Pathology & Oncology Research (2020) 26:371–378
https://doi.org/10.1007/s12253-018-0503-8

ORIGINAL ARTICLE

Do Different Stemness Markers Identify Different Pools of Cancer Stem Cells in Malignancies: *A Study on ER+ and ER-Breast Cancer Cell Lines*

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Received: 6 February 2018 / Accepted: 15 October 2018 / Published online: 25 October 2018

Abstract

In view of popularity of cancer stem cell (CSC) model all events in evolution of cancer are being explained in that context. Breast cancer is first solid tumor in which CSCs were identified. We aimed to compare stemness profile of two major subtypes [Estrogen receptor positive (ER^+) and negative (ER^-)] breast cancer using different sets of markers. Expression of CD44/CD24, CK/Vimentin, E-Cadherin/Fibronectin and percentage of side population (SP) was studied in ER^+ (T47D) and ER^- (MDA-MB-231) cell lines by flow cytometry. Breast CSCs (BCSCs) were sorted using $CD44^+/CD24^{-/low}$ expression and SP analysis and cultured. BCSCs were then compared with Non-CSCs (NCSCs) for response to drugs (Paclitaxel and Cisplatin), Ki67 and ER expression. Results showed higher expression of stemness markers ($CD44^+/CD24^{-/low}$, CK^+/$Vimentin^+$ and E-$Cadherin^-$/$Fibrinectin^{F+}$) in MDA-MB-231 cells. Percentage SP representing BCSCs was found to be significantly more in later (3.20 ± 0.002 cf. T47D $1.25\% \pm 0.0007$). BCSCs were found to be more resistant to drugs as compared to NCSCs in both cell lines. ER expression was weak in BCSCs sorted from T47D as compared to NCSCs. Ki67 was expressed in both BCSCs and NCSCs. Differences in expression of stemness markers help to explain aggressive behavior, higher recurrence rate and metastatic potential of MDA-MB-231 cells. However, no correlation amongst different markers used suggests that they may be identifying varied populations of cells in tumor hierarchy. A weak ER expression in BCSCs may be strategy used by BCSCs to escape effect of hormone therapy in ER^+ breast cancers.

Keywords Stemness · Breast cancer · Cancer stem cells · Estrogen receptor

Introduction

Existence of genetic, epigenetic and phenotypic heterogeneity within cancer cells of same tumour is a well-established now [1]. At the topmost level of tumour hierarchy is believed to be present a small population of cells called cancer stem cells(CSCs) with the ability to undergo self-renewal and generate different lineages of cancer cells. Currently, the CSCs are predicted to mediate tumor non-response and recurrence post chemo-radiotherapy due to the relative inability of these therapies to effectively target them [2, 3]. Amongst the solid cancers, tumours of mammary gland are found to exhibit high levels of both inter as well as intra-tumoral heterogeneity [4]. Clinically, breast cancers have been characterised on the basis of Estrogen receptor (ER) expression into ER^+ and ER^- cancers. Despite advances in therapy of these cancers many difficulties are encountered in their effective treatment. Whereas ER^- cancers are characterized by lack of targeted therapy, tumour recurrence and an overall aggressive course, issues like drug resistance preclude successful targeting of ER^+ tumours [5]. In this context regulators of stemness may serve as important novel therapeutic targets for management of these tumours [6].

Many studies have elaborated upon the distinctive properties of ER^+ and ER^- cancers. Although variety of markers have been proposed for identification and characterisation of breast CSCs (BCSCs), no marker(s) is ideal [7]. Hence, in this study we made an attempt to understand the stemness profile of these two distinct classes of breast cancers using different set of previously described markers in ER^- and ER^+ breast cancer cell lines (BCCLs).

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Materials and Methods

The BCCLs MDA-MB-231 (ER^-) and T-47D (ER^+) were maintained in RPMI-1640 (Gibco) medium supplemented with 10% FCS (Gibco), 100 μg/ml streptomycin (HiMedia) and 100 U/ml penicillin (HiMedia) in a humified incubator (37 °C, 5% CO_2). To obtain high numbers necessary for sorting, the cells were grown in T-25 and T-75 flasks (Corning) respectively and were detached by treatment with trypsin.

