

Altered characteristics of cancer stem/initiating cells in a breast cancer cell line treated with persistent 5-FU chemotherapy

XINQUAN LÜ¹, QING DENG¹, HUIXIANG LI¹ and ZHENHE SUO²

¹Department of Pathology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, P.R. China;

²Division of Pathology, The Norwegian Radium Hospital, Rikshospitalet University Hospital, Montebello, Oslo, Norway

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Abstract. Drug resistance of cancer stem/initiating cells has been considered to be one of the main reasons for tumor relapse. However, knowledge concerning the changes in stem/initiating cells during chemotherapy is limited. In the present study, the breast cancer cell line MDA-MB-468 was cultured with 5-fluorouracil and serially passaged. Six cell generations were collected. Semi-quantitative RT-PCR and flow cytometric techniques were used to evaluate the protein and mRNA expression of stem/initiating factors (CD44⁺/CD24⁻, Oct 3/4, SOX2 and β -catenin), drug-resistance genes (BCRP and MRP1) and an anti-apoptosis gene (survivin). The clone formation rate was also examined in every generation of cells. The results showed that, under conditions of persistent chemotherapy, the factors representing the quantity of stem/initiating cells (β -catenin, Oct 3/4 and SOX2) followed a fluctuating trend of decrease-increase-further increase-decrease-increase-decrease, and factors representing the proportion of stem/initiating cells (proportion of CD44⁺/CD24⁻ and the clone formation rate) demonstrated a fluctuating trend of increase-further increase-further increase-decrease. The drug-resistance genes (BCRP and MRP1) and the anti-apoptosis gene (survivin) demonstrated a wave of increase-further increase-further increase-decrease-increase (MRP1 decrease)-decrease. β -catenin, Oct 3/4 and SOX2 showed a positive correlation ($r=1$, $p<0.01$). Our study confirmed that the drug resistance of cancer cells is mainly due to tumor stem/initiating cells, and that under conditions of persistent chemotherapy, the quantity or function of breast cancer stem/initiating cells increases and decreases alternately.

Introduction

Currently, comprehensive therapy, including surgery, chemotherapy and radiotherapy, is the main method for treating breast cancer, but the curative ratio is low due to frequent relapse. The resistance of cancer cells to chemotherapeutic drugs is thought to be the principal cause for relapse. Numerous theories have attempted to explain this phenomenon, and recently the cancer stem/initiating cell (CSC) theory has attracted much interest.

In 2006, to evaluate the chemosensitivity of glioblastoma multiforme (GBM) stem cells, Eramo *et al* (1) treated stem cell clones derived from different GBM patients with eight chemotherapeutic agents and assessed the rate of cell death in comparison to chemosensitive Jurkat leukemic cells and primary immature erythroblast. They observed a marked resistance of GBM stem cells to all the compounds used, whereas both Jurkat cells and erythroblasts displayed high rates of cell death. After treatment with chemotherapeutic agents, GBM stem cells were able to recover and proliferate. In a study by Phillips *et al*, CD24(-/low)/CD44(+) cancer-initiating cells were isolated from MCF-7 and MDA-MB-231 breast cancer cell lines, and their response to radiation was investigated. The results showed that the cancer-initiating cells were more resistant to radiation than the unisolated cells (2). In another study which included primary breast cancer patients (n=108) treated with neoadjuvant chemotherapy consisting of sequential paclitaxel and epirubicin-based chemotherapy, breast cancer stem cells were identified by immunohistochemical staining of aldehyde dehydrogenase 1 (ALDH1). ALDH1-positive tumors were significantly associated with a low rate of complete response (3). Similar results steadily emerged, urging researchers to relate drug-resistance to cancer stem/initiating cells (4).

