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DNA methyltransferase inhibitor CDA-2 synergizes with high-dose thiotepa and paclitaxel in killing breast cancer stem cells

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TABLE CONTENT

1. ABSTRACT

It has been suggested that breast cancer stem cells (CSCs), which characterized by $CD44^+CD24^{-/low}$, may result in treatment failure in patients with breast cancer. It is possible therefore that that inhibiting such subpopulation might subsequently improve clinical outcome. In the present study, we found that the $CD44^+/CD24^{-/low}$ CSCs, isolated from both human breast cell line MCF-7 and MDA-MB-231, were more resistant to thiotepa, paclitaxel and anthracycline, when compared with the non-breast cancer stem cell subset from the same cell lines, whereas the chemosensitivities were remarkably reversed by higher concentration of thiotepa and paclitaxel except for adriamycin. The percentage of CSCs was significantly decreased with an addition of DNA methyltransferase inhibitor CDA-2 and the expression of Smo, Shh, and Gli-1 of Hedgehog signaling pathway in CSCs was decreased. Of important findings, combination of thiotepa or paclitaxel with CDA-2 could significantly inhibit the proliferation of CSCs regardless of their dosages. These results unveiled that the selection of cytotoxic agents and increasing their dosage might be of great importance in the respect of eliminating CSCs. DNA methyltransferase inhibitor CDA-2 exhibited a synergistic effect with cytotoxic drugs, which might provide a conceptually new therapeutic strategy.

2. INTRODUCTION

Breast cancer is the most common female malignancy and is the leading cause of cancer-associated mortality in women resulted from metastasis or recurrence (1). Despite of advances in treatment, the prognosis of metastatic breast cancer remains poor.

It has been shown that cancer cells in a tumor mass are heterogeneous and only a small subpopulation of cancer cells possesses the ability to proliferate and to be capable of self-renewal. This tumorigenic subpopulation has been called as cancer stem cells (CSCs) or tumor initiating cells (TICs) (2). This subpopulation of CSCs have been identified from some certain tumors such as brain glioma (3), breast cancer (4), prostate cancer (5), melanoma (6) etc.

Breast cancer CSCs, which are characterized by $CD44^+/CD24^{-/low}$, has been isolated from both surgical specimens and breast cancer cell lines, and could be propagated *in vitro* as nonadherent mammospheres (4,7). Recent studies have demonstrated a correlation of CSCs with disease recurrence, metastasis, drug resistance and poor prognosis (8-10). It has been argued that therapies targeting both the proliferating cells and CSCs would be

more effective and improve the treatment of breast cancer. Potential ways to eradiate breast cancer CSCs include immune modulation, blocking the key molecules of stem cell proliferation signaling pathway, cells differentiation treatment (11,12).

Cells differentiation therapies have been regarded as the landmark progress in some malignances since it aims to at differentiating primary initial cells into relatively different normal cells (13). One of the samples of the differentiation therapies is the use of retinoid acid and its analogues which could invert the malignant progression of acute premyelocytic leukemia. In additional to retinoid acid, histone deacetylase inhibitor and human urine extract CDA-2 were also subsequently regarded as cell differentiation agents (14-16). CDA-2 is an inhibitor of DNA methyltransferase, which could suppress tumors through activating tumor suppressor genes, turning on or off genes of certain key signaling regulators via demethylation of their promoter sequences (16-18). Previous studies have unveiled that CDA-2 has shown activities of inhibiting cell proliferation and apoptosis of leukemia, breast cancer, endometrial carcinoma, gastric cancer (19-22). However, it is remains unclear whether CDA-2 may act on CSCs.

The role of high-dose chemotherapy (HDC) with autologous hematopoietic stem cell transplantation in breast cancer has been debated for decades until recently when randomized clinical trials revealed that HDC could significantly prolonged progression-free survival (PFS), and bring marginal benefit to overall survival (OS) of whole population. Interestingly, both PFS and OS were improved dramatically in one of important family, namely triple-negative breast cancer (estrogen receptor (ER) negative, progesterone receptor (PR) negative, Her2 negative, TNBC) (23-25). TNBC is currently assimilated to basal-like breast cancer because of their frequent immunohistochemical negativity for hormone and ERBB2 receptors. Furthermore, Basal-like breast cancer has been shown to have plentiful of $CD44^{+}/CD24^{-/low}$ initial cells, which perhaps makes TNBC an ideal therapeutic model for those patients with refractory or metastatic disease(26,27). To date, little is known whether killing or inhibiting breast cancer CSCs could become a conceptually new therapeutic strategy for metastatic breast cancer (MBC).

We proposed that HDC is a useful method in eliminating breast cancer initial cells or CSCs. In this study, we isolated breast cancer CSCs from MCF-7 and MDA-MB-231 breast cancer cell lines and explored the potential inhibition effects of CSCs to CDA-2 and cytotoxic agents at different concentration. We further analyzed the mRNA expression of down stream molecules of sonic hedgehog signaling pathway (SHH) in CSCs and present evidence here that HDC is a conceptually novel therapeutic strategy for MBC.

