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Expression of cancer stem cell surface markers after chemotherapeutic drug treatment to reflect breast cancer cell regrowth

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【Abstract】 **Objective** To detect the cell viability and the expressions of stem cell surface markers after chemotherapeutic drug treatment. **Methods** We observed the cytotoxic effects of three chemotherapeutic agents [epirubicin (Epi), fluorouracil (5-FU) and cyclophosphamide (Cyc)] in three cell lines, and the cell viabilities after removed these chemotherapeutic agents. Expressions of stem cell surface markers CD44, CD24, CD90, CD14 and aldehyde dehydrogenase1(ALDH1) in breast cancer cells were analyzed by real-time PCR. The post hoc analysis (Tukey's tests) in conjunction with one-way ANOVA was used for statistical analysis. **Results** The initial cytotoxic efficacy was most notable. After the treatment of the same therapeutic agents, cell viability was decreased by 64.8% 35.14%, 32.25% in BT-483 cells, 66.4%, 22.94% and 45.88% in MDA-MB-231 cells, 97.1%, 99.5% and 76.4% in MCF cells. The difference was significant compared with that before treatment ($P=0.000$). However, the inhibitory effects were diminished after chemotherapeutic agent withdrawal. Cell viabilities were increased to 167.9%, 212.04% and 188.66% in MDA-MB-231 cells at 48 h after withdrawal. At 72 h after withdrawal, cell viability was increased with a significant difference in three cell lines (all P values=0.000). Expressions of CD44 and ALDH1 were most prevalent for MDA-MB-231, BT-483 and MCF-7 cells. ALDH1 mRNA level was significant higher in BT-483 (HER-2 overexpression cell line) than MDA-MB-231 (triple negative cell line) ($P=0.012$). CD14 mRNA level in MCF-7 cells were significantly lower than that in MDA-MB-231 and BT-483 ($P=0.003$, 0.001). BT-483 showed significantly higher level of CD44 than MDA-MB-231 and MCF-7 cell line ($P=0.013$, 0.020), and no significant difference was detected between MDA-MB-231 and MCF-7 breast cancer cells ($P=0.955$). CD90 mRNA expressions were detected in MDA-MB-231 cells and MCF-7 cells, but not in BT-483 cells. **Conclusion** Some malignant cells could survive *in vitro* and begin to proliferate again between cycles of chemotherapy.

【Key words】 Neoplastic stem cells; Real-time polymerase chain reaction; Cell line, tumor; Drug therapy; Cell surface makers

Breast cancer is the most frequently diagnosed cancer and one of the leading causes of cancer death in women. Worldwide, over 1.3 million cases of invasive breast cancer are diagnosed, and more than 450 000 women die from breast cancer annually[1]. Metastasis is the main cause of tumor lethality in breast cancer patients[2]. In recent years, the data have showed that cancer stem cells (CSCs) are highly related to cancer recurrence due to their resistance to conventional anti-cancer therapies[3]. The existence of tumor cells which displays stem cell properties accounts for the progression of tumor and thus develops metastasis disease. Most cancer cells do not possess the capability to regenerate a tumor, but CSCs have this regenerative capability. Therefore, it is predicted that CSCs represent only a small fraction of tumor[4].

CSCs are cancer-initiating cells and retain the property of self-protection[5]. When chemotherapy is used for treatment of breast cancer, most cells in tumor are killed due to the cytotoxic effect of drugs. However, CSCs still survive because of their resistance to drugs since CSCs are protected from chemotherapeutic agents by ATP-binding cassette (ABC) drug transporters[5]. In the past ten years, the hypothesis of CSCs model has re-surfaced, and maintained, as a contributing factor of drug resistance to anti-cancer therapies and tumorigenesis. Among other factors, drug resistance has been attributed to the over-expression of certain indicative ABC transporters, generation of cells with stem cell

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like properties through epithelial-mesenchymal transition (EMT) induction, and specific expression of putative CSCs[6].