Expression of surface markers like CD24, CD44 was analysed by incubating the cells with antibodies (20 μl each of PE-CD24 and FITC-CD44) for 30 min at room temperature (RT) in dark. Further, expression of intracellular markers like Vimentin (Vim), Cytokeratin (CK), E-Cadherin (E-Cad), Fibronectin (FN) (BD Biosciences) was analysed by fixing the cells (4% Paraformaldehyde), permeabilization (1%BSA + 0.1% Sodium Azide+0.1% Triton-X-100) and blocking (5% BSA in 1XPBS) followed by incubation with antibodies (20 μL of FITC-CK and 5 μl each of PE-Vim, PE-E-Cad and AlexaFlour647-FN) for 45–60 min at RT in the dark. Side population (SP) was analysed by incubating the cells (10^6 cells/ml) with Hoescht 33,342 (5 μg/ml, Sigma-Aldrich) for 90 min at 37 °C (water bath).

For isolation of BCSCs two protocols were used. In T-47D, isolation of BCSCs was done using SP analysis while in MDA-MB-231, isolation was done by combining cell surface marker and SP analysis. Cells in concentration of 10^7/ 100 μl PBS were first stained with CD44 and CD24 in 5 ml tube. Ten lac cells were then re-suspended in 1 ml of pre-warmed RPMI medium and stained with freshly prepared Hoescht 33,342 dye as per protocol mentioned above.

Sorted BCSCs were seeded in 6/24 well plate in serum-free DMEM-F12, supplemented with 10 ng/ml Fibroblast Growth Factor (FGF), 20 ng/mL Epidermal Growth Factor (EGF), 5 ng/mL insulin and 0.4% bovine serum albumin (all from Sigma) and incubated at 37 °C in 5% CO2 incubator. The non CSC (NCSC) population was collected in separate tube and cultured in DMEM-F12 with FCS (10%). On isolation of BCSCs and NCSCs, expression of Ki-67 was analysed in both cell lines while expression of ER was analysed only in ER^+ sorted cells. Briefly, the cells were seeded on Poly-L-Lysine coated slides and were fixed, permeabilised and blocked followed by incubation with Alexa Flour 488 Mouse- Ki-67 anti human antibody (2 μl) while in case of ER expression, cells were first stained with Rabbit anti human ER-α overnight at 4 °C followed by staining with anti-rabbit FITC-secondary antibody (1:100 dilution each, Invitrogen) for 1 h at RT. Finally, the cells were analysed under florescent inverted microscope (Nikon Eclipse Ti). Quantitative analysis of ER in sorted cells was done using Image J software (NIH) and mean corrected total cell fluorescence (CTCF) was calculated. Cytotoxic effect of drugs like Cisplatin (4 μM) and Paclitaxel (1 μM) was analysed on ER^- and ER^+ sorted cells respectively after incubating for 24 h followed by analysis of viable population through Trypan Blue assay. Statistical analysis was done by *Parametric students t-test* using GraphPad Prism software (version5.00). All the work was carried out after approval from Institute Ethics Committee.

Results

Expression of Different Markers in MDA-MB-231 and T-47D BCCLs

Two gating strategies were used for expression analysis**.** First, to exclude dead cells and debris, the cells were gated on a two physical parameters; Dot plot measuring forward scatter (FSC) vs side scatter (SSC). Second, the expression level for panel of stemness markers was reported in PE vs FITC plots.

CD44/CD24

Percentage of $CD44^+/CD24^{-/low}$ cells were significantly high ($p < 0.0001$) in MDA-MB-231 (96.67% ± 0.012) as compared to T-47D (0.05% ±0.0007) and $CD44^-/CD24^+$ cells were found to be significantly high ($p < 0.0001$) in T-47D (86.7% ± 0.08) cells. In contrast, $CD44^+/CD24^+$ cells were low in both T-47D (4.70% ± 0.03) and MDA-MB-231 (1.87% ± 0.022). However, $CD44^-/CD24^-$ cells in T-47D and MDA-MB-231 were 12.47% ± 0.07 and 1% ± 0.011 respectively (Fig. 1a).

CK/Vim

Percentage of CK^+/Vim^- cells were significantly high ($p = 0.0176$) in T-47D (14.80% ± 0.026) as compared to MDA-MB-231 (0.45% ± 0.004). However, percentage of CK^-/Vim^+ cells were higher in MDA-MB-231 (9.15% ± 0.09) as compared to T-47D (3.65% ± 0.05).

