

ARTICLE

Isolating cancer stem cells in breast cancer models: a study of CD marker expression and Rhodamine 123 as predictors of chemoresistance

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Abstract

It is believed that CSCs, which are resistant to chemotherapy and radiation, develop in certain situations, stay in the tissue niche after treatment, and cause disease recurrence. Targeting these cells, which are thought to be chemoresistant, may enable treatment to eliminate metastasis or recurrence. For this reason, we aimed to isolate CSCs from various breast cancer cell lines using a drug selection method in this study and then identify them via flow cytometry and fluorescent staining. MDA-123, MCF-7, and 4T1 were used as in vitro models, with HUVEC as controls. After isolation of stem cells from these cells by incubation with 5-fluorouracil (5-FU) and cisplatin (Cis), CSCs were identified by flow cytometry using CD24/CD44/CD133/EPCAM by assessing the rhodamine 123 (Rho 123). The results showed that 5-FU and Cis reduced the CD24 receptor in both cancer and normal cells, while the others showed varying expression levels. Administration of 5-FU and Cis significantly decreased Rho 123 fluorescence intensity in MDA-MB-231, MCF-7, 4T1, and HUVEC cells compared to the untreated group. A subpopulation of breast cancer cells with low CD24 and Rho123 expression was cancer stem cells. The 4T1 cells, which expressed low levels of CD24/CD133/EpCAM expression, had significantly lower Rho 123 levels than MDA-MB-231 and MCF-7 cells. This suggests that 4T1 cells may have greater chemoresistance and more aggressive properties. In conclusion, studying surface receptors and rhodamine uptake for the identification of CSCs is a powerful and valuable method for evaluating the efficacy of chemotherapy. The discovery of CSC markers that indicate therapeutic strategies could lead to clinical trials of medicines with potentially better therapeutic outcomes, particularly for recurring malignancies.

Keywords Cancer stem-like cell · CD44-CD24 · CD133 · EpCAM · Rhodamine 123



Introduction

Cancer is a fatal disease that is affected by several factors, including lifestyle choices, environmental influences, internal stress, and heredity (Saller and Boyle 2022; Eala et al. 2022; Cheung and Vousden 2022; Kumari et al. 2022). In the 2020 edition of Globocan, it was estimated that there were 19.3 million new cases of cancer and 10 million cancer-related deaths worldwide. Of these total cases, female breast cancer represents one of the most commonly diagnosed cancers worldwide, with 2.26 million cases (Chhikara BS, Parang K. Global Cancer Statistics 2022). Cancer has traditionally been treated with chemotherapy, radiation, and surgery for solid tumors or a combination of these (Debela et al. 2021). Furthermore, recent research has focused on targeted therapies, including the use of tyrosine kinase inhibitors, monoclonal antibodies, and nanotechnology-based targeting (Chhikara BS, Parang K. Global Cancer Statistics 2022). While these treatments can shrink tumors or prolong life, they do not address the issue of metastasis and relapse (Sauer et al. 2021). This situation led researchers to develop new treatment strategies for cancer.

Drug resistance in breast cancer leads to aggressive progression and recurrence of the disease (Abad et al. 2020) and recurrence of the tumor (Basu et al. 2022). It is supposed that cancer stem-like cells (CSCs) are effective in tumor metastasis and recurrence (Zhang et al. 2023). CSCs living in the tumor microenvironment have shown high tumorigenic properties (Long et al. 2015). These stem cells exhibit characteristics such as self-renewal, multi-lineage differentiation, and clonogenicity (Corbet and Prieur 2020). As with normal stem cells, the dormant nature of cancer stem cells confers resistance to chemotherapeutics. CSCs are thought to be formed via the transformation and self-renewal of differentiated adult cells, tissue-resident stem cells, or their progeny (Hanahan and Weinberg 2011). CSCs, which are resistant to radiation and chemotherapy, are thought to remain in the tissue niche following treatment, emerge in some cases, and are thought to be responsible for disease recurrence (Dean et al. 2005; Malik and Nie 2012). Targeting these cells, which are thought to be chemoresistant (Mitra et al. 2015) treatment may eliminate metastasis or recurrence. Therefore, the isolation of CSCs from the tumor population and the identification of their markers may aid in the development of new therapeutic strategies.

Prior studies on CSCs have been conducted by purifying cells according to specific surface markers. The markers of CSCs are dependent on the specific type of tumour from which they are isolated. Prior studies on CSCs have been conducted by purifying cells according to specific surface markers. The markers of CSCs are dependent on the tumour type. Commonly used CSC markers include Cluster of Differentiation 24 (CD24), CD34, CD44, Interleukin-3 receptor α (CD123), Prominin-1 (CD133), Octamer-binding transcription factor 4 (Oct4), SRY-box transcription factor 2 (Sox2), NANOG homeobox (Nanog), KIT proto-oncogene receptor tyrosine kinase (c-Kit), ATP-binding cassette subfamily G member 2 (ABCG2), and aldehyde dehydrogenase (ALDH) (Bao et al. 2013; Kim et al. 2017; Mohan et al. 2021). The most accurate and consistent way of defining a specific CSC is to use a combination of these markers. Several stem cell markers have been identified in breast cancer stem-like cells (bCSCs), including CD44, CD133, ALDH1, epithelial cell adhesion molecule (EpCAM), C-X-C chemokine receptor type 4 (CXCR4), ABCG2, CD34, integrin $\alpha 6$ (CD49f), CD90, integrin $\beta 3$ (CD61) and breast cancer resistance protein (Li et al. 2017). The genetic instability of CSCs causes their proliferation independently of growth factors and microenvironmental signals, as well as their resistance to chemotherapy and radiotherapy. Therefore, functional selection with drugs could offer alternative approaches to analyzing them, rather than a purification process specific to the receptors (Hamilton and Olszewski 2013; Chiodi et al. 2011). The application of continuous or multi-step drug selection to establish multidrug-resistant cancer cell lines, which are thought to be cancer stem cells, represents a valuable tool for elucidating ATP-binding cassette (ABC) transporter function (Ma et al. 2010). For example, Ma et al. (2010) generated spheroid cells that overexpressed stem cell genes by using a drug selection method with cisplatin and paclitaxel. These spheroid cells were observed to exhibit enhanced tumorigenic potential and robust self-renewal capabilities (Ma et al. 2010).

