# Establishment of Doxorubicin Resistant Stem-like Cell Line from MCF-7 Cells

Syed Zulfiqar Ali Naqvi[1*], Faiza Naz[1] and El-Nasir Lalani[2]

[1]*Department of Molecular Pathology, Liaquat National Hospital and Medical College, Karachi, Pakistan*
[2]*Department of Pathology and Laboratory Medicine, Aga Khan University, Karachi, Pakistan*

## Abstract

**Background:** Conventional therapy of Breast cancer (BCa) is associated with numerous challenges, relapse being the topmost. It has been shown that disease relapse occurs due to the existence of cancer stem cells (CSCs), residing within the tumor.

**Objectives:** To develop a cancer stem cell line model by exploiting the property of stem cells to resist treatment with anti-cancer therapeutic agents and to develop novel therapeutic interventions targeting the elimination of CSCs.

**Methods:** Single-cell clones from MCF-7 cells were established in 96-well plates using culture medium containing 5% FBS. Enrichment of cancer stem cells was carried out from established single cell clones which were initially cultured in a serum-deprived nutrient medium for six weeks, followed by Doxorubicin treatment. Doxorubicin-resistant clones were established and evaluated for their growth and sphere-forming abilities. Further, these clones were characterized based on the presence of stem-cell markers using semi-quantitative reverse transcription-polymerase chain reaction and results were compared with the parental MCF-7 cell line.

**Results:** In complete medium, these spheres differentiated and started growing as a monolayer with differential expression levels of genes involved in stemness. When these spheres were sub-cultured again in stem-cell medium and detached to give rise to single cells, these clones retained sphere-forming ability. Doxorubicin-resistant clones showed a tendency to grow in spheres in a stem-cell medium with serum-deprived culture conditions.

**Conclusion:** Our results provide undeviating evidence of the successful establishment of clones of MCF-7 cell line exhibiting stem cell-like properties. These cell lines have been reprogrammed as stem cell-like models and can be used to delineate recurrent breast cancer attributed to CSCs.

**Keywords:** *Breast cancer, cell line, cancer stem cells, drug-resistance, doxorubicin.*

## INTRODUCTION

Breast cancer is one of the most frequently diagnosed cancers worldwide and a leading cause of cancer-related mortality in females [1]. Regrettably, it is prophesied that its incidence will substantially increase in upcoming decade [2, 3]. Like other cancers, breast cancer is a heterogeneous malady that exhibits intensively divergent clinical responses in each patient [4]. Although tremendous advancements have been made to reduce the incidence and mortality rate of this life-threatening ailment, regardless of the mode of therapy whether hormonal therapy, targeted chemotherapy, or immunotherapy, resistance to therapy has emerged as a major impediment to the complete elimination and recurrence of this complex disease [5].

Emerging evidence suggests that cancer stem cells (CSCs) are a key driver of breast cancer heterogeneity as they differentiate into multiple cell lineages to produce heterogeneous tumor mass [6, 7]. Upon treatment with a therapeutic agent, they rapidly change their phenotype to install heterogeneity in cancer mass and become resistant to the therapy [7]. CSCs not only make breast cancer cells resistant to the therapy but also accelerate the processes of cancer progression, metastasis, and relapse [7]. In 2003, Al-Hajj and coworkers first identified breast CSCs with $CD44^{+}CD24^{-}/LIN^{-}$ phenotype [8]. Later, studies have identified distinctive markers for the identification of breast CSCs in different patients, which further confirms the role of CSCs in establishing breast CSCs heterogeneity and resistance to therapy [9, 10]. Furthermore, some studies have shown that breast CSCs more rapidly establish tumors in xenograft models compared with their non-CSC counterparts [11]. Likewise, CSCs enriched tumor mass exhibits more neoplastic and metastatic potential in both in vitro and in vivo models than non-CSCs [11], which affirms their extensive oncogenic nature.

Doxorubicin is one of the most widely prescribed chemotherapeutic agents that work by producing DNA intercalation, inhibiting topoisomerase II activity, and reducing free radical formation [12]. It is well known that long-term exposure to doxorubicin induces epithelial-mesenchymal transition (EMT) in breast cancer cells to revert them towards stem cell phenotype [12]. These CSCs then rapidly change their phenotype to establish therapy resistance and aid tumor recurrences.