In addition, percentage of CK^+/Vim^+ cells were higher in MDA-MB-231 (88.55% ± 0.125) as compared to T-47D (70.45% ± 0.170). CK^-/Vim^- cells in T-47D and MDA-MB-231 were 11.15% ± 0.146 and 1.85% ± 0.024 respectively (Fig. 1b).

E-Cad/FN

Percentage of $FN^-/E\text{-}Cad^+$ cells were significantly high ($p = 0.001$) in T-47D (96.30% ± 0.0078) as compared to MDA-MB-231 (74.80% ± 0.042). In addition, $FN^+/E\text{-}cad^-$ cells were expressed only in ($p = 0.004$) MDA-MB-231 (0.60% ± 0.0017).

Percentage of $FN^+/E\text{-}Cad^+$ cells were significantly high ($p = 0.008$) in MDA-MB-231 (15.03% ± 0.044) as compared

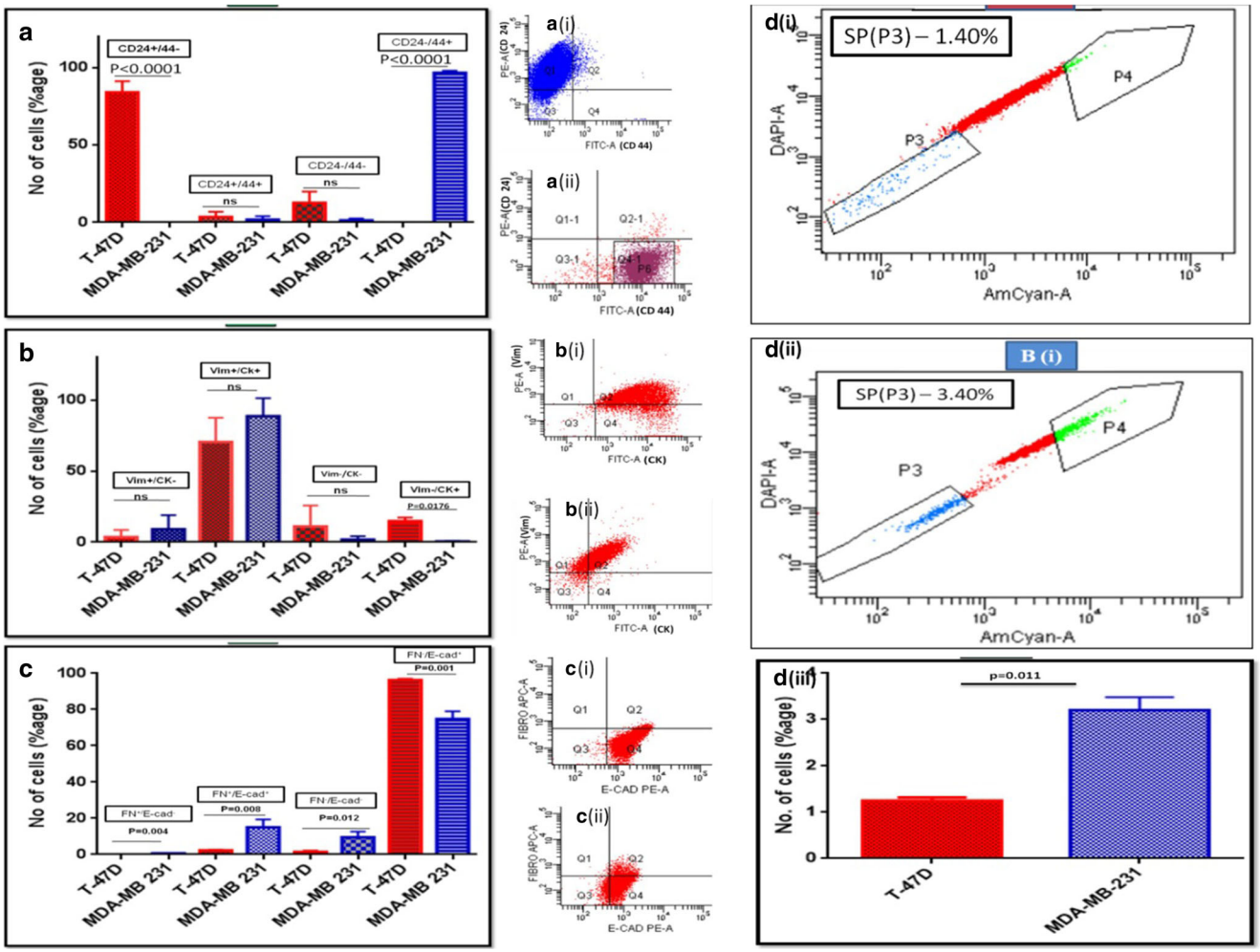

Fig. 1 **a, b & c** Bar charts showing expression of various markers in T47D and MDA-MB-231 cell lines (A) CD44/CD24 (B) CK/Vim (C) E-Cad/FN as analysed by flow cytometry. Representative flow cytometry dot plots for the above markers in T47D (Ai, Bi & Ci) and MDA-MB-231 (Aii, Bii & Cii) cell lines respectively. **d** (i and ii) Side population analysis by flow cytometry in T47D and MDA-MB-231 cell lines respectively. (iii) Bar chart showing percentage of SP in in the two cell lines repectively. Please note that SP population was found to be more in MDA-MB-231 as compared to T-47D

to T-47D (2.33% ± 0.002). Similarly, percentage of $FN^-/E\text{-}cad^-$ cells were considerably high ($p = 0.012$) in MDA-MB-231 (9.57% ± 0.031) as compared to T-47D (1.40% ± 0.007) (Fig. 1c).