CSCs which are not killed by chemotherapy, may become the cause of relapse. However, knowledge concerning the changes manifested in CSCs which are resistant to chemotherapy is limited. Answers to questions concerned with the effects of traditional chemotherapy drugs on CSC, the changes in CSCs treated by chemotherapy, the relationship between CSCs and drug-resistance proteins, and the relationship between CSCs and anti-apoptosis genes may help to understand the mechanism of CSC drug-resistance and to identify more effective methods of therapy which eventually kill CSCs.

In the present study, the MDA-MB-468 breast cancer cell line was cultured and different passages were treated continually

Correspondence to: Dr Huixiang Li, Department of Pathology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, P.R. China
E-mail: huixiang19@yahoo.com.cn

Dr Zhenhe Suo, Division of Pathology, The Norwegian Radium Hospital, Rikshospitalet University Hospital, Montebello, 0310 Oslo, Norway
E-mail: zhenhes@medisin.uio.no

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Table I. Information regarding the PCR primers.

Primer	NCBI ID	Primer sequence	Product size (bp)	Melting temperature (°C)
β -catenin	NM_001098209.1	F: CCCACTAATGTCCAGCGTTT R: AACCAAGCATTTCACCAGG	382	54.0
Oct 3/4	NM_002701.4	F: TTCAGCCAAACGACCATC R: GGAAAGGGACCGAGGAGTA	484	57.4
SOX2	NM_003106.2	F: ACACCAATCCCATCCACACT R: CAAACTTCCTGCAAAGCTCC	223	61.0
MRP1	NM_019898.2	F: CTGAGTTCCTGCGTACCTAT R: TTCTGCGGTGCTGTTGT	212	56.2
BCRP	NM_004827.2	F: GCCATTCTCCAGTCA R: GGGCGTCTATACACCAT	494	49.7
Survivin	NM_001012271.1	F: CTTGCCAGAGCCACGAA R: GGAACCTCACCCATAGCC	632	55.4
β -actin	NM_001101.3	F: GCCCTGAGGCACTCTTC R: GGCCGGACTCGTCATAC	330	54.9

F, forward; R, reverse.

with the traditional chemotherapy drug 5-fluorouracil (5-FU). For every cell generation, certain markers of CSCs, drug-resistance proteins and the anti-apoptosis gene were examined and compared to find a clue with which to resolve the above-mentioned questions. The CSC factors included β -catenin, Octamer binding factor 3/4 (Oct 3/4), SRY-box 2 (SOX2) and CD44⁺/CD24⁻ cells. The markers involved with drug-resistance proteins and anti-apoptosis genes were multidrug resistance protein 1 (MRP1), breast cancer resistance protein (BCRP) and survivin, respectively. Our results suggest that the drug-resistance of cancer cells is mainly due to CSCs, and chemotherapy leads to an alteration in the quantity or function of breast CSCs demonstrating an alternate increase and decrease.

Materials and methods

Cell culture and chemotherapy. The MDA-MB-468 breast cancer cell line was a gift from the Chinese-United Kingdom Medical Laboratory. The cells were cultured at 37°C with 5% CO₂ in DMEM (HyClone, USA) and supplemented with 10% fetal calf serum (HyClone), 100 U/ml penicillin G and 100 μ g/ml streptomycin (Gibco, USA). The cells were cultured in 75 cm² flasks, and a 0.1 μ g/ml (concentration data were obtained from a preliminary experiment) terminal concentration of 5-FU was added to the nutrient fluid when cells grew to a 60-70% base area. Cell cultures were harvested using 0.05% trypsin/EDTA (Gibco) in PBS and subcultured until achieving a 80-90% base area. The second generation was cultured, treated with chemotherapy and passaged in the same manner. Thus, six generations of cells were cultured. The cells in every generation were harvested and used in corresponding procedures in the subsequent experiments. Three independent experiments were performed. The control group was not treated with chemotherapy.