CSCs can cause metastatic spread of tumor cells in other organs of the body as malignant tumor growth may be initiated sometimes[7]. CSCs are also highly related to the resistance to drugs including chemotherapy. Some studies had showed that cells with a $CD44^+/CD24^{-/low}$/epithelial-specific antigen $(ESA)^+$phenotype exists in breast cancer stem cells in human breast cancer[8]. Donnenberg et al[9] has stated that a small population $CD44^+/CD90^+$exists in breast tumors and is localized in the periphery of tumor, near the $CD90^+$stroma. Aldehyde dehydrogenase (ALDH) 1 expression has been reported to be a useful prognostic marker for epithelial cancers[10-16]. The gene expression of stem cell markers in different cell lines indicates that cancer cell line may possess CSCs' property. Continued investigation of CSCs offers the possibility of generating novel target therapies that strive to overcome issues of drug resistance and improve therapeutic efficacy[17]. Optimizing cancer treatment with CSC concept may hopefully prevent cancer recurrence and obviate toxicity.

1 Materials & Methods

1.1 Cell lines

Three breast cancer cell lines were used for analysis: MCF-7, MDA-MB-231 and BT-483. The immune profile of MCF-7 was ER(+), PR(+/−), HER-2(−); MDA-MB-231 ER(−), PR(−), HER-2(−); BT-483 ER(+), PR(+), HER-2(−)[18]. MDA-MB-231 cells were cultured in Leibovitz's (L-15) medium containing 10% FBS (Invitrogen, USA) supplemented with 4 mmol/L glutamine (Sigma) and penicillin-streptomycin (100 U/ml) (Sigma, USA). MCF-7 and BT-483 cell lines were maintained in DMEM supplemented with 10% FBS, 4 mmol/L glutamine and penicillin-streptomycin (100 U/ml) and grown at atmosphere of 5% CO_2, 37 ℃[18].

1.2 Cell viability assay

MDA-MB-231, BT-483 and BT-483 cell lines were given chemotherapy drugs of epirubicin (Epi) at a dosage of 0.05 μg, fluorouracil (5-FU) at a dosage of 10 μg/ml, and cyclophosphamide (Cyc) at a dosage of 60 μg/ml. After treatment for 24 h, chemotherapy drugs were removed. Cell viability was measured using a colorimetric assay for 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent (Roche) and at 450 nm in a microplate reader (Sunrise). Cell viability was measured every 24 h, i. e., at 24, 48, 72, 96, 120, 144 and 168 h after drug withdrawal.

1.3 RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from MDA-MB-231, BT-483 and MCF-7 cells by High Pure RNA Isolation Kit (Roche) as described by the manufacturer. cDNA was synthesized from 1 μg total RNA using Transcriptor reverse transcriptase, random hexamers, dNTPs (Roche) as described by the standard methods. Amplification of the resulting cDNAs was performed using the SYBR GREEN Dye (the Light Cycler® 480 Real-Time PCR System, Roche). The sequence information of primer pairs for CD14, CD44, CD90, CD24, CD29 and GAPDH mRNA was described previously[19]. ALDH1 was designed and purchased from TaKaRa. The primer sequences were as follows: CD14 (forward 5′-CCGC TGGTGCACGTCTCT-3′, reverse 5′-AATCTTCATCG TCCAGCTCACA-3′); CD24 (forward 5′-AGTCCAA TGTGGCAAGGAAAA-3′, reverse 5′-TGTGTCAATA AAAGGTGTGGAATTAGT-3′); CD44 (forward 5′-C AACCGTTGGAAACATAACC-3′, reverse 5′-CAAGT GGGAACTGGAACGAT-3′); CD90 (Thy-1) (forward 5′-CATCTGCGAGTGTGGTGTCT-3′, reverse 5′-CC CCACCATCCCACTACC-3′); GAPDH (forward 5′-ACCCACTCCTCCACCTTTGA-3′, reverse 5′-CTGTT GCTGTAGCCAAATTCGT-3′); ALDH1 (forward 5′-TTGGAATTTCCCGTTGGTTA-3′, reverse (5′-CTGT AGGCCCATAACCAGGA-3′).

1.4 mRNA expression of ALDH1 in breast cancer cell lines

The ALDH families of enzymes are cytosolic isoenzymes that are contributing to the oxidation of retinol to retinoic acid in early stem cell differentiation. In clinic, the expression of ALDH1 detected by immune-staining was correlated with poor prognosis in breast cancer[20]. In this study, we assessed ALDH1 mRNA expression in three breast cancer cell lines.