Presently, drug resistance in breast cancer patients is treated by prescribing other medications [13], without

*Corresponding author: Zulfiqar Ali Naqvi, Department of Molecular Pathology, Liaquat National Hospital and Medical College, Karachi, Pakistan; Email: zulfiqar.naqvi@lnh.edu.pk*
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elucidating the molecular mechanism of resistance. Better recognition of drug-resistance mechanisms would not only decrease the side effects of treatment but also instigate to design novel approaches to achieve an improved therapeutic response. The only hope for controlling cancer lies in learning more about its causes and pathogenesis, and by understanding its molecular basis. Therefore, this study was aimed to establish a doxorubicin-resistant breast CSCs cell line, which will aid researchers to study the mechanism of drug resistance in breast cancer cells and design novel approaches to overcome this disease efficiently.

## METHODS

### Cell Line

Human breast cancer cell line MCF-7 (HTB-22) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The MCF-7 cell line was established from the pleural effusion of breast tissue of a 69-years old Caucasian woman, it is an $ER^+$, $PR^+$, $AR^+$, HER2-non-amplified cell line [14]. The experiments were performed with cells of passage number less than 20. Before any experiment, the cell line was tested and found free from mycoplasma contamination.

### Maintenance of MCF-7 Cell Line

All cell culture work was performed in a Class II Biosafety cabinet (Bakers, Sanford, ME) as described previously [15] with minor modifications. Post UV-treatment, the working surface of the safety cabinet was disinfected with 70% alcohol. Cell culture was maintained in a water-jacketed humidified CO2 incubator (Thermo Fisher Scientific, Germany) at 37°C with 5% $CO_2$. Breast cancer cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) with or without phenol red, supplemented with 10% FBS. Penicillin-streptomycin-amphotericin B mixture was added into the cell culture medium at a working concentration of 100 units, 100 µg, and 0.25 µg per ml, respectively, as described previously [16]. Cell culture medium was replaced with fresh medium after every 72 hours. After attaining 80% confluence, the medium was removed; cells were gently washed with Phosphate buffered Saline (Gibco, Carlsbad, CA) and trypsinized using 0.25% Trypsin EDTA to detach monolayer of cells from flask and plates. Cells were centrifuged and resuspended in 5.0 ml of fresh medium and sub-cultured.

### Testing for Mycoplasma

One of the key problems in cell culture is mycoplasma infection which can extensively affect the metabolism of cells and their physiology. In addition, mycoplasma does not cause turbidity in cell culture media that often occurs in other types of contaminations. To avoid this, at the beginning of each step, it was ensured that the cells were free of mycoplasma during all experiments. For this purpose, the MCF-7cell line was examined for mycoplasma contamination by staining the cells with Hoechst 33258 dye. An aliquot of the fresh suspension was grown on a 22x22 mm sterile cover-slip (Thermo Scientific, Germany) in an antibiotic-free medium and incubated for three days in a six-well plate at 37°C in $CO_2$ incubator. After three days, cells were washed with PBS, fixed with pre-cooled (-20°C) methanol (BDH, Poole, UK) for 3 minutes and, stained with Hoechst 33258 dye (50 ng/ml) in the dark for 30 minutes. Cells were rinsed with de-ionized water and viewed under Olympus BX41 fluorescent microscope with a 330-380 nm excitation.

### Determination of Cell Viability

An aliquot of MCF-7 cell suspension was mixed with an equal volume of 0.4% trypan blue solution to determine cell viability. The trypan blue negative cells were counted using a hemocytometer (Neubauer, Germany) and viable cells were counted using the following formula:

**Number of Cells/ml = Average count per square x dilution factor x $10^4$**

### Determination of $IC_{50}$ of Doxorubicin from Dose-Response Curve MCF-7 Cells

Single Cell suspension (5000 cells / well) of MCF-7 cells was seeded in a 96-well plate. These cells were treated with increasing concentrations of Doxorubicin *i.e.* 14, 21, 35, 50, 100 & 200 ng/ml for 72 hours in triplicates. MTT cell proliferation assay was performed and absorbance was measured. The mean Absorbance ± SEM was calculated using MS Excel. The mean ± SEM value of absorbance (indicative of the % survival) was plotted against the concentration of Doxorubicin and $IC_{50}$ was calculated using the drug response curve.

