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Original Article

Upregulation of C-X-C chemokine receptor type 4 (CXCR4) in the breast cancer stem like cells

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Abstract: Background and objectives: Breast cancer stem like cells (CSCs) as a subset of cancer cells exhibit similar properties with normal stem cells. These cells are responsible for cancer metastasis and recurrence. Pivotal roles of CXCR4 in metastasis, chemoresistance and stemness of tumor cells have been showed previously. Here, we aim to explore the relationship between CXCR4 and CSCs in primary and metastatic breast tumor cells. Methods and Results: Primary and highly metastatic breast tumor cells were isolated in our laboratory. Spheroid formation was used to confirm the presence of CSCs and their self-renewal capability. CXCR4 expression was evaluated using real-time polymerase chain reaction in monolayer culture and multicellular spheroids. Our data showed that in all tested cells, CXCR4 expression was significantly increased in CSCs. In parallel, compared with primary tumor cells, downregulation of CXCR4 in metastatic tumor cells was confirmed. Conclusion: These results provided new insights related to significant alteration of CXCR4 expression in multicellular spheroids. Analysis of molecular properties of spheroids could be used to detect molecular and genetic aspects of CSCs and also created a targeted therapeutic strategy against breast CSCs.

Keywords: Breast cancer, cancer stem cells, metastasis, CXCR4, multicellular spheroids

Introduction

Breast cancer is the most common cancer among women worldwide [1]. Relapse and mortality result in part from primary breast tumor metastasis. The detection of proteins that elevate the spread of breast cancer cells and metastasis is important to realizing molecular mechanisms underlying breast cancer progression. Recent studies have indicated that CXCR4 might contribute in invasion and metastasis of several malignancies, including breast cancer [2, 3].

The cancer stem-like cells (CSCs) concept was originally proposed to reconcile the complex phenotypic heterogeneity of tumors and the fact that only a few cancer cells are actually tumorigenic. CSCs possess the capacity to self-renew, initiate a tumor as well as the potential to differentiate to reconstitute the initial tumor mass, including its heterogeneity [4]. An increasing amount of evidence based on pre-clinical and clinical studies demonstrates the importance of CSCs in tumor progression and relapse suggesting that cancer eradication requires killing of CSCs [5]. Such CSC characteristics imply that they are a crucial driver of tumor formation as well as the initiation of metastatic progression and recurrence. Additionally, CSCs have been shown to be implicated in chemical and radiation resistance of cancer cells [6]. However, how exactly CSCs contribute to the progression of multistage cancer and metastasis yet to be discovered [7].

CXCR4 is a chemokine receptor, binds to stromal cell-derived factor 1 (SDF-1), and plays a role in the progression and metastasis of cancers such as breast cancer [8]. CXCR4 is also called CSC marker since CSCs have the high level of CXCR4 expression on their surface. Cumulative evidence proposes a critical role of CXCR4 as a CSC marker in the metastasis [9].

Recent studies indicate that CXCR4 is involved in chemotaxis, invasion, angiogenesis, and cell proliferation [10]. Genetic knockdown and/or chemical suppression of CXCR4 significantly prevents breast cancer metastasis into the lung [11]. Breast cancer patients who have high CXCR4 level have greater risks of distant metastasis [12]. Consequently, CXCR4 expression is believed to be linked to organ-specific metastasis [13].

Numerous investigations have only found a negligible link between CXCR4 expression and clinical outcome [10]. However, there are not enough data to support the important role of CXCR4 in breast cancer stem cells. This study has been conducted to investigate whether there is a link between expression of CXCR4 and CSCs in primary and metastatic breast cancer tumor cells.