SP Determination

Percentage of SP was found to be significantly high ($p = 0.011$) in MDA-MB-231 (3.20 ± 0.002) as compared to T-47D (1.25% ± 0.0007) (Fig. 1d).

Isolation and Culture of BCSCs

Expression of BCSC marker $CD44^+/CD24^{-/low}$ was negligible in T-47D, so isolation of putative BCSC was done through SP analysis. In contrast, expression of BCSC marker $CD44^+/CD24^{-/low}$ was significantly high in MDA-MB-231 (>95%), therefore, isolation of putative BCSC was done by combining $CD44^+/24^{-/low}$ expression and SP analysis(Fig. 2a).

Three gating strategies were used to sort BCSC from MDA-MB-231. To exclude dead cells and debris, the cells were gated on a two physical parameters: Dot plot for forward scatter (FSC) vs side scatter (SSC). Second, the expression level of CD24 and CD44 was reported in PE vs FITC histograms and $CD44^+/CD24^{-/low}$ population was gated. Third, gated population was further analysed for SP in DAPI vs AmCyan filter. Finally desired population (SP) was gated and sorted in collecting tube containing Stem Cell Media. 3 attempts of sorting were done in each of BCCL and ~80,000 putative BCSCs along with ~10^6 NCSC were isolated in each attempt. These were then cultured in 6–24 well plates with seeding density of 30,000cells/ml.

Putative BCSCs were found to remain viable up to 20 days in serum free medium (till which time they were observed). On the other hand, the NCSCs were found to de-adhere and