### Establishment of Single-Cell Clones and Cancer Stem Cell-like Cells

In a 15-ml culture tube 100 cells were transferred and the volume was made up to 10 ml using DMEM containing 10% FBS, giving one cell/0.1 ml (100 µl). In an Ultra-low

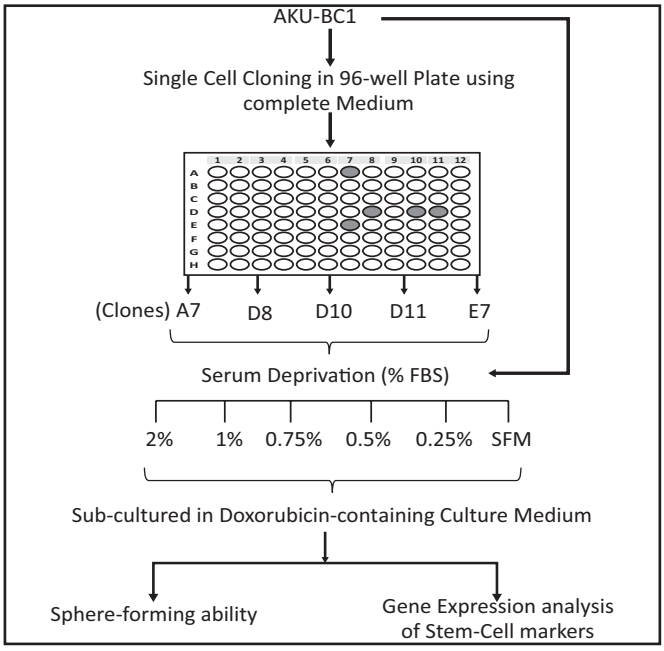


**Fig. (1):**: Methodology depicting establishment of single cell clones, sphere formation and expression analyses.

attachment (ULA) 96-well plate 100 µl cell suspension was added in each well, which theoretically gives a single cell per well. Each well of the 96-well plate was observed under an inverted microscope (Olympus IX70) and the wells with single cells were marked and allowed to grow as single-cell clones (SCCs). To establish CSC-like cells, the cells were cultured in serum-free medium (SFM) and 5% serum-containing medium for six weeks in ULA plates. Then the serum concentration in each well-containing clone was gradually decreased in the order from 2%, 1%, 0.75%, 0.5% to 0.25% for six weeks with each concentration and, finally the serum was removed from the media as the cells were now synchronized with serum-free media. MCF-7 Parental cell line, from which the clones were established was cultured in 5% FCS as control. All clones and parental cell lines were treated with Doxorubicin 100 ng/ml for 48 hours followed by changing the media with no Doxorubicin for 48 hours (one cycle)(**Fig. 1**). Ten cycles of the same treatment were continued then media containing Doxorubicin (100 ng/ml) was used throughout the study.

### Mammosphere Assay

To prepare mammosphere culture, cells from the selected wells containing single-cell clones were grown in serum-free, growth factor enriched conditions containing 20 ng/ml bFGF, 10 ng/ml EGF, and B27 (Invitrogen) in 6-well ultra-low attachment (ULA) plates [4,12,13]. Cells were plated at 10,000 cells/well. Suspension cultures were continued for one week or more. Colonies were observed under Olympus IX70 inverted microscope.

### Growing Mammospheres

The cells growing as spheres (approx. 100µm) were dissociated, passaged, and sub-cultured in the same medium containing Doxorubicin (100 ng/ml) in a six-well ULA plate and sphere-forming ability was assessed. Further, these spheres were characterized for the differential expression of differentiation and stem-cell markers using semi-quantitative RT-PCR, and results were compared with parental cell line MCF-7.