Cancer stem-like cells are capable of protecting themselves from cytotoxic damage by remaining quiescent in the G0 phase of the cell cycle until they receive a stimulus to proliferate and differentiate (Chen et al. 2012).

Additionally, they can pump exposed chemotherapeutic drugs out of the cell via ABC transporters (McIntosh et al. 2016). In addition, overexpression of DNA repair genes (Desai et al. 2014) and ALDH, which detoxifies aldehydes, chemotherapeutic agents, and reactive oxygen species, leads these cells to survive cancer treatment (Vasilidou and Nebert 2005). It can therefore be surmised that these characteristics contribute to the development of drug resistance in CSCs. The targeting of CSCs with proven resistance to chemotherapy and radiotherapy is thought to be useful in providing relevant preclinical cancer models of CSCs and thus in the development of CSC-targeted therapies (Yehya et al. 2023). The aim of this study was therefore to purify bCSCs through a drug selection process.

Rhodamine 123 (Rho 123), a substrate for the ATP-binding cassette subfamily B member 1 (ABCB1)/P-gp transporter, is retained at varying levels within the cell contingent on the metabolic activity and differentiation potential of the stem cells' membrane (Chekwube et al. 2020; Millot et al. 1994). Cancer stem-like cells that are undergoing slow division demonstrate resistance to radiation and chemotherapy (Dean et al. 2005; Malik and Nie 2012). The elevated expression of efflux transporters in CSCs may be a contributing factor to the drug resistance observed in tumours. ABC transport proteins facilitate the efflux of the dye from the cell. An increase in P-gp activity results in the pumping out of Rho 123, leading to a significant reduction in fluorescence (Millot et al. 1994; Abbasi et al. 2021). Such a dye-pumping mechanism is a valuable technique for indirectly identifying stem-like cells (Wu and Alman 2008). Rhodamine 123 displays a diminished phenotype in metabolically inactive primitive cells with long-term transplant potential. In this study, rhodamine 123 dim-stained cells were considered to be stem cells.

In order to simulate relapses, which are likely caused by the proliferation of resistant CSCs in laboratory settings following clinical chemotherapy, *in vitro* studies are preferred for the purification of CSCs and the establishment of effective chemotherapy protocols. For example, Liu et al. (2014) reported a subpopulation of bCSCs that highly express E-cadherin and vimentin and are identified by positive expressions of ALDH, CD44, and the absence of CD24 expression (Liu et al. 2014). Similarly, Shah et al. (2022) also identified the presence of bCSCs carrying the same markers at varying levels in MDA-MB-231 and MCF-7 cell lines (Shah et al. 2022). In light of this information, we aimed to isolate CSCs from various breast cancer cell lines using a drug selection method in this study and then identify them via flow cytometry and fluorescent staining.

In conclusion, the use of a solitary CSC marker is inadequate in itself for the purpose of characterising stem properties. It is hypothesised that the combination of surface receptors and rhodamine 123 staining may provide a more accurate and reliable method for refining the definition of CSCs in breast cancer. In addition, the examination of surface receptors and rhodamine uptake together for the identification of CSCs will represent a valuable method for evaluating the effectiveness of chemotherapy.

Materials and methods

Collection of cancer stem cell subpopulations by drug selection from breast cancer cell lines

This study utilized MDA-MB-231 (MD Anderson Metastatic Breast-231), MCF-7 (Human Breast Adenocarcinoma), and 4T1 (Murine Mammary Carcinoma) breast cancer cell lines as *in vitro* models, and HUVEC (Human Umbilical Vein Endothelial Cells) as controls. These cell lines were obtained from the American Type Culture Collection (ATCC). The cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin in a humidified incubator (Nuair-Nu 5500 E US) containing 5% CO₂ at 37 °C. To obtain sufficient cells for testing, the cells were passaged once they reached 80% confluency as a monolayer. For drug selection, we seeded 10⁵ cells/mL in the wells of 6-well plates and treated them with 5-fluorouracil and cisplatin (Sigma). The concentrations used were 0.001 µg/mL for each drug for 2 weeks. These cells were replenished every 3 days by removing 50% of the medium. The number of dead and live cells was counted by using trypan blue dye on the floating cells in

the medium. After each drug refresh, floating dead cells were removed. Remaining adherent cells were washed and counted using trypan blue exclusion. Only trypan blue-negative viable cells were used for further experiments. After two weeks, resistant cells were detected using the Rho 123 uptake assay. Additionally, the group of untreated cells was analyzed in the same manner. The resistant cells were defined as cancer stem cells.

Functional identification by Rhodamine 123 staining

Rhodamine 123 staining commenced immediately after the processes outlined in Sect. "[Collection of cancer stem cell subpopulations by drug selection from breast cancer cell lines](#)". That is, for drug selection, experimental and control group cells were treated with 0.001 µg/ml 5-fluorouracil (5-FU) and cisplatin (Cis) (Sigma) for 2 weeks. The cells were incubated at a concentration of 10^5 cells/ml in a 6-well plate in a humidified incubator (Nuair-Nu 5500 E US) with 5% CO₂ at 37 °C. After incubation, the cells were washed twice with 1.000 µL of 1 × PBS. Next, the cells were exposed to 10 µM rhodamine 123 (Sigma-Aldrich) for 30 min at 37 °C in 5% CO₂. These cells were then washed three times with ice-cold 1 × PBS to remove excess Rho 123. Following the washes, the cells were fixed with a 3% glutaraldehyde buffer for one hour and then washed again with ice-cold 1 × PBS. After dehydrating and mounting, we investigated Rho 123 accumulation using fluorescence microscopy and analyzed it with ImageJ, taking advantage of the ability of Rho 123 to emit fluorescence. In each image, 20 fields were scanned, and the mean fluorescence intensities and standard deviation values were calculated. All experiments were conducted with three independent biological replicates, each containing three technical replicates.