3. MATERIALS and METHODS

3.1. Cell lines and Cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-231 was obtained from cell culture center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). MCF-7 cells were cultured with Dulbecco's Modified Eagle's Medium-high Glucose (DMEM, Invitrogen/Gibco, USA) medium, supplemented with 10% heat-inactivated fetal calf serum(FCS, Invitrogen/Gibco, USA) at 37C in a humidified atmosphere (5% CO2). MDA-MB-231 cells were cultured with Leibovitz's L-15 (Invitrogen/Gibco, USA) medium, supplemented with 10% FCS at 37C in a humidified atmosphere (100% air). Cells were routinely maintained in 75 cm^2 tissue culture flasks (Corning, USA) and harvested by 0.05% trypsin/0.025% EDTA (Invitrogen/Gibco, USA) treatment when they were in logarithmic phase of growth for Flow cytometry analysis or drug sensitivity assay.

CDA-2 was purchased from Hefei Everlife Pharmaceutical Co. Ltd. (Anhui, China). CDA-2 was added to cells 24 hours after seeding which include the following treatment: negative control, 1mg/ml, 2mg/ml, 3mg/ml, and 4mg/ml. Medium in each culture flask (Corning; USA) was adjusted to same hydrogen ion concentration and environment. PE labeled anti-human CD44 antibody and FITC labeled anti-human CD24 antibody or their respective isotope controls were from BD Pharmingen (San Diego, CA, USA). The percentage of CSCs was analyzed on Flow cytometry a MoFlo High-performance cell sorter (FortCollins, Florida, USA).

3.2. ATP Bioluminescence Assay

The sorted $CD44^{+}/CD24^{-/low}$ breast CSCs and non-breast CSCs were immediately incubated in polypropylene round-bottom 96-well plates(Corning, USA) at a concentration of 1×10^3 cells per well. Cytotoxic agents, paclitaxel, adriamycin, and thiotepa, were purchased from Sanofi-aventis, Pfizer and Xudong Haipu (Shanghai, China) respectively. Concentrations of CDA-2 were set as 0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 4mg/ml, and 8mg/ml. Chemotherapeutic compounds were at six different dilutions (25–800%) of the test drug concentrations (TDC/Cmax). The 100% TDC of paclitaxel (PTX), adriamycin (ADM) and thiotepa (PSTA) were 10ug/ml, 1ug/ml and 10ug/ml, respectively. Cultured cells without any drugs were set as negative control (M0), while blank media without tumor cells were positive control (M1). The individual TDC for each cytotoxic drug was predetermined by reference to the published pharmacokinetic and response data. The light output corresponding to the level of ATP present was measured in a luminometer (BHP9504-02, HAMAMATSU, Beijing, China). Data was transferred automatically to an Excel spreadsheet where the inhibitory rate achieved at each tested concentration was calculated by the equation: (test-M1) / {100(M0-M1)}.

3.3. Detection the Activity of Hh Pathway by RT-PCR Method

Total RNA of sorted cells was extracted using Trizol reagent (Invitrogen-life Technology) according to standard protocol. mRNA of Smo, Ptch, Shh, and Gli-1 were amplified using the HotStarTaq® kit according to the manufacturer's instructions. Primers specific to each gene of interest were synthesized (GAPDH: sense

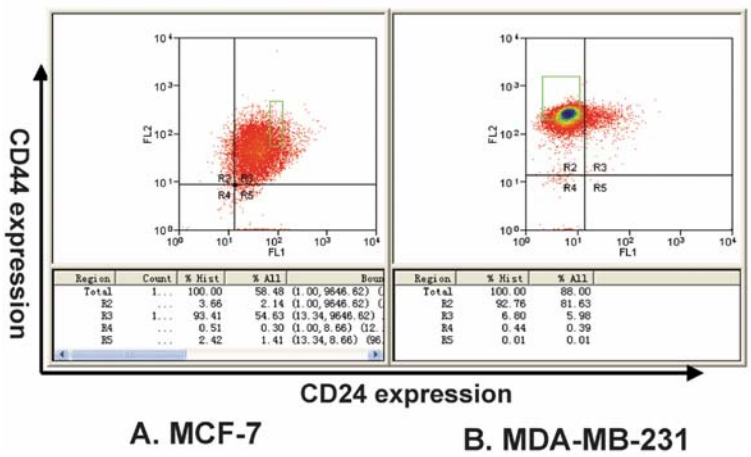

Figure 1. Cancer initiating cells in MCF-7 and MDA-MB-231 cell lines. A. The percentage of $CD44^{+}$/ $CD24^{-/low}$ subset in MCF-7 cell line was 1.70±1.43%. B. The percentage of $CD44^{+}$/ $CD24^{-/low}$ subset in MDA-MB-231 cell line was 94.2±1.2%.