Materials and methods

Cell culture

Primary and metastatic breast tumor cells were isolated and characterized in our previous works [14]. Briefly primary tumor, lung and brain of cancerous mice were excised after 35 days of tumor induction in female BALB/c mice, and surface blood was removed by rinsing it in PBS. After mincing with scissors, fragments were placed to 50 ml conical tube. For enzymatic digestion, primary tumor, lung and the brain were digested in 10 mg/ml collagenase type IV at 37°C for 75 min on a platform rocker. All enzymes were purchased from Sigma (St. Louis, MO, USA). The digested organ filtered through 70-um cell strainers, and washed with PBS. In the next step, washed cells were resuspended in medium containing 10% FBS, 100 U/ml Penicillin, and 100 ug/ml Streptomycin (all from Gibco, USA). Ultimately, the cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 2% Penicillin-Streptomycin (Gibco, USA) in humidified atmosphere of 5% CO_2 at 37°C.

2D monolayer and 3D multicellular spheroids culture of tumor cells

Primary (4T1T) and metastatic breast tumor cells isolated from brain and lung (4T1B and 4T1L, respectively) were harvested and cultured. Briefly, 1) 2D monolayer: The cells were cultured in 6-well plates (1×10^4 cells/well) in high glucose DMEM with 10% FBS and 2% Penicillin-Streptomycin and incubated at 37°C. 2) 3D multicellular spheroids: The cells were cultured in 6-well plates (1×10^4 cells/well) in an ultra-low-attachment 6-well plate in spheroid forming media containing high glucose DMEM with 0.5% FBS and 2% Penicillin-Streptomycin for 6 days.

Molecular assessment of CXCR4 expression

RNA extracted from tumor cells cultured in both 2D monolayers and 3D spheroids using TrizoLEX reagent (DNA Biotech). Quality and quantity analysis of isolated RNA performed by gel electrophoresis and NanoDrop, respectively. The first strand cDNA synthesis was performed using reverse transcription system (Easy cDNA Synthesis Kit for RNA or mRNA to cDNA - pars tous). Real-time PCR procedure was executed based on the 1 ul cDNA in all samples. Quantization of all gene transcripts was done by SYBR Green Real time PCR Master Mix (Amplicon A/S, Denmark) using StepOnePlus™ Real-Time PCR System, according to the manufacturer's instruction. The amplification procedure was as follows: 1 cycle of 95°C for 15 min, 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The exact mRNA expression was normalized to the expression level of GAPDH. Relative fold changes of gene expression were calculated according to $2^{-\Delta\Delta Ct}$ method.

The primers were designed using AlleleID version 6: CXCR4-F: 5'-TGTAGGACTGTAGAACTGTAGAG-3'; CXCR4-R: 5'-CCTCGGAATGAAGAGATTATGC-3'; GADPH-F: 5'-CCTGGAGAAACCTGCCAAGTA-3'; and GADPH-R: 5'-GGCATCGAAGGTGGAAGAGT-3'.

Statistical analysis

The Paired Samples t-test was used to analyze the data (GraphPad Software, V.6.0, La Jolla, CA, USA). Results are shown as Mean ± SD. *P*-value less than 0.05 was considered statistically significant.

Results

Formation of 3D multicellular breast cancer spheroids

In-vitro 3D spheroid formation can indicate the presence of CSLCs and their renewal ability. As