Identification of subpopulations of cancer stem cells through flow cytometric analysis

Flow cytometric measurements were performed according to the manufacturer's instructions. The cells were incubated with 0.001 µg/ml 5-fluorouracil and cisplatin for 2 weeks. After an incubation period, the cells were trypsinized and then centrifuged for 5 min. The viable cells were counted using a hemocytometer after staining with trypan blue. Subsequently, 1×10^5 viable cells per sample were pelleted by centrifugation at 1500 rpm for 5 min.

Then, 5 µL of CD24-FITC (Santa Cruz Biotechnology), CD44-FITC (Santa Cruz Biotechnology), and EPCAM-FITC (BioLegend) antibodies, along with CD133-PE (BioLegend), were carefully mixed with 100 µL of viable cells using pipetting. The mixture was incubated in the dark for 15–20 min at +4 °C. After incubation, 100 µL of 1 × PBS was added. Then, after centrifugation and removal of the supernatant, the cells were analyzed using a Beckman Coulter flow cytometer. A total of 30.000 cells were counted. The results were evaluated in the NaviosEX software program (Beckman Coulter, Navios, IN, USA).

Results

Collection of cancer stem cell subpopulations by drug selection from breast cancer cell lines

The cancer stem cells were obtained through functional selection using 5-FU and Cis. MCF-7, MDA-MB-231, 4T1, and HUVEC cells were treated with 5-FU and Cis for 15 days, using a dose of 0.001 µg/ml for each drug.

Figure 1 presents microscopic images of breast cancer cells treated with and without the drug. Figure 1 shows the morphological changes in MCF-7, MDA-MB-231, and 4T1 breast cancer cells after 15 days of treatment with 0.001 µg/ml 5-FU and 0.001 µg/ml Cis. According to the figure, the days indicate that sensitive cells die, resulting in a decrease in cell number.

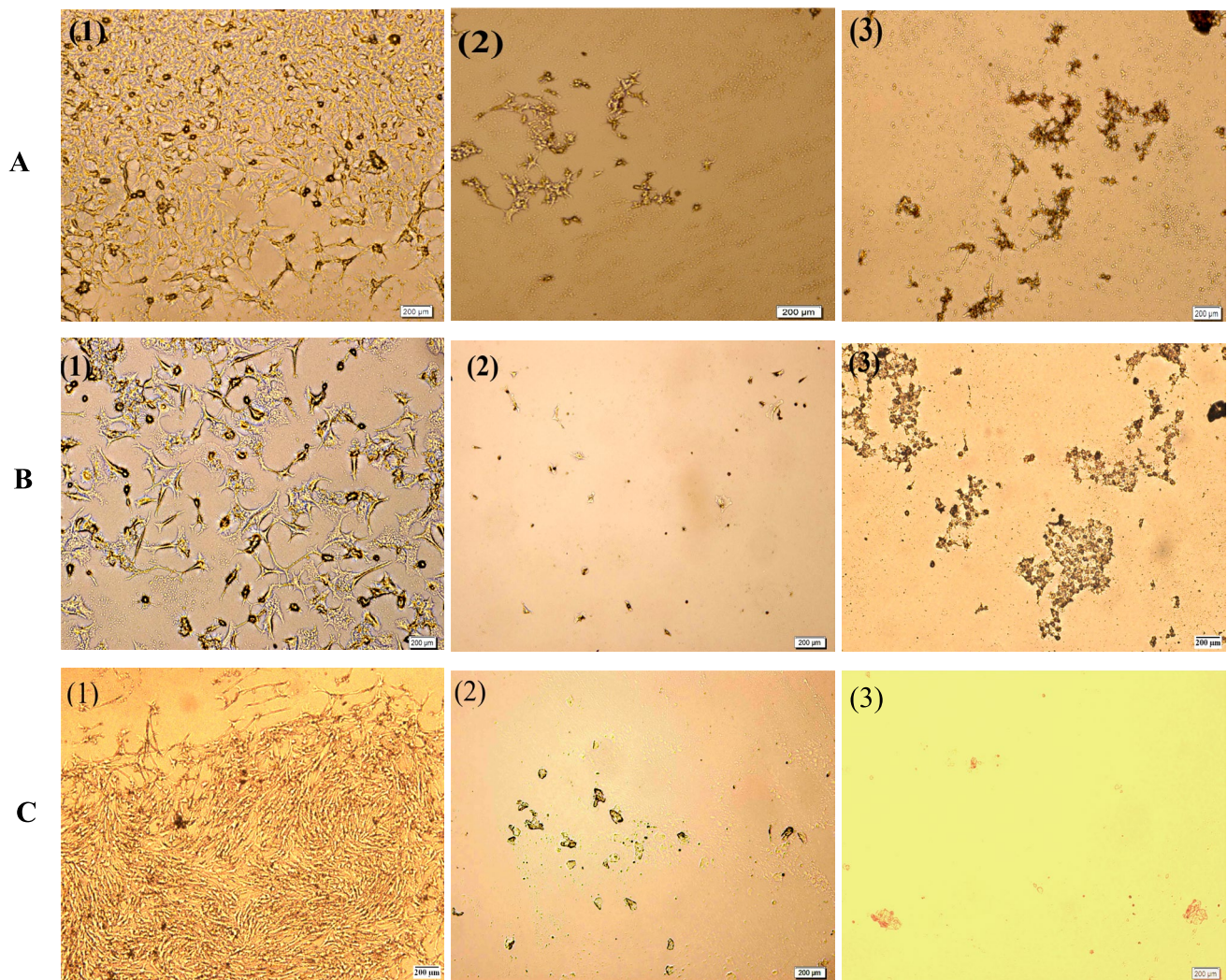


Fig. 1 Images of breast cancer cells treated and untreated with 5-FU and Cis. **(A)** MDA-MB-231 Cell Line, **(B)** MCF-7 Cell Line, **(C)** 4T1 Cell Line. 1 indicates untreated cells, 2 indicates the cells treated with 5-FU, and 3 indicates the cells treated with Cis. The drugs were administered for 15 days. Each drug dose was $0.001 \mu\text{g/mL} \times 20$ objectives

Functional identification by rhodamine 123 staining

Rhodamine 123 accumulation was detected by fluorescence microscopy. ImageJ software was used for analysis. Drug treatment with 5-FU and Cis resulted in a decrease in Rho123 fluorescence intensity in all cells compared to the control groups.