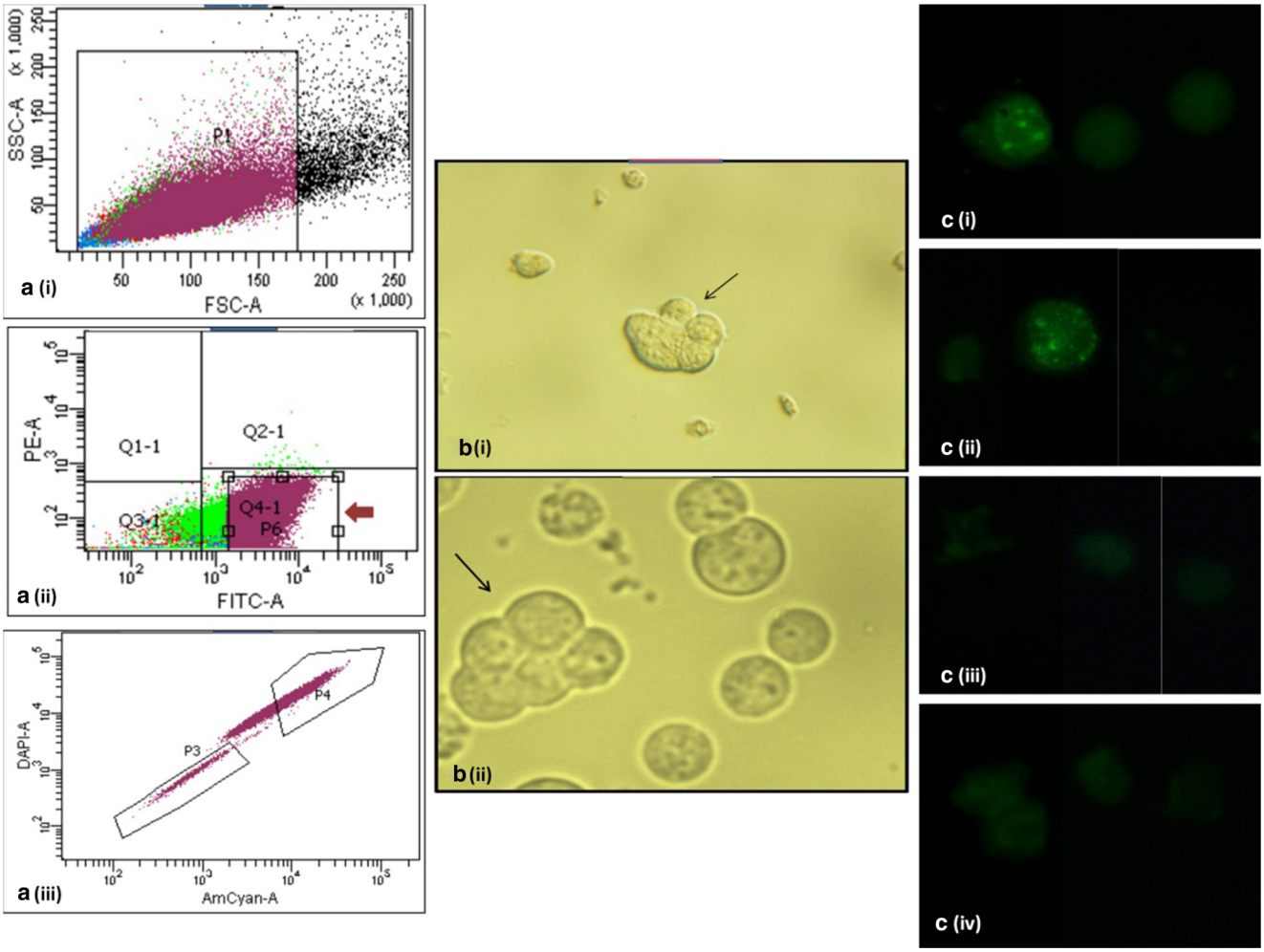

Fig. 2 **a** Sorting of putative BCSCs in MDA-MB-231 cells by [A(i & ii)] Gating of CD44+/CD24−/low cells followed by [A(iii)] Side population analysis of the later. **b** Mammosphere formation by BCSCs isolated from T47D [B(i)] and MDA-MB-231 [B(ii)] cells respectively.**C.** Diffuse and dot like pattern of Ki-67 expression in T-47D and MDA-MB-231 cells [C(i&ii)] NCSCs [(iii & iv)] BCSCs respectively(Fluorescence microscope)

die in serum free medium. Mammospheres (3–6 cells) were observed on 4th day after seeding in T-47D whereas in MDA-MB-231 they (2–6 cells) were noticeable by 6th day only (Fig. 2b).

Analysis of Cultured BCSCs

Ki-67 Expression

No difference was observed in Ki-67 expression in both BCSCs and NCSCs isolated from both the cell lines. However, whereas expression of Ki-67 was diffuse in MDA-MB-231 both diffuse and dot like patterns were observed in T47D (Fig. 2c).

ER Expression

Expression of ER in T-47D BCSCs was significantly weak as compared to NCSCs. The CTCF values in NCSCs and BCSCs were found to be 0.895 ± 0.085; 0.359 ± 0.279 under 20X ($p = 0.007$) and 1.80% ± 1.008; 0.705% ± 0.196 under 40X ($p = 0.0462$) respectively (Fig. 3a).