GTCAACGGATTTGGTCGTATT, antisense AGTCCTCTGGGTGGCAGTGAT; Ptch: sense GGTGGCACAGTCAAGAACA, antisense ACCAAGAGCGAGAAATGG; Shh: sense ACCGAGGGCTGGGACGAAGA, antisense GGAAAGTGAGGAAGTCGCTG; GIL-1: sense GGGTTACATACCTGTCCTTC, antisense CTCAACAGGAGCTACTGTGG; Smo: sense CACCTCCAATGAGACTCTGTCC, antisense CTCAGCCTGGTTGAAGAAGTCG). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were set to normalize cDNAs and PCR conditions were optimized for the transcripts to remain in the linear amplification range of the reaction. PCR products were analyzed on a 2% agarose gel and semi-quantitative analysis was performed with Labworks and Image J (NIH image). To reduce sources of variability that may be introduced between different PCR runs, comparative RT-PCR was carried out with each experimental group to be analyzed run in triplicate, normalized to an internal control gene (GAPDH) on the same plate.

3.4. Statistical Analysis

The statistical analyses were conducted with SPSS software (version 15.0). All probabilities were two tailed, with a P value less than 0.05 considered statistically significant (non-parametric test).

4. RESULTS

4.1. $CD44^{+}$/ $CD24^{-/low}$ CSCs in MCF-7 and MDA-MB-231 Cell Lines

The mean percentage of $CD44^{+}$/ $CD24^{-/low}$ CSCs from MCF-7 and MDA-MB-231 were 1.70±1.43% and 94.2±1.2% respectively (Figure 1).

4.2. CDA-2 Inhibiting Proliferation of $CD44^{+}/CD24^{-/low}$ CSCs

Breast cancer cells of MCF-7 and MDA-MB-231 were incubated with CDA-2 of increased concentration of 0 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml to 4mg/ml. The percentage of $CD44^{+}$/ $CD24^{-/low}$ CSCs were significantly reduced from 1.70±1.43%, 0.74±0.42%, 0.49±0.28%, 0.35±0.16%, to 0.18±0.06% in MCF-7 cells (P=0.011). The similar phenomena was also observed in MDA-MB-231 cells and the percentage of $CD44^{+}$/ $CD24^{-/low}$ CSCs decreased from 94.2±1.2%, 72.2±8.1%, 46.6±0.0%, 6.8±3.9%, to 2.9±1.0% (P=0.01) (Figure 2) .

When $CD44^{+}/CD24^{-/low}$ CSCs isolated from MCF-7 and MDA-MB-231 were cultured with CDA-2 of increased concentration from 0.25mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml to 8mg/ml for 72h, the proliferation of $CD44^{+}/CD24^{-/low}$ CSCs was significant inhibited and exhibited a dose-dependent elimination. (Figure 2).

4.3. CDA-2 Down-regulating Activity of SHH Pathway

As shown in figure 3, the mRNA expression of SHH and GIi-1 were over-expressed in $CD44^{+}/CD24^{-/low}$ CSCs while they was not expressed in NCSCs of both MCF-7 and MDA-MB-231 cell lines. Ptch was not expressed in any subpopulations of both cell lines, while Smo was only expressed in $CD44^{+}/CD24^{-/low}$ CSCs subpopulation of MCF-7 cell line.

After incubated with CDA-2 at a concentration of 1 mg/ml, the mRNA expression of SHH, GIi-1 and Smo in $CD44^{+}/CD24^{-/low}$ CSCs subpopulation of both MCF-7 and MDA-MB-231 cell lines were significantly decreased, indicating that CDA-2 may inhibit proliferation of CSCs by down-regulating the activity of SHH signaling pathway. (Figure 3)

4.4. Higher Concentration Thiotepa and Paclitaxel Except for Anthracycline Inhibiting the Proliferation of CSCs

After exposure to paclitaxel (PTX) at less than 200% Cmax concentration, the proliferation rate of $CD44^{+}/CD24^{-/low}$ CSCs subpopulation was markedly higher than that of NCSCs subpopulation in both MCF-7 (P=0.026) and MDA-MB-231 cell lines (P=0.01). However, when treated with higher concentrations of