The mean value of Rho 123 fluorescence intensity (MFI) is presented in Table 1. The mean fluorescence intensity was 78.15 ± 14.75 for untreated MDA-MB-231 cells (control), and 38.14 ± 5.08 and 24.52 ± 9.01 for 5FU and Cis-treated MDA-MB-231 cells, respectively.

The fluorescence intensity of Rho 123 was 61.32 ± 7.2 in the control group for MCF-7 cells, while it was 27.28 ± 6.1 and 27.87 ± 5.9 in the 5FU and Cis-treated groups, respectively. Finally, when this density was measured for 4T1 cells, it was found to be 59.95 ± 18.5 in the control group and 7.67 ± 3.47 to 4.75 ± 1.576 in the 5FU and Cis-treated groups, respectively. When normal endothelial HUVEC cells were analysed, the MFI value was 28.88 ± 3.23 in the control group, while it was 6.68 ± 1.9 and 8.067 ± 3.45 in 5FU and Cis-treated cells, respectively.

Table 1 Mean fluorescence intensity values of Rhodamine 123 on MCF-7, MDA-MB-231, 4T1 breast cancer and normal HUVEC cells

Group	MFI $\bar{X} \pm S.D$	Min–Max	p
MDA-MB-231-Control	78.15 $\pm$ 14.75	46–98.04	
MDA-MB-231-5FU	38.14 $\pm$ 5.08 ^a	25–48	0.000
MDA-MB-231-Cis	24.52 $\pm$ 9.01 ^b	21–41.4	0.002
MCF-7-Control	61.32 $\pm$ 7.2	47.4–74.6	
MCF-7-5FU	27.28 $\pm$ 6.1 ^c	14.35–41.12	0.000
MCF-7-Cis	27.87 $\pm$ 5.9 ^d	14.545–27.30	0.000
4T1-Control	59.95 $\pm$ 18.5	34.963–99.54	
4T1-5FU	7.67 $\pm$ 3.47 ^e	3.80–13.90	0.000
4T1-Cis	4.75 $\pm$ 1.57 ^f	2.71–8.95	0.000
HUVEC-Control	28.88 $\pm$ 3.23	32.96–28.88	
HUVEC-5FU	6.68 $\pm$ 1.9 ^g	3.85–10.9	0.000
HUVEC-Cis	8.067 $\pm$ 3.45 ^h	11.02–16.86	0.000

†Means and standard deviations ($\bar{X} \pm SD$). Statistical significance between Control and 5FU ^{a,c,e,g}; Control and Cis ^{b,d,f,h}. p-value was calculated according to control by the Mann–Whitney U test using SPSS (p-values < 0.05). MFI: Mean Fluorescence Intensity

Figure 2 shows the fluorescence staining images of Rho 123 on normal HUVEC and MDA-MB-123, MCF-7, and 4T1 breast cancer cells. Accordingly, in all cell groups, Rho 123 showed remarkably higher fluorescence in untreated control groups, while decreased fluorescence was observed in 5FU and Cis-treated groups ($p \leq 0.001$, Table 1).

Table 2 presents a comparison of Rho 123 fluorescence intensities in drug-treated HUVEC cells and drug-treated breast cancer cells. The MFI of Rho123 in healthy HUVEC cells treated with 5FU and Cis was statistically lower than in MDA-MB231 and MCF-7 cells ($p < 0.01$). The MFI value in the 4T1-5-FU cell group was similar to that of HUVEC-5FU cells, and no statistically significant difference was identified ($p > 0.05$). However, in the 4T1-Cis cell group, the MFI value was significantly lower than that of HUVEC-Cis cells, and this value was 4.75 ± 1.57 ($p \leq 0.01$).

Identification of subpopulations of cancer stem cells through flow cytometric analysis

In this study, cancer stem cells were identified using surface markers via flow cytometry. Figure 3 shows the levels of monoclonal antibodies in both the control, 5-FU-treated, and Cis-treated groups. According to the results, both drugs (5-FU and Cis) reduced the CD24 receptor in cancer and normal cells. In MDA-MB-231 cells, both drugs lowered the levels of CD44, CD133, and the EpCAM receptor. While the CD44 and CD133 receptors were reduced in MCF-7 cells after Cis treatment, the EpCAM receptors increased considerably. Although CD44 and CD133 antibody levels remained unchanged in the same cells after 5-FU treatment compared to the control group, a significant increase in EpCAM levels was observed. 5FU-treated 4T1 cells exhibited higher growth in the CD133 marker compared to Cis-treated 4T1 cells. In HUVEC cells, CD133 marker expression decreased less in 5-FU-treated cells compared to Cis-treated cells. CD44 expression remained constant in 5-FU-treated cells but increased in Cis-treated cells. EpCAM exhibited a similar reduction level in both treatments.