### RNA Extraction

RNA was extracted from spheres grown to approximately 70% confluence in 6-well plates. The growth medium was aspirated; cells were washed three times with PBS and 1 ml TRI Reagent (Sigma-Aldrich) was added to the wells. Cells were lysed by repeated pipetting and lysate was collected in a 1.5 ml tube (Eppendorf, Hamburg, Germany). After the addition of 0.2 ml Chloroform (Sigma-Aldrich), the reaction mixture was vortexed for 15 seconds and incubated for 20 minutes at room temperature, followed by centrifugation at 12,000xg for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube, and RNA was precipitated by adding 0.5 ml of isopropanol (Sigma-Aldrich) followed by incubation at room temperature for 20 minutes. RNA was harvested by centrifugation at 12,000g for 10 minutes at 4°C. The supernatant was discarded and the pellet containing RNA was washed with 1 ml of 75% ethanol (Sigma-Aldrich), vortexed, centrifuged, and air dried. Finally, the RNA pellet was dissolved in an appropriate volume (20-50µl) of RNase-DNase-free water. To facilitate solubility, RNA samples were incubated at 55°C for 15 minutes with occasional mixing by repeated pipetting. RNA was quantitated using Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and used immediately or stored at -70°C. The integrity of RNA was determined by resolving 2 µg total RNA samples on 1% agarose by gel electrophoresis.

**Table 1:** List of primers and their respective annealing temperatures.

| Gene | Primer | Annealing temperature |
|---|---|---|
| CD44 | Forward: 5'-TCCAACACCTCCCAGTATGACA-3'<br>Reverse: 5'-GGCAGGTCTGTGACTGATGTACA-3' | 55°C |
| ABCG2 | Forward: 5'-GGGTTCTCTTCTTCCTGACGACC-3<br>Reverse: 5'-TGGTTGTGAGATTGACCAACAGACC-3' | 58°C |

### Reverse Transcription-PCR (RT-PCR)

1 µg RNA was subsequently used to synthesize cDNA. RNA was reverse transcribed using 200 units of MMLV reverse transcriptase (RTase) (Invitrogen, Carlsbad, CA) in the presence of 1X PCR buffer (100mM Tris-HCl, 500mM KCl, pH 8.3), 3mM MgCl2, 10U RNase inhibitor, 200µM dNTPs, 2.5µM oligo (dT)16 and 10mM dithiothreitol for 1 hour at 42°C in a final volume of 25µl. The reaction was stopped by denaturing RTase at 94°C for 5 minutes, and the resultant cDNA was stored at 4°C until use.

Semi-quantitative RT-PCR for genes involved in stemness was carried out using 2 µl cDNA in PCR buffer (100mM Tris-HCl, 500 mM KCl, pH 8.3), 1.5mM $MgCl_2$, 200µM dNTPs, 0.5 units Taq polymerase and 0.2 µM of each primer set in a final volume of 20 ul. Primer sequence for CD44 was:
Forward 5'-TCCAACACCTCCCAGTATGACA-3',
Reverse 5'-GGCAGGTCTGTGACTGATGTACA-3'
and for ABCG2:
Forward 5'-GGGTTCTCTTCTTCCTGACGACC-3',
Reverse 5'-TGGTTGTGAGATTGACCAACAGACC-3'.

Thermocycling conditions followed were: Denaturing step at 94°C for 5 min, followed by 21 cycles of 94°C for 30 sec, 55°C or 58°C for respective gene, (**Table 1**) for 30 sec and 72°C for 1 min and a final extension at 72°C for 7 min. PCR products were loaded on 2% agarose gel containing 0.5 µg/ml ethidium bromide. The electrophoresis was performed at 100 volts in 1X TBE buffer for approximately 40 min, and bands were visualized under UV light.

## RESULTS

### MCF- 7 Cell Line was Free of Mycoplasma

MCF-7 cell line was a mycoplasma negative cell line as detected after staining the cell with Hoechst 33258 dye (**Fig. 2**).