Effect of Drugs on Cell Viability

Viability of T-47D BCSCs was found to be higher i.e. 62.7% ± 0.054 after 24 h of incubation with Paclitaxel in contrast to 37.30% ± 0.067 in case of NCSC population. However, in MDA-MB-231 BCSCs had 57.5% ± 0.113 viability after 24 h of treatment with Cisplatin in contrast to 42.50% ± 0.23 viability in case of NCSCs (Fig. 3b).

Discussion

Breast cancer is the second leading cause of cancer related deaths among women worldwide. It is one of the highly heterogeneous cancers in terms of histological, molecular and

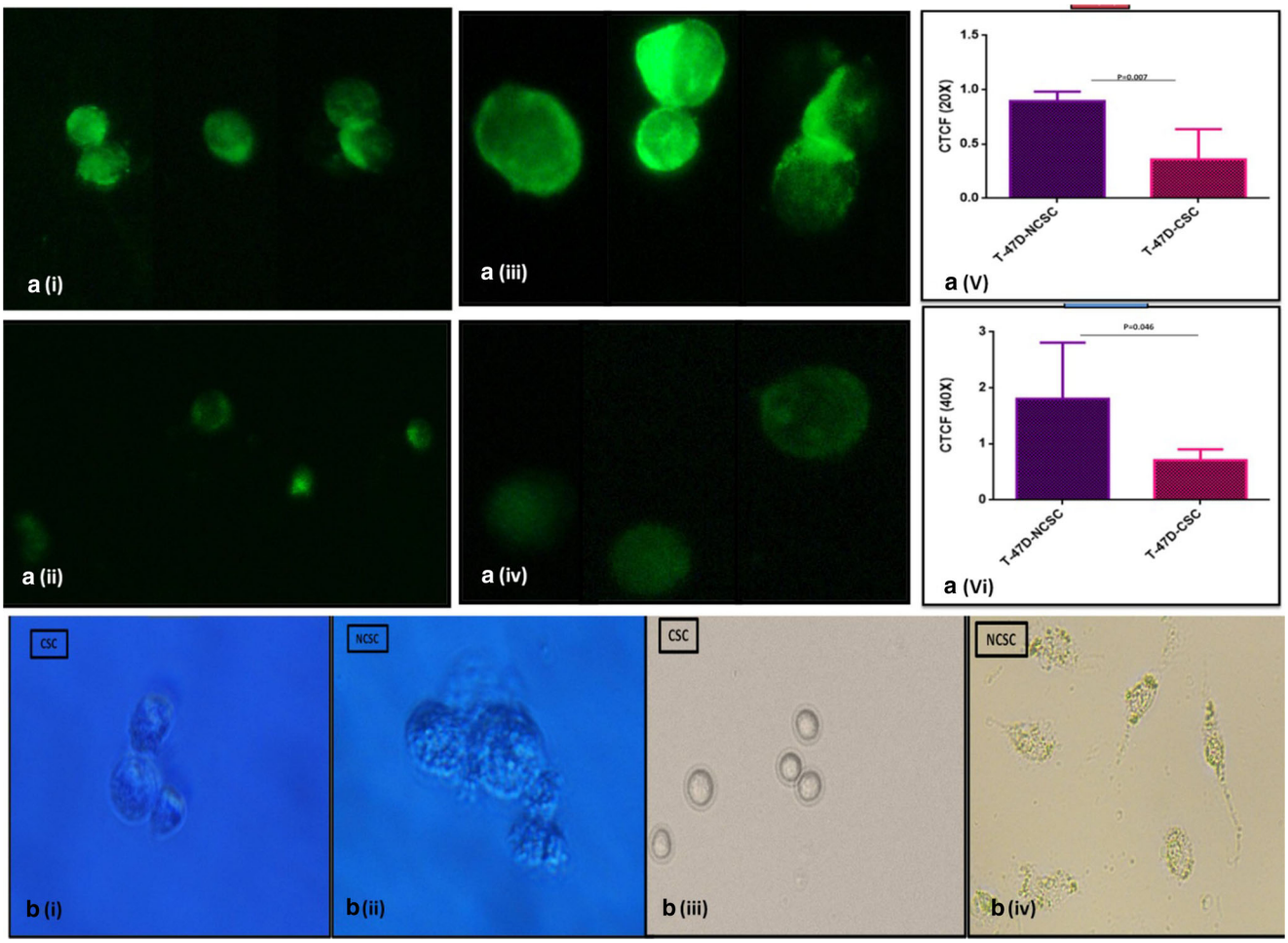

Fig. 3 **a** Comparison of ER expression in NCSCs [A(i &iii)] and [BCSCs] A(ii &iv) in 20X and 40X respectively in T47D cell lines. Please note weak expression of ER in BCSCs (Fluorescence microscope) as compared to NCSCs. Bar chart showing differential expression of ER in 20X [A(v)] &40 X [A(vi)] respectively. **b** Cell viability in BCSCs [B(i)] and NCSCs [B(ii)] sorted from T47D after treatment with Paclitaxel and BCSCs [B(iii)] and NCSCs [B(iv)] sorted from MDA-MB-231 after treatment with Cisplatin. Please note the granularity in NCSCs after drug treatment indicating compromised viability Inverted microscope 40X

clinical features leading to differences in behaviour and therapeutic response in different tumour subtypes [8]. Several hypotheses have been put forward to explain intertumoral heterogeneity; like various genetic and epigenetic aberrations as well as distinct subtype-specific tumor cells. However, to explain intratumoral heterogeneity CSC model has been proposed according to which cancers arise from CSCs displaying self-renewal and differentiation potentials and the ability to give rise to phenotypically diverse malignant cell populations. The origin of CSCs is believed to be ambiguous as they may either originate from the malignant transformation of a stem/progenitor cell through the deregulation of the normally tightly regulated self-renewal program, or through transformation of committed cells by dedifferentiation of mature cells which resume stem cell-like features including a self-renewal potential. Self-renewal, tumorigenicity, multilineage differentiation, and increased resistance to radio and chemotherapy are the properties which make CSCs critical targets in breast cancer therapy [3, 8].