Discussion

The identification of CSCs may necessitate the analysis of multiple markers. For example, CD20, CD44, CD133, CD24, and EPCAM have been extensively employed in the context of solid tumors, while CD133, CD34, and CD38 have been utilized in the investigation of hematological malignancies (Kim et al. 2017; Fang et al. 2005; Patrawala et al. 2006; Herrmann et al. 2020). The expression of these markers is either absent or present at varying levels in some cancers when analyzed by flow cytometry. In a study conducted by Li et al. (2007), CD44+/CD24+/ESA+ positive cancer stem cells were isolated from tumor tissue derived from a total of 10 human pancreatic adenocarcinoma xenografts. These cells demonstrated the capacity for self-renewal, the production of differentiated progeny, and highly tumorigenic properties (Li et al. 2007). In a 2014 study, CD133, CD90, and

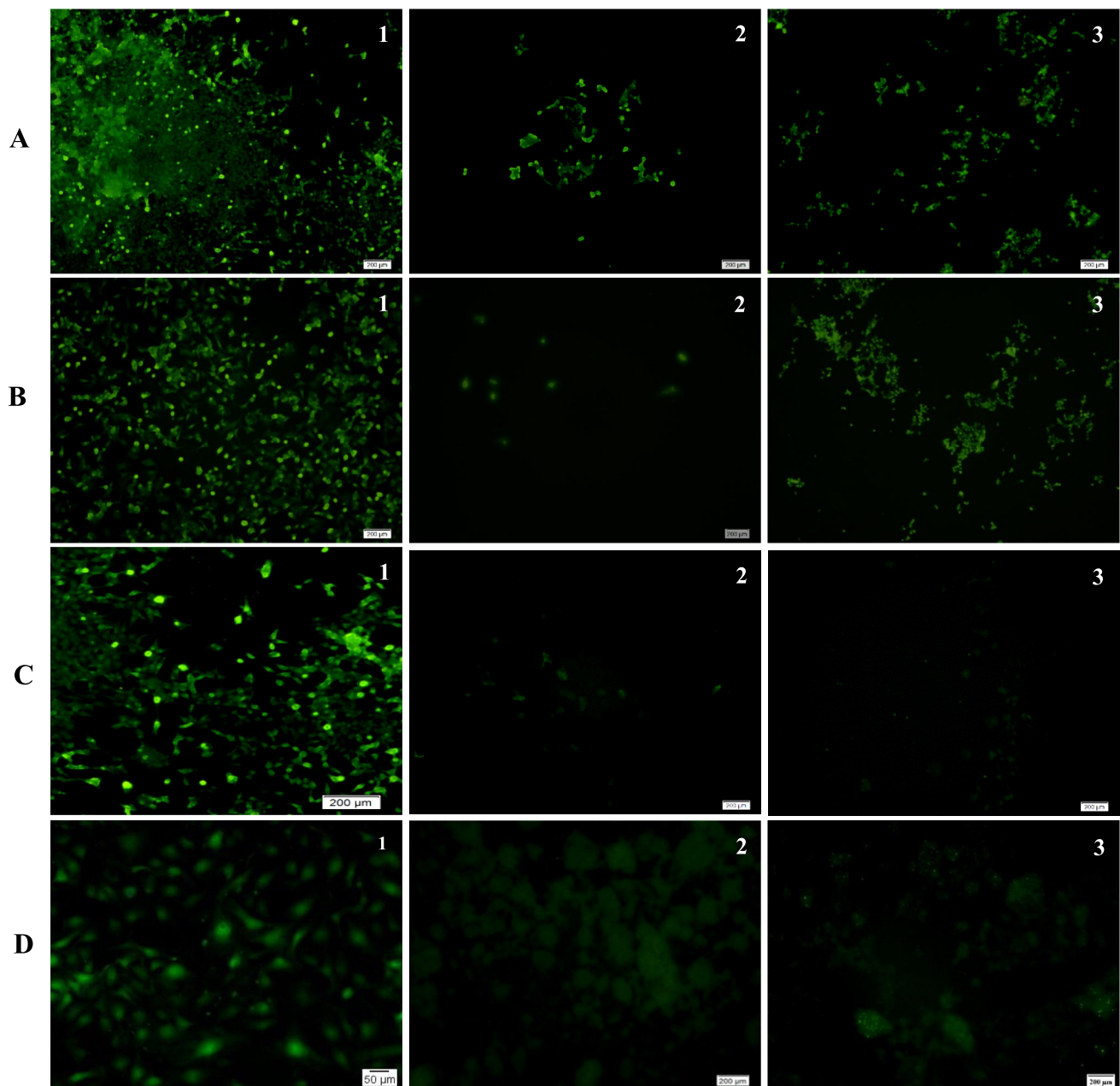


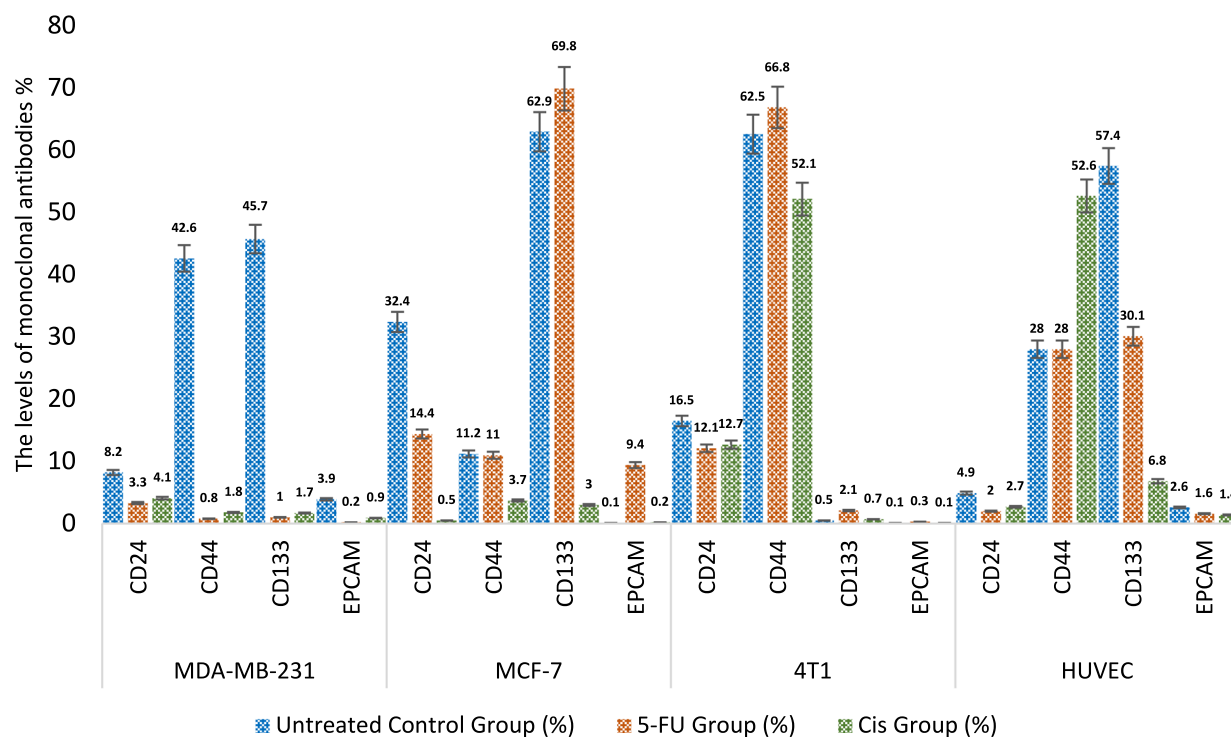
Fig. 2 Fluorescence staining images of cells stained with Rhodamine 123. (A) MDA-MB-231 cell line (B) MCF-7 cell line (C) 4T1 cell line (D) HUVEC cell line. 1: Untreated Rho 123^{bright} cells, 2: Rho 123^{dim} cells treated with 5-FU, 3: Rho 123^{dim} cells treated with Cis. $\times 20$ objective