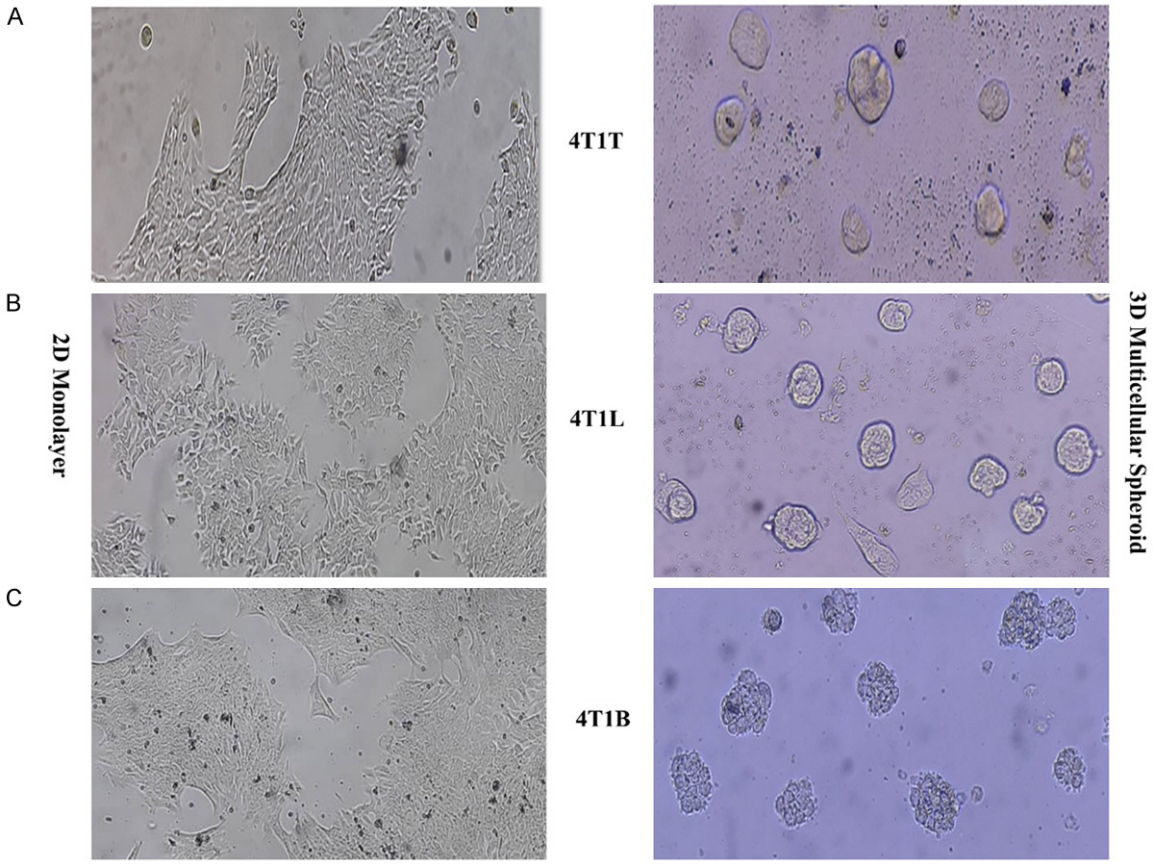

Figure 1. Spheroid conduction in primary and metastatic breast tumor cells. A. 2D monolayer and 3D multicellular spheroids of primary breast tumor cells (4T1T). B and C. 2D monolayer and 3D multicellular spheroids of lung metastatic (4T1L) and brain metastatic (4T1B) breast tumor cells respectively.

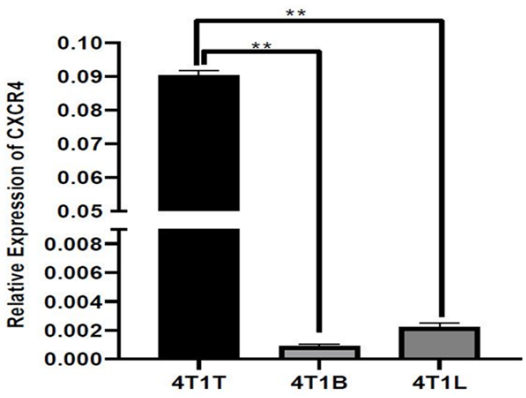

Figure 2. Reduced expression of CXCR4 in metastatic tumor cells using real-time PCR. CXCR4 was significantly downregulated in Metastatic Tumor Cells. All results are expressed as mean ± SD from at least three independent experiments analyzed by Two-tailed T test. **P < 0.001.

showed in **Figure 1**, after six days, the spheroids formed from the all 3 evaluated breast tumor cells (4T1T, 4T1B, and 4T1L) (**Figure 1**).

Significant down-regulation of CXCR4 in metastatic breast tumor cells

As showed in **Figure 2**, compared to primary tumor (4T1T) the relative expression of CXCR4 was significantly down regulated in both metastatic brain (4T1B), and metastatic lung (4T1L) tumor cells.