1.5 Statistical analysis

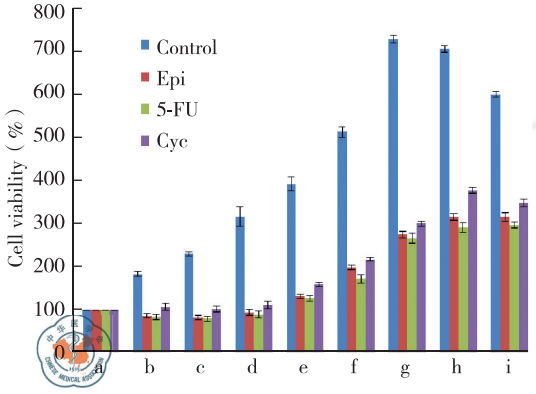
Statistical analysis was performed by SPSS 16.0. Significant differences between groups were evaluated by one-way ANOVA followed by Tukey's multiple comparisons testing. Differences were regarded as statistical significance at $P<0.05$.

2 Results

2.1 Viability of breast cancer cells after chemotherapy treatment

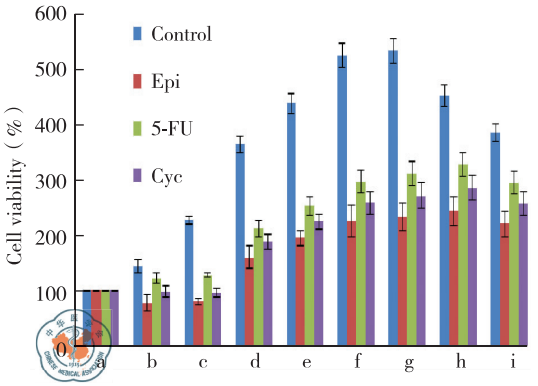
As illustrated from assay data, cell viability was detected in all three breast cancer cell lines used. In the control group treated with fresh culture medium for 24 h, cell viability was increased by 40.91% in BT-483, 44.43% in MDA-MB-231, and 83.33% in MCF-7 cell lines, respectively. While cell viabilities of three cell lines treated with three chemotherapeutic agents, i.e., Cyc at the dosage of 60 μg/ml, Epi at the dosage of 0.05 μg and 5-FU at a dosage of 10 μg/ml were significantly decreased. Viability of BT-483 cells treated with Epi, 5-FU and Cyc was decreased by 64.80%, 35.14% and 32.25% respectively compared with control group. After the treatment of the same therapeutic agents: Epi, 5-FU and Cyc, cell viability was decreased by 66.40%, 22.94% and 45.88% in MDA-MB-231 cells, 97.10%, 99.50% and 76.40% in MCF-7 cells. (Figures 1–3).

Overall initial cytotoxic efficacy was most notable for Epi, 5-FU and Cyc. At 24 h after withdrawal of 5-FU and Cyc, relative cell viability increase was observed in BT-483 with significant difference ($P = 0.010$, 5-FU withdrawal *vs* 5-FU treatment; $P = 0.020$, Cyc withdrawal *vs* Cyc treatment), while no significant difference between Epi withdrawal and Epi treatment group ($P = 0.453$). At 48 h after Epi withdrawal, significant increase in relative cell viability was observed in BT-483 cell lines (compared with 24 h after withdrawal, $P = 0.001$). For MDA-MB-231 cells, there was no significant change in cell viability in the group at 24 h after withdrawal of three chemotherapeutic agents (Epi: $P = 0.533$; 5-FU: $P = 0.125$; Cyc: $P = 0.719$). Cell viabilities were increased to 167.90%, 212.04% and 188.66% at 48 h after withdrawal of Epi, 5-FU and Cyc in MDA-MB-231 cells. Unlike BT-483 or MDA-MB-231, MCF-7 exhibited cell inhibition during intervals of 48 h after withdrawal of chemotherapy agents. At 72 h after withdrawal of Epi, 5-FU and Cyc, cell viability was increased with