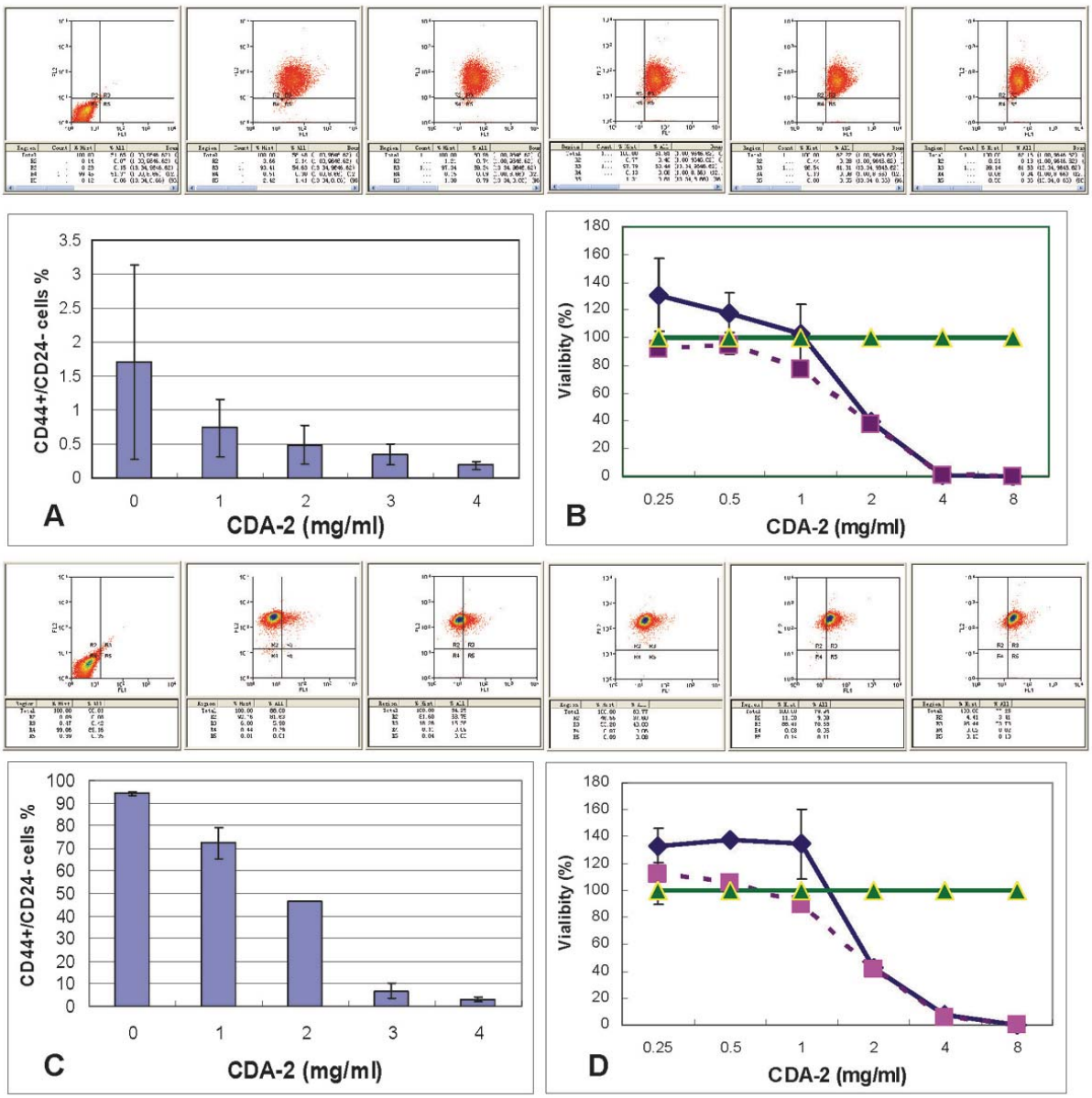

Figure 2. CDA-2 eliminating $CD44^{+}/CD24^{-/low}$ breast cancer stem cell. CSC: $CD44^{+}/CD24^{-/low}$ cells; NCSC: non- $CD44^{+}/CD24^{-/low}$ cells; CDA-2: cell differentiation agent 2. A. The percentage of CSCs of MCF-7 reduced from 1.70±1.43% to 0.18±0.06% as the CDA-2 concentration increased from 0mg/ml to 4mg/ml, (P=0.011). C. The percentage of $CD44^{+}$/ $CD24^{-/low}$ cells of MDA-MB-231 decreased from 94.2±1.2% to 2.9±1.0% as the CDA-2 concentration increased from 0mg/ml to 4mg/ml (P=0.01). In ATP assay, CSCs of MCF-7 (B) and MDA-MB-231 (D) were significant inhibited by CDA-2 with concentrations from 0.25mg/ml to 8mg/ml.

paclitaxel (>200% Cmax), $CD44^{+}/CD24^{-/low}$ CSCs could restored sensitivity to paclitaxel in both cell lines and were almost completely eliminated in MCF-7 cell line (Figure 4A).

For the situation of the anthracycline, adriamycin (ADM), $CD44^{+}/CD24^{-/low}$ CSCs exhibited similar resistance at lower concentration of less 200% Cmax compared with that of NCSCs in both MCF-7 (P=0.01)and MDA-MB-231 cell lines (p=0.002). To be much surprised, when the concentration of adriamycin increased to 400% or 800% Cmax, the proliferation rates of both CSCs and NCSCs significantly rebounded with the contrary to paclitaxel and thiotepa.(Figure 4B).