EpCAM-expressing cells were isolated from patients with hepatocellular carcinoma (HCC). These cells were found to be associated with a shorter survival time and an earlier recurrence of the tumor. It was hypothesised that these cells were cancer stem cells (Guo et al. 2014). In breast cancer, the CD44⁺/CD24^{low} population was identified as cancer stem cells (Gaio et al. 2020). A subsequent study discovered that CD44⁺/CD24⁻ cells were breast cancer stem cells (Pervan et al. 2022). In another study, it has been shown that the expression of SOX2, OCT4, and NANOG markers increases in CD44-positive breast cancer stem cells (Karalis et al. 2022). The present study demonstrated a reduction in CD24 levels in both healthy and breast cancer cells following the administration of 5-FU and Cis, as evidenced in the published literature. A reduction or absence of the CD24 marker is

Table 2 Mean Fluorescence Intensity Values of Rhodamine 123 in MCF-7, MDA-MB-231, 3T3 breast cancer, and normal HUVEC cells treated with 5-FU and Cis

Group	MFI $\bar{X} \pm S.D$	Min–Max	p
MDA-MB-231-5FU	38.14 $\pm$ 5.08	25–48	0.000
HUVEC-5FU	6.68 $\pm$ 1.9	3.85–10.9	
MDA-MB-231-Cis	24.52 $\pm$ 9.01	21–41.4	0.005
HUVEC-Cis	8.067 $\pm$ 3.45	11.02–16.86	
MCF-7-5FU	27.28 $\pm$ 6.1	14,35–41,12	0.000
HUVEC-5FU	6.68 $\pm$ 1.9	3.85–10.9	
MCF-7-Cis	27.87 $\pm$ 5.9	14,545–27.30	0.000
HUVEC-Cis	8.067 $\pm$ 3.45	11.02–16.86	
4T1-5FU	7.67 $\pm$ 3.47	3,80–13,90	0.91
HUVEC-5FU	6.68 $\pm$ 1.9	3.85–10.9	
4T1-Cis	4.75 $\pm$ 1.57	2.71–8.95	0.001
HUVEC-Cis	8.067 $\pm$ 3.45 ^h	11.02–16.86	

^hMeans and standard deviations ($\bar{X} \pm S.D$). Statistically significance between MDA-MB-231, MCF-7, 4T1/5-FU and HUVEC-5FU; MDA-MB-231, MCF-7, 4T1/Cis and HUVEC-Cis. p-value was calculated according to control by the Mann–Whitney U test using SPSS (p-values < 0.05). MFI: Mean Fluorescence Intensity

**Fig. 3** The alterations in marker expression of stem cells in both breast cancer and healthy cells before and 15 days after drug administration

associated with cancer stem cells (Li et al. 2017). These findings are corroborated by existing literature. However, the levels of other surface markers exhibited variability in both healthy and breast cancer cells in response to the antineoplastic drugs. The malignant cell population comprises subpopulations with different phenotypes, which are associated with cell proliferation or cell migration. This subpopulation has been defined as cancer stem cells exhibiting epithelial-to-mesenchymal transition (EMT) or mesenchymal-to-epithelial transition (MET) characteristics (Brabletz et al. 2005; Mani et al. 2008). It has been reported that CD44 expression levels may change in these transition cells. For example, Biddle et al. (2011) classified cancer stem cells based on CD44 expression levels. The researchers distinguished between two types of cancer stem cells based on CD44 expression levels:

