.001). The above results suggested that over expression of CXCL12 could boost the proliferation, migration and invasion of MCF-7 cells.

Over expression of CXCL12 confers CSC-like phenotype on MCF-7 cells

To address the effect of CXCL12 on the CSC-like phenotypes formation in MCF-7 cells, we
used chromatometry to detect ALDH activity in mammosphere cell suspension. The result proved that over expression of CXCL12 significantly enhanced ALDH activity compared with parental (Figure 3A, P<0.001). In addition, RT-PCR and Western blot analysis showed that over expression of CXCL12 in MCF-7 cells remarkably increased both mRNA and protein levels of OCT-4, Nanog, and SOX2 compared with parental (Figure 3B and 3C, P<0.01). These results demonstrated that over expression of CXCL12 conferred CSC-like phenotypes on MCF-7 cells.

**Over expression of CXCL12 promotes EMT of MCF-7 cells**

To evaluate the effect of CXCL12 on EMT in MCF-7 cells, we observed that over expression of CXCL12 significantly down regulated E-cadherin expression and notably up regulated vimentin, N-cadherin and α-SMA expressions in MCF-7 cells by Western blot analysis (Figure 4A-C, P<0.01). Similarly, E-cadherin, distributed in the connections among cells and cytoplasm, was slightly stained in CXCL12 over expressed cells and dispersed stained in parental and pEGFP-N1 transfected cells (Figure 4D). Our results revealed that over expression of CXCL12 promoted EMT of MCF-7 cells.

**Wnt/β-catenin pathway is involved in CXCL12-induced EMT in MCF-7 cells**

To explore whether Wnt/β-catenin pathway is involved in CXCL12 induced EMT of MCF-7 cells, we employed Western blot to examine the
alterations of Wnt/β-catenin pathway related protein levels. We detected the significantly increased Wnt and β-catenin levels in CXCL12 over expressed MCF-7 cells compared with parental (Figure 5A, P<0.01). Meanwhile, immunofluorescence study revealed that a substantial proportion of CXCL12 over expressed MCF-7 cells exhibited β-catenin transfer from cytoplasm to the nucleus (Figure 5B), suggesting the activation of Wnt/β-catenin pathway. We further silenced β-catenin by siRNA interference, and the western blot results showed that...
β-catenin expression was decreased obviously by 3.33-fold than those in control siRNA cells (Figure 5C, \(P<0.001\)), confirming the successfully silenced β-catenin. Simultaneously, the expression of E-cadherin was remarkably upregulated after silencing β-catenin in CXCL12 over expressed MCF-7 cells (Figure 5C, \(P<0.001\)), which indicated that overexpression of CXCL12 induced EMT in MCF-7 cells through Wnt/β-catenin pathway.

Discussion

The chemokine CXCL12 is highly expressed in multiple carcinomas and plays a decisive role in tumor progression by mediating pro-proliferation and pro-metastatic effects. However, the effect of CXCL12 on the progression of EMT in breast carcinoma and the related mechanism has rarely been reported. In this study, we over expressed CXCL12 in MCF-7 cells, since MCF-7
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cells could co-express CXCR4 and CXCR7 but with non-invasive [16]. We found that CXCL12 promoted the proliferation, migration, and invasion by inducing EMT and CSC-like phenotypes formation in MCF-7 cells, which may be mediated by Wnt/β-catenin pathway.

CXCL12/CXCR4 axis could increase microvesSEL density, and stimulate the growth, migration, and invasion in various tumors containing gallbladder carcinoma, hepatoma, colorectal carcinoma, and breast carcinoma [17-20]. Our study demonstrated that over expression of CXCL12 increased the proliferation, migration, invasion of MCF-7 cells, which was consistent with previous studies.

CD44, CD24 and ALDH1 are recognized as the biomarkers of breast cancer stem cells (BCSCs). CD44 is positively correlated with stem cell-like characteristics and CD24 is related to differentiated features of epithelial cells [21]. Huang and his colleagues demonstrated that CAFs increased the proliferation of CD44⁺/CD24⁻ cells through CXCL12/CXCR4 axis [22]. We found that over expression of CXCL12 increased the subpopulation of CD44⁺/CD24⁻ in MCF-7 cells, suggesting that CXCL12 may be associated with the formation and proliferation of BCSCs. ALDH1 is a detoxifying enzyme with undetectable or extremely low levels in normal breast tissue, over expression of ALDH1 is discovered in various tumors and significantly correlated with stem cell differentiation and prognosis [23, 24]. Notably, the stem cells related genes OCT4 and SOX2 are preferentially expressed in ALDH1 positive cells [25]. Here, we confirmed that over expression of CXCL12 elevated ALDH activity significantly in MCF7 mammosphere cells. In addition, the expression of OCT4, Nanog, and SOX2 were remarkably up regulated in CXCL12 over expressed MCF-7 cells. Overall, the above results suggested that over expression of CXCL12 conferred CSC-like phenotypes on MCF-7 cells.

It has been proved that ALDH1 expression was obviously correlated with the expression of E-cadherin, vimentin, and snail, indicating a close association between CSCs formation and EMT [26]. E-cadherin is an epithelial marker exclusively expressed in epithelium, whereas N-cadherin and vimentin are mesenchymal markers responsible for maintaining cellular integrity and resisting against cellular stress [27]. α-SMA is a frequent biomarker of myofibroblasts to monitor the dynamic behaviors of myofibroblasts [28]. Prior studies pointed out that CXCL12 could promote EMT in colorectal, hepatocellular, and pancreatic carcinoma cells [29-31], but has rarely been reported in breast cancer cells. Our results illustrated that over expressing CXCL12 induced EMT in MCF-7 cells as evidenced by the down regulation of E-cadherin and up regulation of N-cadherin, vimentin and α-SMA.

β-catenin is a most potent member in cadherins/catenin compound. Wnt pathway could inhibit the degradation of β-catenin in cytoplasm, thereby the increased β-catenin translocates into nucleus and stimulates the transcription of various downstream genes to involve in EMT [32]. On the other hand, E-cadherin can bind to the cytoplasmic domain of β-catenin and sequester it to prevent its nuclear translocation [33]. It has been reported that CXCL12/ CXCR4 axis promotes EMT in human colon cancer cells by activating Wnt/β-catenin signaling pathway, and silencing CXCR4 blocks the activation of the Wnt/β-catenin pathway in human colon cancer cells [29, 34]. Similarly, we detected the activation of Wnt/β-catenin pathway after over expressing CXCL12 in MCF-7 cells. Furthermore, the expression of E-cadherin was elevated significantly by siRNA interference of β-catenin gene, indicating that Wnt/β-catenin pathway is involved in CXCL12 induced EMT of breast cancer cells.

In conclusion, over expression of CXCL12 activated Wnt/β-catenin pathway to trigger EMT of MCF-7 cells and induced CSC phenotypes formation, ultimately promoted the metastasis of MCF-7 cells. These results further explained the mechanism of CXCL12-mediated metastasis in breast cancer cells. CXCL12 may serve as a potential target for the treatment of breast cancer.

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Disclosure of conflict of interest

None.
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