RNA extraction and semi-quantitative RT-PCR assay. Cells were harvested and stored at -70°C in TRIzol[®] (Invitrogen, USA). Total RNA was isolated by extracting the TRIzol

samples with chloroform followed by separation and purification according to the manufacturer's instructions. RNase inhibitor mix (1 μ l) was added per 50 μ l total RNA prior to treatment with DNase (Promega, USA) for 20 min. The RNA concentration and purity were quality controlled by analysis using a spectrophotometer (U-3010, Japan). First-strand cDNA was synthesized from the total RNA (3 μ g) using the First-Strand Synthesis system (Promega) and used as a template for PCR amplification with *Taq* polymerase (Qiagen, Germany). Specific primer sequences are listed in Table I. PCR conditions included an initial denaturation at 94°C for 5 min, and 35 cycles at 94°C for 30 sec, annealing (Table I) for 45 sec, 72°C for 45 sec and a final extension step at 72°C for 5 min. RT-PCR products were separated by 1.2% agarose gel electrophoresis in a 0.5% Tris-acetate buffer and stained with ethidium bromide. The bands were photographed using a digital camera (Canon, Japan), and the absorbance of every band was calculated using Software Quantity One (Bio-Rad, USA). The absorbances of the inspected bands were divided by the absorbance of β -actin in the same sample to obtain the relative value of the marker in the sample. At least six independent experiments were performed.

Flow cytometry. To identify the CD44⁺/CD24⁻ cell proportion in every generation, cells were harvested with 0.05% trypsin/EDTA (Gibco) and then suspended (2x10⁶ cells/100 μ l) in Stain Buffer containing 1% FBS (EBioscience, USA). Phycoerythrin (PE)-conjugated mouse against human CD44 monoclonal antibody (EBioscience) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD24 monoclonal antibody (EBioscience) were added to the cell suspension at the concentrations recommended by the manufacturer, and incubation was carried out at 4°C in the dark for 60 min. Proper isotype controls were used for each cell labeling experiment. The labeled cells were fixed in 100% methanol on ice for 5 min. Flow cytometric analysis was performed in triplicate using a flow cytometer (Partec, Germany). Four generations were assessed.

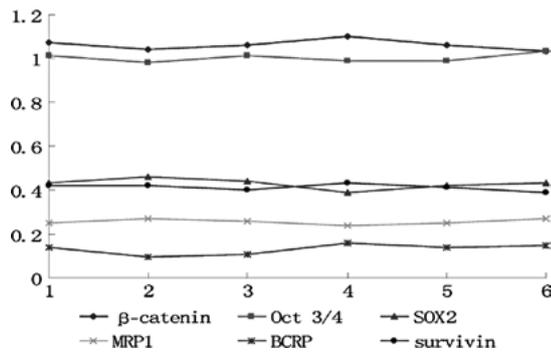


Figure 1. Trend of mRNA expression of the relative markers in each cell generation in the control group. The x-axis indicates cell generation, whereas the y-axis indicates the relative value of mRNA expression.

Colony formation experiment. Single cells were prepared in each generation and seeded in culture dishes (60 mm in diameter); each dish initially had 10×10^3 cells. The cells were cultured in the same culture medium as the former (but without 5-FU) for 2-3 weeks and fixed with methanol when colonies were visible by the naked eye. The colonies were stained for 2-3 min with haematoxylin and those that consisted of ≥ 50 cells were counted under a microscope. Four generations were assessed. At least six independent experiments were performed.

Statistical analysis. Data are expressed as the means \pm standard deviation (SD). The statistical significance of differences between groups was analyzed by one-way analysis of variance, and the relationship between variables was calculated by two-tailed Pearson correlation analysis using the Statistical Product and Service Solutions version 13.0 program for Windows (SPSS, USA). Differences with p-values < 0.05 were considered significant.

Results

mRNA expression of CSC factors, drug-resistance genes and an anti-apoptosis gene. In the experimental (treated with 5-FU) and control groups (without 5-FU), the mRNA expression of the markers, β -catenin, Oct 3/4, SOX2, MRP1, BCRP, survivin and actin, in six cell generations was investigated.