In this work, we have used different markers to identify the putative BCSCs. CD44 is a transmembrane glycoprotein receptor for hyaluronic acid which is believed to play a key role in tumour initiation, progression and metastasis [9–13], while CD24 is a glycosyl phosphotidyl inositol anchored glycoprotein which is involved in increased proliferation and adhesion of tumour cells to components of extracellular matrix (ECM) like fibronectin, collagen, and lamin [12]. $CD44^{+}/CD24^{-/low}$ expression has been used to identify BCSCs in various studies and some studies even consider it to be a superior prognostic marker to ALDH [14, 15]. In contrast, some studies suggest that $CD44^{+}CD24^{-}$ antigenic phenotype is a marker of Epithelial to Mesenchymal Transition (EMT) rather than stem cells and is involved in induction of EMT which in turn results in the gain of stemness properties [16, 17]. In our study when $CD44^{+}/CD24^{-/low}$ was used as a marker to identify stem cells more than 95% of population in MDA-MB-231 was found to express the above phenotype. On the other hand, a significantly ($p < 0.05$) high proportion of cells in T47D were found to

show $CD44^{-}/CD24^{+}$ profile. Presence of such high percentage of stemness marker in MDA-MB-231 may correlate with its high tumorigenicity and aggressiveness in contrast to T-47D. In literature, several studies also showed the increased expression of stemness markers in MDA-MB-231 (as compared to T-47D) consistent with its basal nature [7, 18]. Tae Gyu Oh et al., have correlated increased expression of $CD44^{+}/CD24^{-/low}$ in MDA-MB-231 with decreased expression of RORγ nuclear receptors that regulate several genes involved in cell migration, viability and EMT [19].

Recent studies suggest a close link between EMT and stemness in cancers. Expression of markers associated with enhanced mesenchymal properties in breast cancer cells have been found to correlate with increased expression of $CD44^{+}/CD24^{-/low}$ and mammosphere formation efficiency [16]. Therefore, we used CK / Vim and E-Cad/FN to assess stemness properties in our cells. Tumour cells undergoing EMT are marked by decrease of CK (epithelial marker) and increase of vim (mesenchymal marker) [20]. In addition to higher expression of CK^{-}/Vim^{+} cells in MDA-MB-231, we also observed a significantly high proportion of dual (CK^{+}/Vim^{+}) positive cells in both the cell lines albeit more in MDA-MB-231. Whereas the cells expressing former have been shown to be more prevalent in basal type of cancers in general, existence of transitional cells with dual positivity has been correlated with increased plasticity and more malignant and stem-like behaviour in previous studies [21, 22].