Significant up-regulation of CXCR4 in breast CSLCs

The relative expression of CXCR4 in both 2D monolayer and 3D multicellular spheroids was evaluated in our work. The presence of cancer stem-like cells (CSLCs) and their ability to self-renew is determined by in vitro 3D multicellular spheroid formation. Compared to 2D monolayers, CXCR4 expression was significantly elevated in 3D spheroids (**Figure 3**). Accordingly, significant upregulation of CXCR4 in CSLCs was confirmed in all 3 evaluated breast cancer cells (4T1T, 4T1B, and 4T1L).

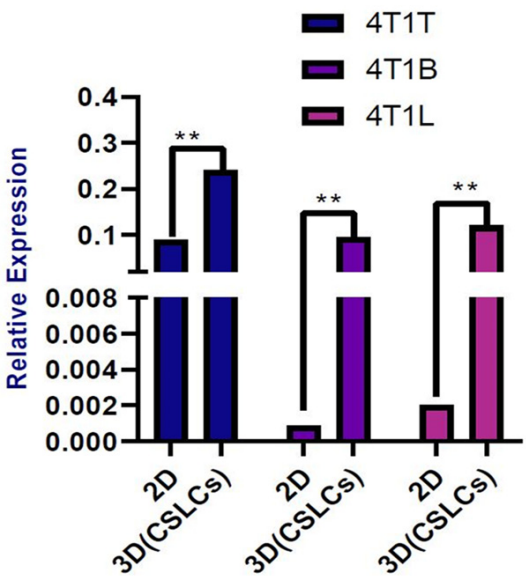

Figure 3. Evaluation of CXCR4 gene expression in multicellular breast cancer spheroid with real-time PCR. CXCR4 was significantly upregulated in multicellular breast cancer spheroids. All results are expressed as mean ± SD from at least three independent experiments analyzed by Two-tailed T test. **P < 0.005.

Discussion

The results of our study can be interpreted in two parts. In part one, our results indicated that the CXCR4 expression was significantly downregulated in metastatic tumor cells. Numerous earlier studies have shown the significance of the CXCR4 in the spread of tumors and development of cancer [15-17]. According to recent studies, the CXCR4 has a crucial role in breast cancer cells spreading to particular organs [18, 19]. Krohn et al worked on murine breast cancer cell lines. They reported that 4T1 cells contain a higher numbers of $CXCR4^+$ cells than the less invasive cells, *e.g.* 4TO7, 167FARN and 67NR [20]. Contrary to these results, we founded that compared with 4T1T as a primary tumor cell, the expression of CXCR4 was significantly downregulated in 4T1B and 4T1L as highly metastatic tumor cells. That means primary tumor cells (4T1) have high level of CXCR4 expression that mediate metastatic migration of these cells. After migration to metastatic location the expression of CXCR4 was significantly downregulated in metastatic tumor cells (4T1B and 4T1L).

In part two, our data revealed that the CXCR4 expression was elevated in all multicellular cancer stem-like cells. CSCs comprise a small part of a tumor's cells and propagate tumor cells. They present a challenge to cancer therapeutics. CSCs can only proliferate in a particular microenvironment to support the growth of primary and metastatic tumors [21]. CXCL12-CXCR4 signaling is involved in the homing of normal stem cell [22]. Note that CSCs also express CXCR4 receptor. This suggests that CXCL12-CXCR4 might guide the CSCs trafficking to other organs expressing high levels of CXCL12 ligand *e.g.* lungs and lymph nodes [23]. Krohn et al in their work on breast cancer, showed that $CXCR4^+$ cells are extremely enriched in mammosphere [20]. In line with their results, our results also indicated that cells in multicellular spheroids have a higher expression of CXCR4. A recent study indicated that the CXCR4 receptor maintains CSCs in tamoxifen-resistant breast cancer [24].

Conclusion

Our results provide a new perspective about significant alteration of CXCR4 expression in multicellular spheroids and metastatic tumor cells. Analysis of molecular aspects of multicellular spheroids and metastatic cells could be used for the understanding of molecular and genetic properties of CSCs and also designing a targeted strategy to treat metastatic breast cancer.

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Disclosure of conflict of interest

None.

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