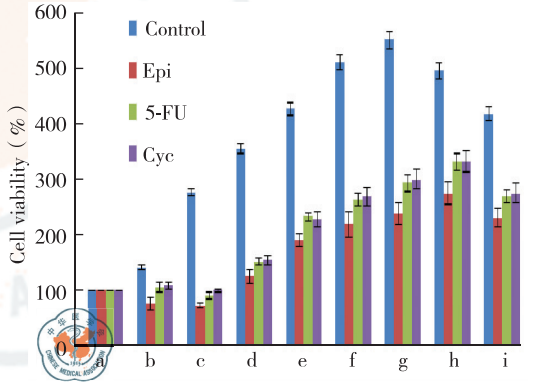
a: before; b: treated for 24 h; c: 24 h after drug withdrawl; d: 48 h after drug withdrawl; e: 72 h after drug withdrawl; f: 96 h after drug withdrawl; g: 120 h after drug withdrawl; h: 144 h after drug withdrawl; i: 168 h after drug withdrawl. Cell viabilities were assessed every 24 h by WST-1 assay. The viability was calculated relative to the negative control (taken as 100%). Error bars indicate standard deviation

Figure 1 Cell viability of MCF-7 at pretreatment of chemotherapeutic agents and chemotherapeutic agent withdrawal



a: before; b: treated for 24 h; c: 24 h after drug withdrawl; d: 48 h after drug withdrawl; e: 72 h after drug withdrawl; f: 96 h after drug withdrawl; g: 120 h after drug withdrawl; h: 144 h after drug withdrawl; i: 168 h after drug withdrawl. Cell viabilities were assessed every 24 h by WST-1 assay. The viability was calculated relative to the negative control (taken as 100%). Error bars indicate standard deviation

Figure 2 Cell viability of MDA-MB-231 at pretreatment of chemotherapeutic agents and chemotherapeutic agent withdrawal



a: before; b: treated for 24 h; c: 24 h after drug withdrawl; d: 48 h after drug withdrawl; e: 72 h after drug withdrawl; f: 96 h after drug withdrawl; g: 120 h after drug withdrawl; h: 144 h after drug withdrawl; i: 168 h after drug withdrawl. Cell viabilities were assessed every 24 h by WST-1 assay. The viability was calculated relative to the negative control (taken as 100%). Error bars indicate standard deviation

Figure 3 Cell viability of BT-483 at pretreatment chemotherapeutic agents and chemotherapeutic agent withdrawal

significant difference (all P values = 0.000).

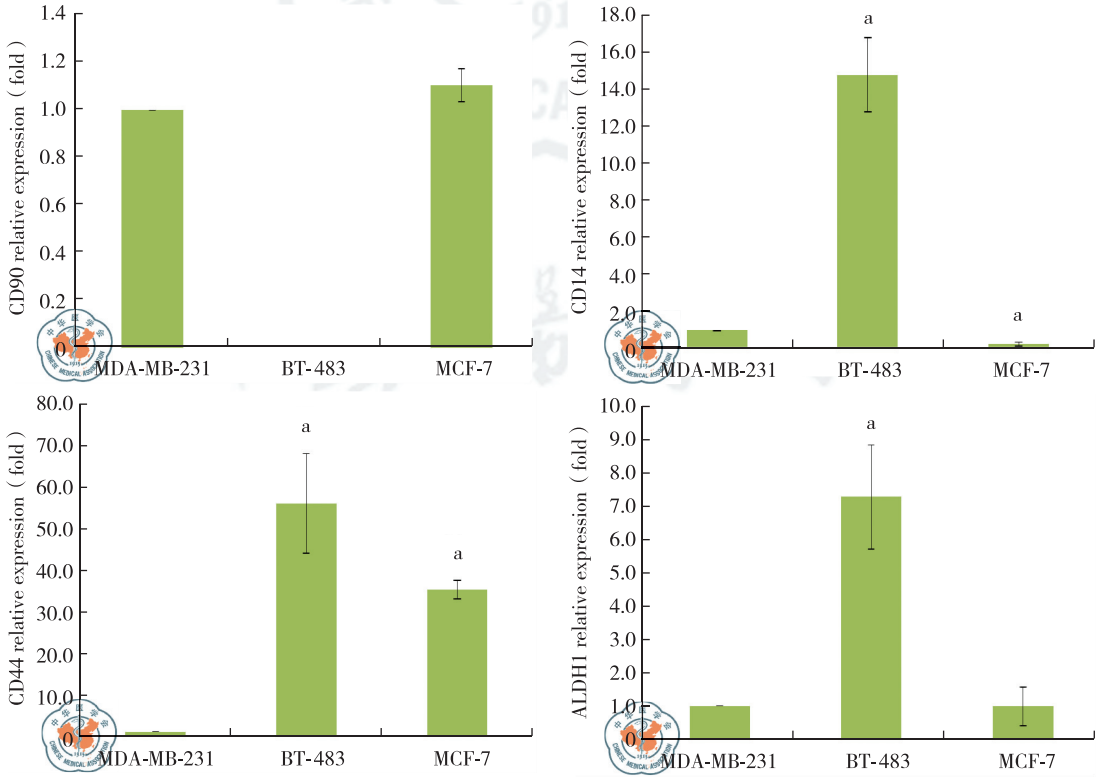
2.2 mRNA expression of CD14, CD44 and CD90 in breast cancer cell lines