E-cad is a transmembrane glycoprotein which mediates homotypic cell-cell contacts between epithelial cells. It is a tumour suppressor that inhibits cell proliferation, invasiveness and metastasis [23, 24]. FN is a glycoprotein scaffold for fibrillar ECM and is involved in transmitting ECM signals to cells by binding to integrin receptors [25]. Cellular FN has not only been shown to be involved in cell migration and invasion but it has also been found to regulate the expression of EMT markers through PI3K and Erk pathway in other cancers [26]. In our study we have found FN^{+} cells only in MDA-MB-231 cell line and a higher percentage of $E\text{-}cad^{+}$ in T-47D which further helps to explain higher propensity of the former to undergo EMT and metastasize [25, 26].

Further, we tried to assess the SP which is based upon the principle that CSCs display high expression of ABC transporters resulting in efflux of fluorescent Hoescht 33,342 which can be analysed by flow cytometry [7, 27]. Our results showed that SP was more in MDA-MB-231 as compared to T-47D. Other studies have also shown higher percentage of SP along with greater expression of ABCG2 protein in ER^{-} as compared to ER^{+} BCCL [28]. However, in contrast, some studies have reported high percentage of SP in luminal type cancers also [29]. SP level has been found to be positively associated with adverse tumour outcome in breast cancer in previous studies [30].

When we tried to see any correlation between either the populations identified by different set of markers or by different methods used no consensus was observed amongst them. A previous study by Liu et al. has also shown similar results [31]. These results point towards the presence of heterogeneous pools of malignant cells which may be sub-serving different set of functions within the tumours.

After isolation, putative BCSCs were cultured in defined culture conditions [32, 33]. Mammospheres were obtained earlier in T-47D as compared to MDA-MB-231. This may be accounted for the fact that there is high expression of E-cad in T-47D as compared to MDA-MB-231, which is necessary for cell to cell adhesion in sphere formation [34].

Further in our study, we have found that there is increased resistance against anti cancer drugs in BCSCs as compared to NCSC in T-47D and MDA-MB-231 cell lines. Previous studies have also reported that CSCs are responsible for Cisplatin resistance in tumours [35]. Alteration in expression of ABC drug transporter, enhanced DNA repair mechanism, inhibition of apoptosis are some of the mechanisms suggested to be responsible for enhanced therapeutic resistance of CSCs [36, 37].

We have also tried to compare the proliferative potential of sorted cells from both the cell lines using expression of proliferative marker Ki-67. However, we didn't find any significant difference in BCSCs and NCSCs. The expression of Ki-67 in BCSCs further suggests that they may not be as quiescent as their normal counterparts (normal stem cells). Further, some of the studies have suggested that Ki-67 may be required for maintenance of stem cell niche and CSC survival [38].

Lastly, we tried to determine the expression of ER in CSC isolated from T-47D through immunocytochemistry. Interestingly in our study, there was weak expression of ER in isolated BCSCs as compared to NCSCs which may relate to the fact that CSCs undergoing EMT are accompanied by loss of ERs [39, 40]. In addition, decrease in ER expression can be the survival strategy adopted by BCSCs to protect them from anti-hormonal therapy resulting in disease relapse after treatment.

In conclusion, our work shows higher expression of stemness and EMT markers in ER^{-} BCCL which not only may help to explain the aggressive nature, but also, higher recurrence and metastasis potential of ER^{-} breast cancers. However, no correlation among different sets of BCSCs markers used in our study suggests that they may be identifying different subsets of malignant cells in tumour hierarchy. However, the exact position of these cells in tumour hierarchy and their functional significance remain to be investigated. Further, weak expression of ER in BCSCs observed in the study in ER^{+} cancers suggests that down regulating the expression of ER may be one of the mechanisms utilised by them to escape the effect of hormonal therapy. Although, $CD44^{+}/CD24^{-/low}$ expression alone was not much useful for

isolation of CSCs, we feel that clubbing it with SP analysis may help in yielding fairly reasonable populations. The study findings, therefore, raise some pertinent questions in the field of BCSC research the answers to which may help in better understanding patho-physiology of ER^+ and ER^- subtypes. This may in turn aid in planning more effective therapeutic strategies against breast cancer.

Acknowledgements We acknowledge the funding support provided by PGIMER for this work.

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