a CD44-low cancer stem cell, which is non-EMT and forms a paraclone whose self-renewal ability is lost, and a CD44-high cancer stem cell type, which is proliferative and metastatic (Biddle et al. 2011). The present study demonstrated that the CD44 receptor remained unaltered following 5-FU treatment in MCF-7, 4T1, and HUVEC cells. Nevertheless, a notable decline was evident in MDA-MB-231 cells. Upon examination of the impact of Cis application on the CD44 receptor, a significant increase was observed in HUVEC cells (see Fig. 3). A reduction in CD44 levels may be indicative of the differentiation of cancer stem cells in response to drug treatment. It can therefore be postulated that the drugs employed are capable of inactivating cancer stem cells in MDA-MB-231 and MCF-7 cells. The observation that this receptor level remains unchanged in 4T1 cells may be indicative of a greater propensity for these cells to metastasise or to proliferate. The observed elevation of CD44 in HUVEC cells may be indicative of epithelial-to-mesenchymal transition. CD133, also known as Prominin-1, is a biomarker that plays a role in cell growth, development, and tumour biology. Both CD133+ and CD133- cells have been identified as tumour-initiating cells (Shmelkov et al. 2005). In general, cells that are positive for CD133 and cells that are negative for CD133 exhibit distinct characteristics. To illustrate, glioma cells that are CD133+ are derived from primordial CD133+ cancer stem cells. Moreover, CD133-CSCs maintain their stem-like characteristics and capacity for tumour initiation, and can re-express CD133 in vivo (Lottaz et al. 2010; Campos and Herold-Mende 2011). Several studies have shown that CD133+ cells are linked to metastasis and resistance to chemotherapy and radiotherapy. In their 2009 study, Bertolini et al. (2009) reported that treatment of lung cancer cells in vitro with cisplatin resulted in enrichment of the CD133+ fraction, both after acute exposure to the drug and in cells with a stable cisplatin-resistant phenotype (Bertolini et al. 2009). In a separate study, Piao et al. (2012) observed that irradiated CD133+ Huh-7 hepatocellular carcinoma cells induced tumour formation in an in vivo xenograft model. It was hypothesized that these cells were resistant to radiotherapy (Piao et al. 2012). Zhang et al. (2012) demonstrated that CD133+/CXCR4+ cancer cells exhibit a high metastatic capacity both in vitro and in vivo (Zhang et al. 2012). In our study, the administration of 5-FU resulted in an increase in CD133 receptor levels in 4T1 cells (Fig. 3). These findings indicate that 4T1 cells exhibit a high metastatic capacity and chemoradioresistance. In light of the results above, it can be posited that 4T1 cells evince a greater degree of resistance to both 5-FU and Cis in comparison to MDA-MB-231 and MCF-7 cells. Furthermore, it is proposed that 4T1 cells may display greater aggressiveness than other cells.

Some studies have indicated that the EpCAM is associated with cancer stemness, metastasis, and chemo-/radio-resistance (Zheng et al. 2017; Wang et al. 2018). The present study demonstrated that EPCAM, a receptor associated with primitive stem cells, exhibited elevated levels in MCF-7 and 4T1 cells following 5FU treatment. This observation suggests that these cells may have developed resistance to the drug. Moreover, the elevation of the EpCAM receptor, particularly in CD24^{low}/CD133^{high} 4T1 cells, indicates that these cells possess a high metastatic potential. It has been established in the scientific literature that the 4T1 cell line serves as an animal model for stage IV human breast cancer (ATCC). Additionally, it has been documented that the prognosis for metastatic cancer at this stage is unfavourable (Mahar et al. 2024). In both MDA-MB-231 and HUVEC cells, treatment with 5FU and Cis resulted in a decrease in EPCAM levels, as evidenced in Fig. 3. The therapeutic drugs employed may demonstrate varying degrees of specificity for different cells and different CSC markers. Our findings suggest that the effects of the medicines 5-FU and Cis may be contingent on the cell type. Consequently, variations in receptor levels (i.e., CD24, CD44, CD133, and EPCAM) of the cells can be observed.

The data presented above suggest that the purification of cancer stem cells may not be achieved solely through the use of receptors such as CD24, CD44, CD133, and EPCAM. The level of surface markers present on these cells varies according to the specific drug being administered and the tissue of origin. Furthermore, their genetic instability (Hamilton and Olszewski 2013; Chiodi et al. 2011) affects the levels of the markers above. It would therefore be beneficial to employ additional methods beyond those based on surface markers. Normal stem cells (NSCs) are defined according to their source of origin. To characterise these cells, specific markers are employed, including Endoglin (CD105), Thy-1 (CD90), Ecto-5'-nucleotidase (CD73), adhesion molecule ITGAM (CD11b), neural cell adhesion molecule (CD56), Paired box 7 (PAX7), Cluster of Differentiation 19, 29, 31, 38, 45, 166 (CD19, CD29, CD31, CD38, CD45, CD166), CD24 CD44, SOX2, CD34, and CD133, (Berry and Rodeheffer

2013; Shin et al. 2021; Fonseca et al. 2023; Brunet et al. 2023; Soleimani and Naghani 2023). It is also noteworthy that some of these markers are employed in the characterisation of cancer stem cells. The presence of surface antibodies is a common feature of both NSCs and CSCs, with expression levels varying in specific cancer types, thereby posing a challenge in characterizing CSCs. The expression of the same antibodies in healthy and breast cancer cells presented a challenge in identifying cancer stem cells. Consequently, alternative methodologies are required in conjunction with antibody-based identification.

It is believed that mitochondrial mutations occur before nuclear mutations (Beneyto-Calabuig et al. 2023). According to this information, molecular changes in mitochondria that begin early in cancer cells can facilitate the detection of CSCs. Several methods can be used to detect mitochondrial changes. One of these methods is the measurement of mitochondrial membrane potential, which is proportional to the accumulation of fluorescent dye in the mitochondria (Rickard et al. 2023). Rhodamine 123 is frequently utilized as a fluorescent dye (Hanif et al. 2023; Ernst et al. 2023). Rhodamine 123 exhibits low fluorescence in metabolically inactive primitive stem cells (Bertoncello et al. 1985), allowing for easy differentiation between stem cells and other cells. The use of Rho 123 may be the gold standard for detecting CSCs. In a study, the isolation of cancer stem cell-like cells from the MHCC97 cell line was evaluated using the Rho123 accumulation assay (Rho low and Rho high). In this study, Rho 123 low cells were shown to have similar characteristics to SP (side population) cells, which have a high proliferative capacity and express high levels of CD133 (Liu et al. 2010). Vu et al. (2013) reported that hepatocellular carcinoma stem cells strongly expressed CD13, CD133, CD90, and CD326, effluxed rhodamine 123, and formed tumor spheres in suspension (Vu et al. 2013). It was observed that both drugs (i.e., 5-FU and Cis) resulted in a significant decrease in Rho123 fluorescence intensity in MDA-MB-231, MCF-7, 4T1, and HUVEC cells compared to the control group in the present work.