qPCR was utilized to compare and analyze the relative mRNA expression of the selected stem cell markers among three breast cancer cell lines in this study. As Figure 4 illustrates, the expressions of CD44 and ALDH1 were most prevalent for MDA-MB-231, BT-483 and MCF-7 cells. ALDH1 mRNA level was significantly higher in BT-483 (HER-2 overexpression cell line) than that in MDA-MB-231 (triple negative cell line) (P = 0.012). The results revealed that CD14 was expressed in MDA-MB-231, BT-483 and MCF-7 breast cancer cells and the highest CD14 mRNA level was found in BT-483. CD14 mRNA expression in BT-483 was around 14-fold higher than that in MDA-MB-231. CD14 mRNA level in MCF-7 cells was significantly lower than that in MDA-MB-231 and BT-483 cells (P = 0.003, 0.001).

CD44 mRNA expression was detected in MDA-MB-231, BT-483 and MCF-7 breast cancer cells. Similar as CD14 expression, BT-483 showed significantly higher level of CD44 than that in MDA-MB-231 and MCF-7 cell line (P = 0.013, 0.020, Figure 4). No significant difference was detected in CD44 between MDA-MB-231 and MCF-7 breast cancer cells (P = 0.955). In our study, CD90 mRNA expressions were detected in MDA-MB-231 and MCF-7 cells. However, no CD90 expression was detected in BT-483 cells (Figure 4).

3 Discussion

Among other factors, drug resistance has been attributed to the over-expression of certain indicative ABC transporters, generation of cells with stem cell-like properties through EMT induction, and specific expression of putative cancer stem cell subpopulations. The discovery of CSCs in solid tumors including breast cancer has been a hot topic in recent years and has changed oncologists' view on chemotherapy. CSCs are proposed to be cells capable of giving rise to a new tumor which is the cause of cancer[21]. They are cells within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor[4,22]. CSCs have active DNA-repair capacity to self-renew. After exposure to carcinogens, they have the potential to accumulate mutations[4]. Distinctive properties of stem cells include self-renewal,



Breast cancer cell lines were maintained as described in Methods section. Upon reaching 80% confluence, total RNA was extracted and expression levels of stem cell markers were analyzed by qPCR. The normalization gene used was GAPDH. a: P<0.05, compared with MDA-MB-231 cells.

Figure 4 Analysis of stem cell markers in mammary cancer cell lines by real-time PCR

proliferation, resistance to drugs, and the potential to proliferate extensively[22]. de la Mare et al[23] found that MCF-7 can re-grow under serum-free culture after removing chemotherapeutic paclitaxel (Ptx) and the proportion of $CD44^{high}/CD24^{low}$ marker-bearing cells in breast cancer cells MCF-7 was increased.

In this study, three chemotherapeutic agents—Epi at a dose of 0.05 μg, 5-FU at a dose of 10 μg/ml and Cyc at a dose of 60 μg/ml showed significantly cytotoxic effects on MDA-MB-231, BT483 and MCF-7 cell lines. Although the cell viabilities of three breast cancer lines were inhibited by Epi, 5-FU and Cyc after 24 h treatment, the prohibitory effects were attenuated after drug withdrawal. Based on the results, the prohibitory effect faded out, and cell viability started to increase during 48 – 72 h after drug withdrawal and the increased cell proliferation was observed until 144 h after drug withdrawal. The WST-1 assay result showed that MDA-MB-231, BT483 and MCF-7 breast cancer cells re-initiated cellular growth when chemotherapeutic agents were removed. Our findings raised the possibility that at least some malignant cells could survive *in vitro* and begin to proliferate again between cycles of chemotherapy.