In the control group, each marker exhibited no differences among the cell generations ($p > 0.05$) (Fig. 1). In the experimental group, the markers fluctuated in the different cell generations ($p < 0.05$) (Figs. 2 and 3). All markers selected in the present study showed a similar wave of increases and decreases (Fig. 3). The factors related to CSCs (β -catenin, Oct 3/4 and SOX2) exhibited a fluctuating trend of increase-increase-further increase-decrease-increase-decrease. The drug-resistance genes (BCRP and MRP) and the anti-apoptosis gene (survivin) exhibited waves of increase-further increase-further increase-decrease-increase (MRP1 decrease)-decrease.

The correlation among the markers was also analyzed and most exhibited a positive correlation (Table II).

Proportion of CD44⁺/CD24⁻ cells. The proportion of CD44⁺/CD24⁻ cells in the experimental group also exhibited a trend of decrease and increase in each cell generation ($p < 0.05$) (Fig. 4). The proportion of CD44⁺/CD24⁻ cells in each cell generation

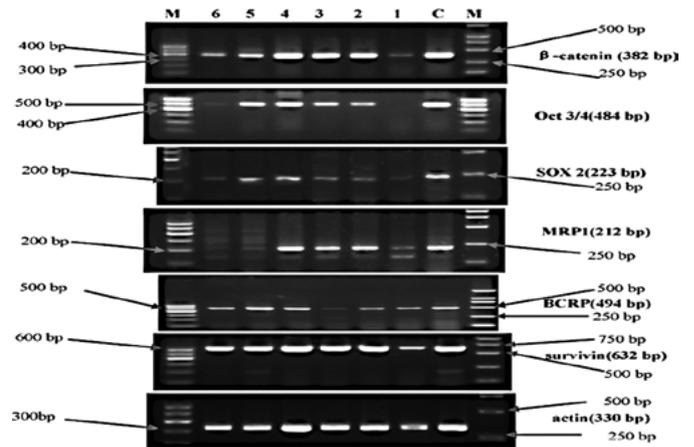


Figure 2. Electropherogram of mRNA expression of the relative markers in each cell generation in the experimental group. M, molecular weight marker; the left marker is the 100-bp ladder, the right marker is DL2000 (apart from Oct 3/4, whose marker was the 100-bp ladder). C, control group. Lanes 1, 2, 3, 4, 5 and 6 represent the first, second, third, fourth, fifth and sixth cell generation, respectively.

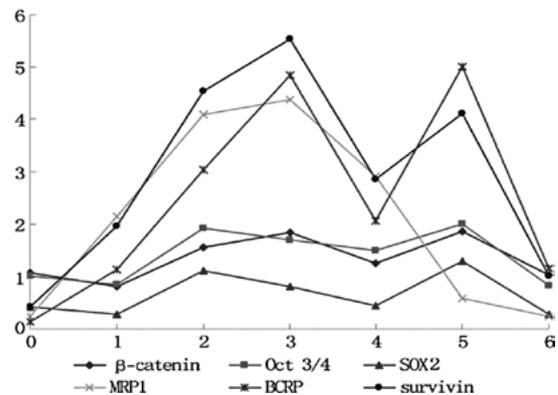


Figure 3. Trend of mRNA expression of the relative markers in each cell generation in the experimental group. The x-axis indicates the cell generation, whereas the y-axis indicates the relative value of mRNA expression.

in the control group showed no difference among the different cell generations ($p > 0.05$; data not shown).

The proportion of CD44⁺/CD24⁻ cells in the experimental group showed a positive correlation with other markers (Table III).

Colony formation. In the different cell generations, the colonies formed per 10^4 seeded cells varied ($p < 0.05$) (Fig. 5). The decrease and increase fluctuation was very similar to that shown in Fig. 4. The number of colonies formed in the experimental group exhibited a positive correlation with several of the other markers (Table IV).