As for thiotepa (PSTA), the proliferation rate of $CD44^{+}/CD24^{-/low}$ CSCs subpopulation was also significantly higher than that of NCSCs at concentration less 200% Cmax in both MCF-7 (P=0.005) and MDA-MB-231 cell line (P=0.002). Similarly, the sensitivity of

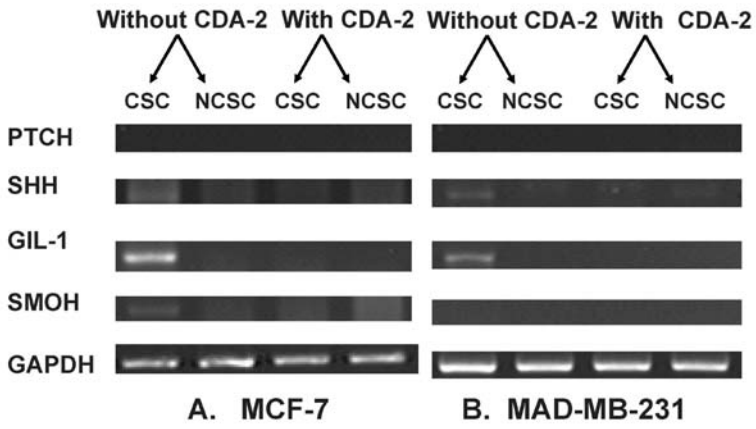

Figure 3. CDA-2 down-regulating activity of Hedgehog pathway. CDA-2: cell differentiation agent 2; CSC: $CD44^{+}/CD24^{-/low}$ cells without CDA-2; NCSC: non-$CD44^{+}/CD24^{-/low}$ cells without CDA-2; CSC: $CD44^{+}/CD24^{-/low}$ cells treated with 1mg/ml CDA-2; NCSC: non-$CD44^{+}/CD24^{-/low}$ cells treated with 1mg/ml CDA-2. A. In MCF-7: Smoothen (Smo), Shh and GIi1 were expressed in $CD44^{+}/CD24^{-/low}$ CSCs and their expression were significantly inhibited by 1 mg/ml CDA-2. B. In MDA-MB-231: Shh and Gli1 was expressed in $CD44^{+}/CD24^{-/low}$ CSCs and their expression were also significantly inhibited by 1 mg/ml CDA-2.

$CD44^{+}/CD24^{-/low}$ CSCs to thiotepa could be recovered at higher concentrations (>200% Cmax), The proliferation rates of CSCs were remarkably reduced in both cell lines and nearly 100% inhibited in MDA-MB-231 cell line (Figure 4C).

4.5. CDA-2 Synergized the Activity of Thiotepa and Paclitaxel to Inhibit Proliferation of CSCs

Co-culture experiments of 1mg/ml CDA-2 combined with thiotepa, paclitaxel, and anthracycline respectively at various concentrations (25%, 50%, 100%, 200%, 400%, and 800% of Cmax) could result in reduction of the proliferation rates of CSCs and NCSCs in both MCF-7 and MDA-MB-231 cell lines respectively by ATP-TCA assay.

Encouraging findings supported that the proliferation of $CD44^{+}/CD24^{-/low}$ CSCs subpopulation was dramatically inhibited by CDA-2 combined with thiotepa, paclitaxel and anthracycline regardless of their concentration in both MCF-7 and MDA-MB-231 cell lines. The proliferation rates of CSCs isolated from MCF-7 cells were 20±1.0%, 18±1.0%, 16±1.0%, 22±2.0%, 18±1.0%, and 15±1.0% when treated with 1mg/ml CDA-2 and thiotepa of 25%, 50%, 100%, 200%, 400%, and 800% Cmax respectively, compared with proliferation rates of 145±9.0%, 139±4.0%, 123±11.0%, 111±1.0%, 87±12.0%, and 27±2.0% with thiotepa alone (P=0.000). The similar result was observed in MDA-MB-231 cell line interfered with CDA-2 and thiotepa (P=0.020). CDA-2 combined with anthracycline and paclitaxel also exhibited similar synergistic effects with significant statistic difference (MCF-7: P<0.001, P=0.039; MDA-MB-231: P<0.001, P<0.001). (Figure 5)

5. DISCUSSION

Using different systems, investigators have demonstrated that only a minority of "stem-like cells" in breast cancer is capable of self-renewal and tumorigenesis. Through examining the ability of tumorigenesis of different subpopulations isolated from primary breast cancer or metastatic lesions when transplanted into immunosuppressed NOD/SCID mice, breast cancer stem cells (CSC) were identified by cell surface markers $CD44^{+}CD24^{-/low}$. (2,4,7) Furthermore, it has also been observation that CSCs are able to be serially passaged, each time generating a stem cell population, as well as the more differentiated non-tumorigenic cells forming the bulk of breast cancer (28).