To evaluate prospective identification of tumorigenic markers, also known as CSCs, different stem cell markers were analyzed in three human breast cancer cell lines in this study. Lobba et al[24] analyzed the expression of 10 stem cell markers in 5 breast cancer cell lines seeking for markers which may be associated with the malignant grade of the respective cell lines. They found that two stem cell markers (CD90 and CD14) were reported to be differentially expressed in breast cancer cell lines, when compared with non-tumorigenic lines of the same tissue origin. The CD90 marker was highly expressed in malignant cell lines while CD14 molecule displayed higher expression in non-tumorigenic cell line[24]. Furthermore, Liu et al[25] demonstrated that high level of CD90 mRNA was highly expressed in CD133 positive brain tumor stem cells from primary culture and resistant to several chemotherapeutic agents. Recent studies demonstrated that the CD90 marker was expressed on hepatic stem progenitor cells during liver development.

However, stem cell markers used to identify stem cells from one organ are frequently not useful for identifying stem cells in other tissues. Our study revealed that CD90 mRNA level was high in MDA-MB-231 and MCF-7 but not detectable in BT-483 cells. The CD90 markers should be further analyzed in human breast cancer samples to confirm the results. Although breast cancer stem cells have not yet been identified directly, a subpopulation of tumor cells that strongly express CD44 but not CD24 (the $CD44^{+}$ $CD24^{-/low}$ phenotype) was considered as breast cancer stem cell markers by many oncologists. For breast cancer, the vast majority of cells in culture were $CD44^{+}CD24^{-/low}$, and 10% to 20% of these retained the ability to self-renew[8,26]. Tiffany et al[27] isolated a cell population characterized by high CD44 expression and low or undetectable levels of CD24 ($CD44^{+}CD24^{-/low}$) in MDA-MB-231 and MCF-7 cells. These cells were highly tumorigenic when injected into immune compromised NOD/SCID mice and shared classic features of normal stem cells, including the capacity for self-renewal and generation of heterogeneous progeny. Our study showed that among all markers analyzed, high CD44 expression were detected in MDA-MB-231, BT-483 and MCF-7 cell lines, while the levels of CD24 were not detectable in this study.

The breast CSCs marker ALDH1 has been described as a marker of both normal and malignant breast stem/progenitor[20,28-29]. Remarkably, ALDH1 positive cells (putative CSCs) are significantly resistant to cytotoxic drugs. It has been reported that ALDH1 positive population is significantly associated with poor clinical outcome[28] and certain histological and clinical characteristics, such as high tumor grade, HER-2 positivity and Ki67 proliferation status when compared with ALDH1 negative population[30] in breast cancers. It seems that the ALDH1 positive is a marker of basal-like or HER-2 overexpressing tumors, putatively originating from luminal committed progenitors. MDA-MB-231 breast cancer cell, classified as an ER-negative, PR-negative and HER-2-negative (so-called "triple-negative") phenotype, might have smaller population of ALDH1 positive cells compared with ER-positive, PR-positive, and HER-2-negative cells, BT-483 cells. Based on our results, mRNA expression of ALHD1 in MDA-MB-231 cells was significantly lower than that in BT-483. Further study might evaluate the activity of ALDH1 enzyme in breast cancer cells, and percentage of the putative CSC fraction obtained in the different breast cancer cell lines.

Although the existence of tumor-initiating cells in solid human tumors is widely accepted, CSCs markers for breast cancer have not yet been identified directly. Some stem cell markers are successfully enriched for tumor stem cells, but also present in normal stem cells and many non-stem cells

in various tumors and tissues. However, our study supported that breast cancer cell lines contain stem-like cells that possess self-renewal ability. Use of cancer cell lines as models for the development and testing of novel therapeutic target aimed at eradicating cancer stem cells might be a possible way to identify and prevent the breast cancer-initiating cells from increasing and contributes to translate the CSC concept to clinical practice for overcoming drug resistance and preventing cancer recurrence.

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