Discussion

In the present study, three factors were selected to represent the existence or quantity/functional change in CSCs: mRNA expression of β -catenin, Oct 3/4 and SOX2. β -catenin is the key component in the Wnt/ β -catenin signaling pathway. The Wnt/ β -catenin signaling pathway plays an important role in the differentiation and proliferation of stem cells (5). Experiments

Table II. Correlation among the stem/initiating-related factors, chemoresistance and anti-apoptotic factors.

Factors		β -catenin	Oct 3/4	SOX2	MRP1	BCRP	Survivin
β -catenin	r-value	1	0.922	0.882	0.389	0.936	0.859
	p-value		0.003	0.009	0.389	0.002	0.013
Oct 3/4	r-value	0.922	1	0.933	0.486	0.861	0.860
	p-value	0.003		0.002	0.269	0.013	0.013
SOX2	r-value	0.882	0.933	1	0.279	0.824	0.759
	p-value	0.009	0.002		0.544	0.023	0.048
MRP1	r-value	0.389	0.486	0.279	1	0.436	0.753
	p-value	0.389	0.269	0.544		0.329	0.051
BCRP	r-value	0.936	0.861	0.824	0.436	1	0.917
	p-value	0.002	0.013	0.023	0.329		0.004
Survivin	r-value	0.859	0.860	0.759	0.753	0.917	1
	p-value	0.013	0.013	0.048	0.051	0.004	

Table III. Correlation between the proportion of CD44⁺/CD24⁻ cells and the other factors.

	β -catenin	Oct 3/4	SOX2	MRP1	BCRP	Survivin
Proportion of CD44 ⁺ /CD24 ⁻						
r-value	0.857	0.804	0.889	0.846	0.891	0.921
p-value	0.063	0.101	0.043	0.071	0.042	0.026

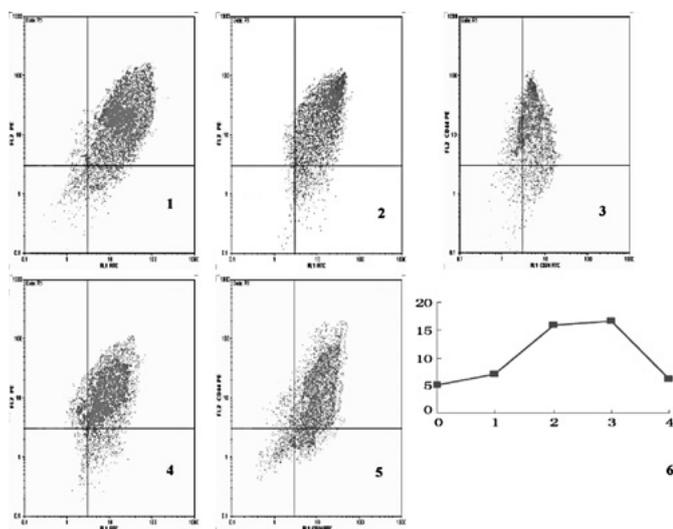


Figure 4. Proportion of CD44⁺/CD24⁻ cells in the experimental group. Images 1, 2, 3, 4 and 5 are the proportion of CD44⁺/CD24⁻ cells in the non-treated group, the first, second, third and fourth generation of cells, respectively. The CD44⁺/CD24⁻ cells were located in the upper left quadrant. Image 6 is a line graph showing the fluctuation in the proportion of CD44⁺/CD24⁻ cells in different cell generations. The x-axis indicates the cell generation, whereas the y-axis denotes the percentage.

have confirmed that activation of the Wnt/ β -catenin signaling pathway maintains stem cell renewal capacity and restrains stem cell differentiation in normal tissue (6-9), whereas it prompts tumor stem cell amplification in tumor tissue (9). Oct 3/4 is an embryo-specific transcription factor, which maintains totipotency (10) of the embryo stem cells and participates in

the renewal of stem cells (11). Oct 3/4 is thought to be one of the markers of totipotency or stem cells. Its expression was found to be extremely low or absent in differentiated cells, but was increased in normal stem and cancer stem cells (12,13). SOX2 was found to be expressed in totipotency cells and early multipotential cells in the embryo, regulating the self-renewal process (14,15) of embryo stem cells. SOX2 has been associated with the maintenance of cell pluripotency (16,17) and is also considered as a marker of stem cells (18). In light of the above findings, the mRNA expression of β -catenin, Oct 3/4 and SOX2 may be considered to represent the quantity of activated stem/initiating cells.