However, the percentage of breast cancer stem cell subpopulation, identified by cell surface makers, varied largely in different breast cancer cell lines (2,29,30). In our study, the percentage of $CD44^{+}/CD24^{-/low}$ CSC subpopulation were 1.70±1.43% and 94.2±1.2 in MCF-7 and MDA-MB-231 cell lines respectively, which were similar to previous studies (29,30). The large variation of CSCs population identified by cell surface maker raised puzzles to the feature of this subpopulation.

Breast cancer cell lines could be classified into five subtypes: luminal, basal, mesenchymal, ErbB2-positive, and myoepithelial, based on gene expression profiling of them (31-34). MDA-MB-231 cell line comes from mesenchymal group while MCF-7 was luminal group (29,34). As for gene expression profile, basal and mesenchymal cells are similar except for differential expression of 227 genes. Some studies suggested that basal-like or mesenchymal subtype might contained higher percent of primary initial cells (25,26). Therefore, the heterogeneous of breast cancer cell subtype may partially be responsible for the variation of percentage of CSCs subpopulation. Meanwhile, better characterization of specific breast cancer stem cell markers is required for more exactly identifying this subpopulation.

Increased evidences showed that CSCs were more resistant to chemotherapy and radiotherapy, which may shrink the size of the tumor, but their effects are often

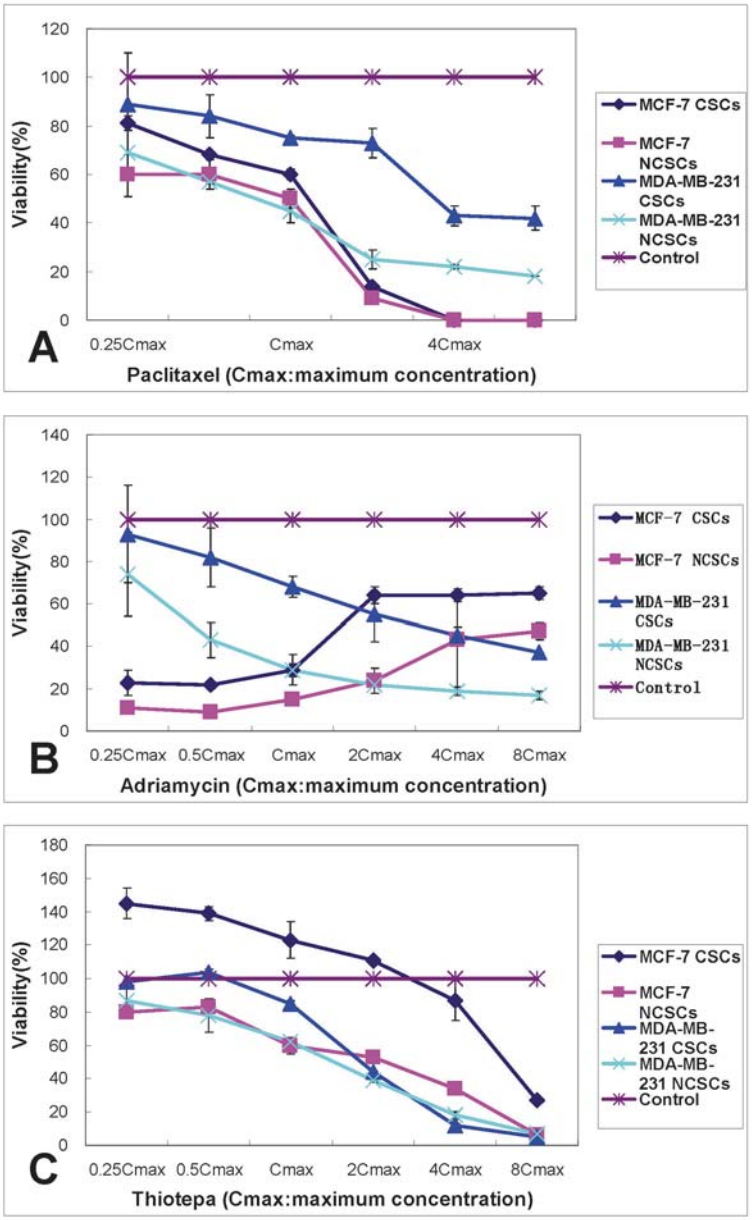

Figure 4. Breast cancer CSCs exhibit different sensitivity to cytotoxic agents of different concentration. The proliferation of $CD44^{+}/CD24^{-/low}$ CSCs sorted from MCF-7 and MDA-MB-231 were tested by ATP Bioluminescence Assay after treated with increasing concentration of cytotoxic agents for 72h. PTX: paclitaxel; ADM: adriamycin; PSTA: Thiotepa; CDA-2: cell differentiation agent 2. CSCs: CD44+/CD24- cells; NCSCs: non-CD44+/CD24- cells. A. MCF-7: PTX group, CSCs versus NCSCs, P=0.378; But when PTX<2Cmax,P=0.026; MDA-MB-231: PTX group, CSCs versus NCSCs, P=0.010; B. MCF-7: ADM group, CSCs versus NCSCs. P=0.010; MDA-MB-231: ADM group, CSCs versus NCSCs, P=0.002; C. MCF-7: PSTA group, CSCs versus NCSCs, P=0.005; MDA-MB-231: PSTA group, CSCs versus NCSCs, P=0.478