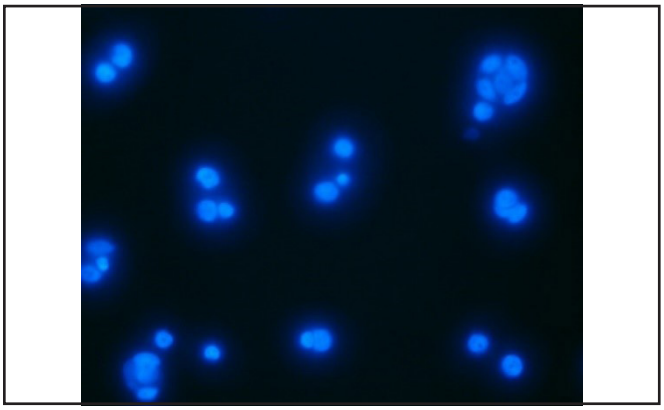

**Fig. (2):** Hoechst staining for detection of mycoplasma in MCF-7 cells

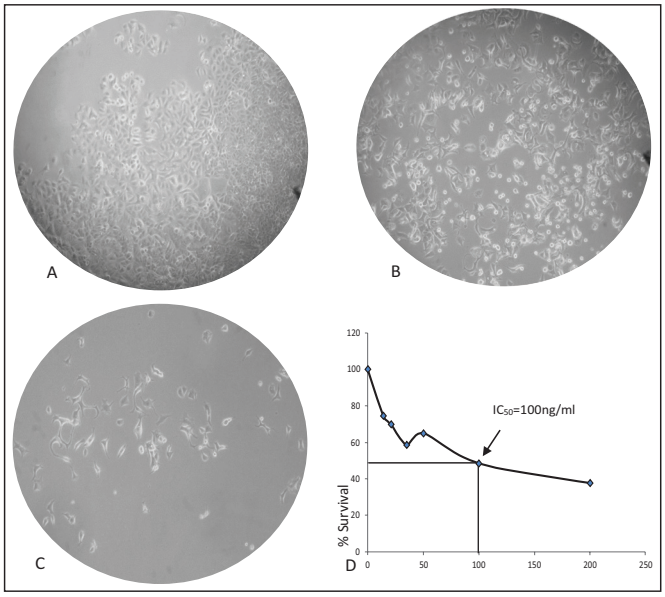


**Fig. (3):** Determination of $IC_{50}$ **of Doxorubicin from dose-response curve on MCF-7 cells**: MCF-7 cell line untreated **(A)**, doxorubicin 50ng/ml treated **(B)**, 100ng/ml treated **(C)** and dose response curve to calculate $IC_{50}$ value, Mean ± SEM **(D)**.

### Determination of $IC_{50}$ of Doxorubicin on MCF-7 Cells

For the treatment of MCF-7 cells and enrichment of stem cell population, the $IC_{50}$ value of Doxorubicin on MCF-7 cell line was determined which came out to be 100 ng/ml, killing all the cells significantly over a due course of time (**Fig. 3**). As we were interested in creating Doxorubicin-resistant cell lines and clones, we used 100 ng/ml doxorubicin which kills the cell a bit slowly as compared to its $IC_{50}$, but was effective enough.

### Establishment of Single-Cell Clone and Cancer Stem Cell-like Cells

Five Single-cell clones namely A7, D8, D10, D11, and E7 were established successfully from MCF-7 cells (**Fig. 4**). These names are given to the clones according to the well number of the 96-well plates in which they were established. The established clones were passaged and stored frozen in liquid nitrogen. The clones were gradually serum-deprived to synchronized cells for reprogramming and serum starvation.

### Doxorubicin Treatment Promotes Mammosphere Formation in Selected Clones of MCF-7 Cells

Long-term Doxorubicin (100 ng/ml) treatment cycles of SCCs in serum-deprived, growth factor enriched medium resulted in the development of doxorubicin-resistant cells with mammosphere forming ability (**Fig. 5**). All clones exhibited mammosphere formation except the clones D11, E7, and parental MCF-7 cells, which failed to make mammosphere and did not survive in a Doxorubicin-treated, serum-deprived, growth factor enriched medium. When the spheres from A7, D8, and D10 clones were dispersed and grown in a complete medium containing 5% FBS, the cells started growing as a monolayer.