The other two markers of stem/initiating cells are CD44⁺/CD24⁻ cells and the rate of colony formation. According to a study by Al-Hajj *et al.* (19) as well as other previous studies (20-23), CD44⁺/CD24⁻ cells in breast cancer tissue exhibit certain stem cell-like properties, such as self-renewal and multi-directional differentiation, therefore they are considered to be breast cancer stem/initiating cells. The capacity to form colonies reflects renewal ability, so under equivalent seeding conditions, the rate of colony formation reflects the proportion of stem/initiating cells in the primitive seeded cells. In the present study, these two markers represented the proportion of stem/initiating cells.

In the experimental group, the mRNA expression of the above markers in the six generations exhibited an evident fluctuation with statistical significance, whereas the mRNA expression in the control group did not fluctuate. The results indicate that chemotherapy surely affects the quantity or function of CSCs.

The mRNA expression of three markers representing the quantity of CSCs (β -catenin, Oct 3/4 and SOX2) exhibited the following trend of fluctuation: decrease-increase-further increase-decrease-increase-decrease (1-6 generation). The two

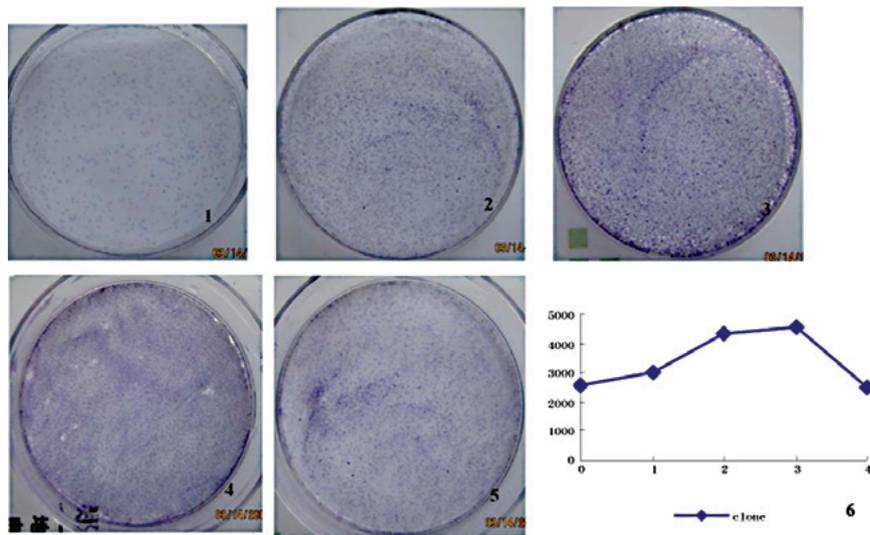


Figure 5. Colony formation in the experimental group. Images 1, 2, 3, 4 and 5 show the colony formation in the non-treated group, and first, second, third, fourth generation cells, respectively. Image 6 is a line graph showing fluctuations in the colony forming rate (colonies/10⁴ seeding cells) in the different cell generations. The x-axis indicates the cell generation, whereas the y-axis denotes the quantity of colonies.

Table IV. Correlation between the rate of clone formation and the other stem cell-related factors.