transient and often fail to improve survival (2,14,30,35,36). Therefore, therapies targeting breast CSCs may be more effective to improve clinical outcome. Targeting signal transduction pathways that controls cancer stem cell proliferation and differentiation might be useful to transform malignancies into benign diseases (11,12). It have been demonstrated that CSCs do not appear to have the hyper proliferation signals activity such as tyrosine kinase, while other stem cell proliferating associated molecular signaling pathways such as Wnt, Hh and Notch may play an important role in the development of CSCs(37,38). The Hedgehog signaling pathway has been implicated in many different kinds of cancers. More recently, studies have revealed the relationship between hedgehog pathway and CSCs in multiple myeloma (39), breast cancer (40) and glioma (41,42). Our results showed

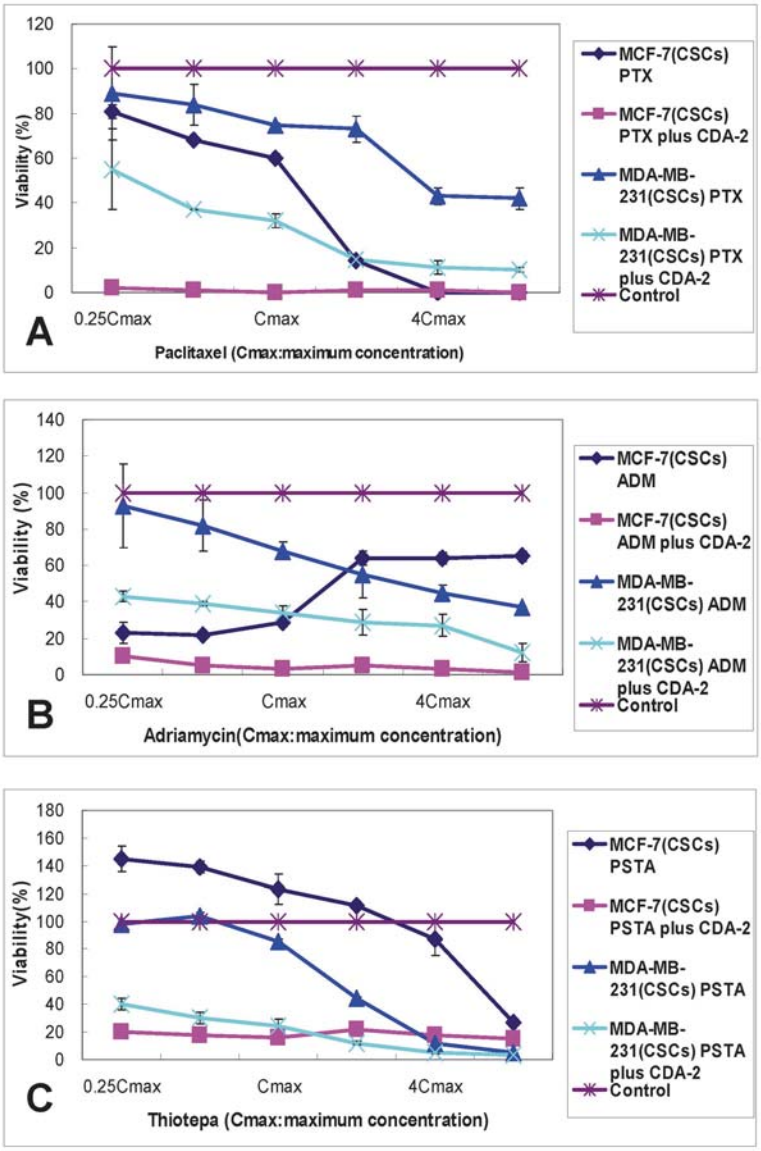

Figure 5. CDA-2 combined with cytotoxic agents significantly inhibited breast cancer CSCs proliferation. The proliferation of $CD44^{+}/CD24^{-/low}$ CSCs sorted from MCF-7 and MDA-MB-231 were tested by ATP Bioluminescence Assay after treated with increasing concentration of cytotoxic agents plus with 1mg/ml CDA-2 or not. PTX: paclitaxel; ADM: adriamycin; PSTA: Thiotepa; CDA-2: cell differentiation agent 2 CSCs: CD44+/CD24- cells. A. MCF-7(CSCs): PTX group versus (PTX plus CDA2) group, P=0.039; MDA-MB-231(CSCs): PTX group versus (PTX plus CDA2) group, P<0.001; B. MCF-7(CSCs): ADM group versus (ADM plus CDA2) group: P<0.001; MDA-MB-231(CSCs): ADM group versus (ADM plus CDA2) group: P<0.001; C. MCF-7(CSCs): PSTA group versus (PSTA plus CDA2) group: P<0.001; MDA-MB-231(CSCs): PSTA group versus (PSTA+CDA2) group: P=0.020

that the Hh signaling pathway was active in CSCs of breast cancer accompanied with over-expression of Shh, Smo, and Gli-1, while the expression of these molecules were negative in NCSCs.