In the introduction, it was explained that cancer stem cells survive by becoming resistant to chemotherapy, which can lead to cancer recurrence. Studies of drug resistance have focused on the mechanisms that pump drugs out of the cell. For example, one study found that Rho 123 accumulation was significantly reduced in P-gp-expressing R7 cells, RVC, and RDC (multidrug-resistant models) compared to K562 control (Chen et al. 2020). In particular, in some cancers, the high expression of molecules that pump drugs out of cells, including those in cancer stem cells and normal stem cells, may explain the drug resistance observed in tumors. Kuo et al. (2023) concluded that the expression of the ABCG2 transporter protein was significantly higher in CD44+ and EpCAM+ CSCs of PDAC cell lines. This work demonstrates that cancer stem-like cells display chemoresistance (Kuo et al. 2023). Recently, candidate cancer drugs have been evaluated by measuring the accumulation of the Rho123 fluorescent dye. For example, Bortolozzi et al. (2020) performed a Rho 123 dye accumulation assay using flow cytometry to assess the ability of newly synthesized ecdysteroids to modulate the ABCB1 multidrug resistance pump (Bortolozzi et al. 2020). Haiyat et al. (2021) discovered that the fluorescence intensity of Rho 123 and the expression of CD24 increased in bCSCs as drug resistance decreased (Haiyat et al. 2021). Dehghanzad et al. (2024) conducted a study to characterise cancer stem cells from a human colorectal cancer cell line (Dehghanzad et al. 2024). The LS174T cells (LS174T/DR) showed only 0.66% and 2.46% double positivity for CD133 and CD44 markers in sensitive and oxaliplatin-resistant cells, respectively. Rho 123 accumulation was observed to be low in these resistant cells. In another study, it was found that Cis-resistant lung cancer cells (A549/CDD), which were abundantly overexpressing cancer stem cell markers, also overexpressed embryonic stem cell markers OCT-4 and NANOG, as well as the drug resistance-associated protein MRP1. Additionally, Rhodamine 123 fluorescence intensity was measured to examine the transport capacity of MRP1, revealing lower fluorescence intensity in A549/CDD cells compared to non-resistant A549 cells (Ye et al. 2024).

Our findings indicate a notable decline in Rho 123 fluorescence intensity among MDA-MB-231, 4T1, and MCF-7 cells with low CD24 levels following treatment with 5-FU and Cis when compared to the control group ($p < 0.05$; see Table 1 and Fig. 2). It can therefore be posited that Rho 123^(dim) cells that have developed resistance to anticancer agents may be considered to exhibit stem-like characteristics. The fluorescence intensity of Rho123 in healthy HUVEC cells treated with 5FU and Cis was found to be statistically lower than in MDA-MB231 and MCF-7 cells ($p < 0.05$, as shown in Table 2). This may indicate that ABC transporter proteins, such

as pg-p, are more highly expressed in HUVEC-5FU and HUVEC-Cis group cells than in MDA-MB-231-5FU and MDA-MB-231-Cis and MCF-7-5FU and MCF-7-Cis group cells. The results suggest that healthy cells may possess the ability to protect themselves from chemotherapeutic agents through a drug-pumping mechanism, whereas breast cancer cells appear to be more sensitive. The measurement of fluorescence intensity in 4T1-5FU and 4T1-Cis cells at a level comparable to that observed in HUVEC cells may suggest that 4T1 cells exhibit enhanced chemoresistance and aggressive characteristics compared to MDA-MB-231 and MCF-7 cells. In conclusion, the measurement of fluorescence intensity, which indicates the accumulation of Rho 123 in cells, may prove to be a promising and practical method for the biological research of CSCs and the development of targeted therapies resistant to drugs. Furthermore, this approach is considered advantageous in studies related to drug resistance and the effectiveness of anticancer treatments.

Drug selection is a valuable tool in obtaining CSCs with drug resistance (Ma et al. 2010). The measurement of Rho 123 fluorescence intensity represents a universal method for the assessment of the effect of P-gp associated with drug resistance (Millot et al. 1994). Consequently, measuring Rho 123 accumulation following the selection of a drug may prove to be a valuable method for determining cancer stages and identifying cancer stem cells.

Conclusion

The identification and characterization of cancer stem cells are of great importance for the development of therapies, especially in cancers with a tendency to metastasize and recur. In this study, CSCs were successfully isolated from various breast cancer cell lines (MDA-MB-231, MCF-7, and 4T1) using 5-fluorouracil (5-FU) and cisplatin (Cis). In our study, we demonstrated a significant decrease in the expression of surface markers CD24, CD44, CD133, and EPCAM, as well as a reduction of the fluorescence intensity of rhodamine 123, which collectively indicate the presence of CSCs.

According to our findings, it is clear that CSCs exhibit high resistance to chemotherapy, hence the need for therapies that eliminate these cells and prevent disease relapse. Therefore, this study emphasizes the importance of developing drug therapies tailored to the specific cell type and receptors. In addition, our study highlights the genetic instability and heterogeneity of CSCs, thus emphasizing the necessity of functional selection with drugs in the analysis of CSCs, as conventional receptor-specific purification techniques may be insufficient. In conclusion, this research provides valuable insights into the mechanisms of drug resistance in breast cancer and offers a comprehensive method to identify and study CSCs. Future clinical trials focusing on CSC markers have the potential to benefit patients with recurrent malignancies.

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Author contribution S.E.K., N.O., and I.N.K. designed the study. I.N.K., M.S., N.O. and A.E. performed the experiments. F.D.K., N.O., and D.Ö. data analyzing. N.O., D.Ö., S.E.K. and F.D.K. writing. S.E.K., F.D.K., D.Ö., N.O. revised the manuscript. All authors contributed to the data analysis and the manuscript's writing.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors confirm that they have no competing interests.

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