	β -catenin	Oct 3/4	SOX2	MRP1	BCRP	Survivin	CD44 ⁺ /CD24 ⁻
Clone formation							
r-value	0.811	0.722	0.850	0.792	0.858	0.880	0.992
p-value	0.096	0.168	0.068	0.110	0.063	0.049	0.001

markers representing the proportion of CSCs exhibited the following fluctuating trend: increase-further increase-further increase-decrease (1-4 generation). In the first cell generation, the quantity of CSCs decreased slightly since some of them were killed by 5-FU (even though they had drug-resistance properties). More non-stem/initiating cells were killed compared to CSCs, therefore the proportion of CSCs increased in the first generation cells. CSCs are activated and proliferate through stimulation of 5-FU, a decrease in cell number, and factors released from dead cells. Thus, the quantity and proportion of CSCs increased significantly in the second and third generations. In the fourth generation, most of the CSCs differentiated to relatively mature tumor cells, leading to the quantity and proportion of CSCs to decrease. The differentiated tumor cells had no or low drug-resistance, so a large portion of them were killed and the CSCs were again activated and proliferated, leading to an increase in the quantity and proportion of CSCs in the fifth generation cells. The situation in the sixth generation was similar to the fourth generation, reflecting the alteration resulting from CSC differentiation.

The results indicate that the quantity and proportion of CSCs increase and decrease in turn in different cell generations under a condition of persistent chemotherapy. This finding has not been previously reported.

The fact that the mRNA expression of β -catenin, Oct 3/4 and SOX2 exhibits a nearly perfect positive correlation ($p < 0.01$) suggests that they come from the same population of cells, proving their reliability to represent CSCs. The percentage of CD44⁺/CD24⁻ cells exhibited nearly a perfect

positive correlation ($r = 0.99$, $p < 0.01$) with the rate of colony formation, confirming their reliability as indicators of the proportion of CSCs.

Another key point in the present study is the drug-resistance of CSC. The MRP1 gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. This full transporter is a member of the MRP subfamily which is involved in multi-drug resistance (24-27). MRP proteins markedly increase in side population (SP) cells (28-30), in which CSCs are rich. The BCRP gene is also included in the superfamily of ABC transporters. BCRP has been proven to be involved in the multi-drug resistance of many types of cancers (particularly breast cancer) (31-33) and in CSCs (24-27).

mRNA expression of BCRP fluctuated along with an increase and decrease in the mRNA expression of β -catenin, Oct 3/4 and SOX2 in the second, third, fourth, fifth and sixth generation, showing a positive correlation between them. The results suggest that BCRP expression is a property of CSCs, and there is low BCRP expression in differentiated cells. This finding is consistent with that of Doyle *et al* (32), who argued that the number of SP cells (abundant in CSCs) in MCF-7/AdrVp (a multidrug-resistant human breast cancer subline) was higher than in parental MCF-7 cells, and BCRP was overexpressed in the former. A similar study (34) showed that CD133-positive cells (assumed markers of glioblastoma cancer stem cells) isolated from human glioblastoma expressed higher levels of BCRP1 mRNA. MRP1 did not demonstrate a positive correlation with CSC-related factors; thus, MRP1 may be expressed in both CSCs and differentiated cells.

Survivin is a member of the inhibitor of apoptosis gene family, which encodes negative regulatory proteins that prevent apoptotic cell death (35,36). In the present study, the expression of survivin mRNA was positively correlated with factors of CSCs, suggesting that the expression of survivin is associated with the drug-resistance of CSCs. In fact, overexpression of anti-apoptosis genes in cancer stem cells has been previously reported (34,37,38). 'Survivor cells' surviving chemotherapy in acute myelocytic leukemia, for example, were found to be enriched in CD34⁺ stem cells and accompanied by overexpression of many anti-apoptosis genes. BCRP may cooperate with survivin to maintain CSC survival.

In summary, the overexpression of drug-resistance genes and anti-apoptosis genes in CSCs is an important cause of traditional chemotherapy failure and cancer relapse. To cure cancer, effective ways to eliminate CSCs must be developed.

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