Targeting Hh pathway might be a potential treatment for tumors. Cyclopamine, a lily-derived relevant chemical, could inhibit Hh ligand-dependent and independent Hh pathway activation through direct interaction with Smo. It has been demonstrated that cyclopamine could inhibit the proliferation of tumor cells such as breast cancer, pancreas cancer, glioma and lung cancer both in vivo and *in vitro* (43-45).

As an inhibitor of DNA methyltransferase, CDA-2 has been reported that has remarkable effect of anti-cell-proliferation on tumor cells *in vitro* and interfering the activity of signaling pathway such as ERK and Akt

phosphorylation had been reported (16,17). In this present study, CDA-2 dramatically exhibited anti-tumor activity by inhibiting proliferation of $CD44^{+}/CD24^{-/low}$ CSCs. Meanwhile, the expression of Shh, GIi-1 and Smo of $CD44^{+}/CD24^{-/low}$ CSCs were all significantly down-regulated by CDA-2. Thus, the anti-tumor or differentiation-inducing activity of CDA-2 may through down-regulating the hedgehog signaling pathway in breast cancer CSCs.

High-dose chemotherapy (HDC) has been proved to give significantly prolonged progression-free survival (PFS), while the improvement of over-all survival (OS) were only observed in some certain subgroup such as TNBC. TNBC, which most manifest features of the basal-like histological subset, have many similarities to *BRCA1*-associated breast cancers. *In vitro* studies, *BRCA1*-associated breast cancers have shown extreme sensitivity to the alkylating agent, DNA double-strand breaks, such as those induced by etoposide and cisplatin, whereas resistant to mitotic-spindle poisons, such as taxanes and vincas (46,47). Therefore the selection of sensitivity cytotoxic agents is closely associated with clinical outcome. Previous studies have demonstrated the efficiency of high-dose cytotoxic agents such as alkylating and anthracycline on resistant cancer cells both *in vitro* and in animal models (48). However it is not known the effects of high-dose cytotoxic agents on CSCs, which possess the same characteristic of multi-drug resistance.

We explored the sensitivity of breast cancer CSCs to three prevalently used anti-breast cancer drugs: paclitaxel, anthracycline, and alkylating agent thiotepa of different concentrations. The results revealed that high-dose alkylating agent and paclitaxel could significantly inhibited the proliferation of $CD44^{+}/CD24^{-/low}$ breast CSCs in both MCF-7 and MDA-MB-231 cell lines, which were primarily resistant to them at low concentrations. However, increasing the dose of anthracycline was failed to restore the sensitivity of breast CSCs. The discordance between drug sensitivity and dosage indicated that the selection of conditioning regimen of HDC may be crucial to clinical outcome.

Be of important findings in our study, combination of CDA-2 and cytotoxic agents exhibited encouraging anti-tumor activity by dramatically inhibiting the proliferation of both $CD44^{+}CD24^{-/low}$ CSCs and non-CSCs regardless of their dosage.

Alkylating agents are frequently used in chemotherapy of breast cancer, which the molecular mechanism were mediating cell death by damaging DNA. Therefore, cellular DNA repair mechanisms can influence their anti-tumor efficacy. It has been demonstrated that DNA methyltransferase such as O(6)-methylguanine-DNA methyltransferase (MGMT), may play an important role in the action of DNA repair (49). Therefore, block the DNA repair induced by alkylating agents might dramatically improve the efficacy (50). Therefore the synergistic effects of DNA methyltransferase inhibitor CDA-2 with alkylating agent thiotepa and paclitaxel may be the interference of DNA repair system of CSCs. CDA-2 might inhibit the activity of some DNA methyltransferase which involved in DNA repair. Furthermore, the inhibition of proliferation-dependant signal transduction pathway such as Hh, Akt and Erk caused by CDA-2 would also resulted in increased frangibility of CSCs to DNA damages caused by cytotoxic agents.

This study revealed that the selection of cytotoxic agents and increasing their dosage might be of great importance in the respect of eliminating CSCs. DNA methyltransferase inhibitor CDA-2 exhibited a synergistic effect with cytotoxic drugs. These results might establish one innovation model that CDA-2 synergized thiotepa and/or paclitaxel for targeting CSCs, which will provide a conceptually new therapeutic strategy for clinical treatment of metastatic breast cancer.

6. ACKNOWLEDGMENTS

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