### Doxorubicin-Resistant Clones Express Stem Cell Marker CD44 and Increased ABCG-2

Reverse transcription-polymerase chain reaction revealed that, the spheres from SCCs of A7, D8, and D10 express markers of the stemness CD44 and ABCG-2 to varying degrees. The expression of CD44 was equally expressed in all SCCs while it was absent in the parental MCF-7 cell lines from which the SCCs were established (**Fig. 6A**). On the other hand, Clones D8 and D10 have the highest expression of ABCG-2 in all clones, while SSC A7 has a higher expression of ABCG-2 as compared to the parental MCF-7 cell line (**Fig. 6B**).

## DISCUSSION

The cancer stem cell model explicates that within tumor bulk a small population of stem-like cells exists that aid tumorigenesis, metastasis, therapy resistance, and

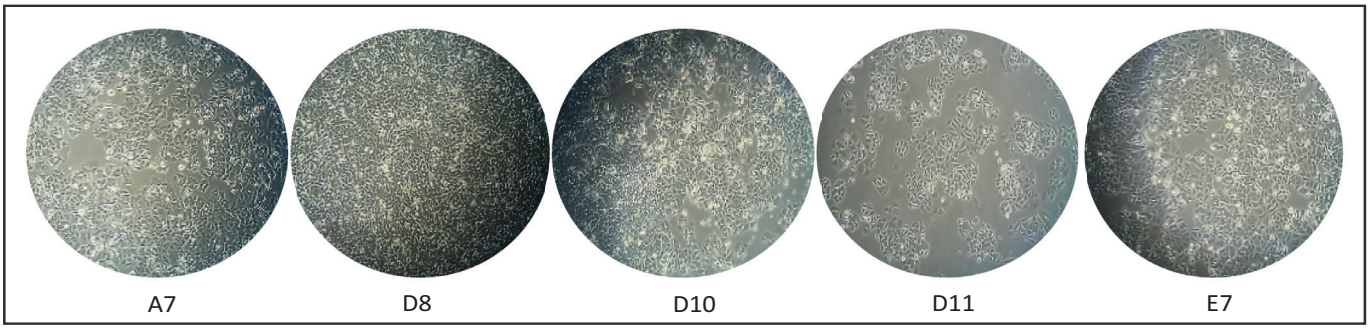


**Fig. (4):** Single Cell Clones established from MCF7 breast cancer cell line, clones are named against the well of the 96-wel plate, they were grown in and established.

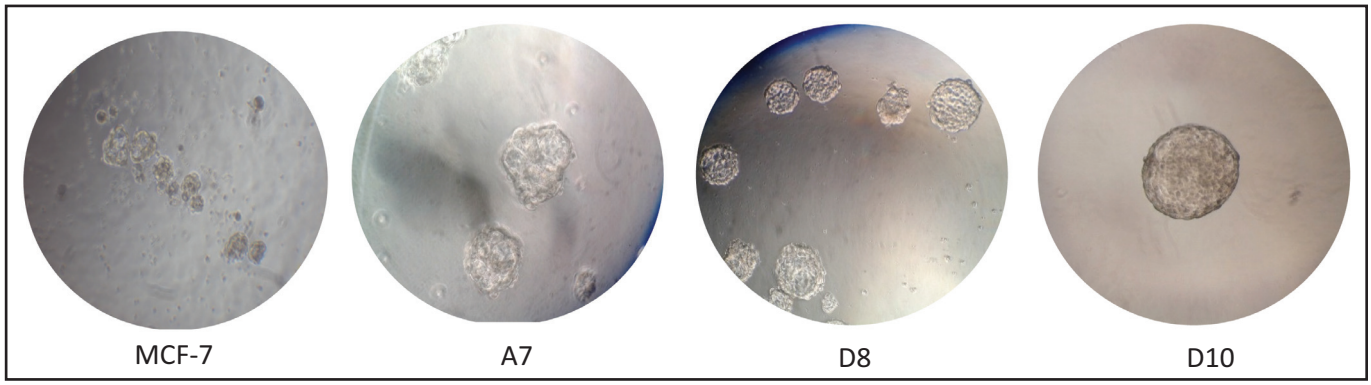


**Fig. (5):** Spheres established from Doxorubicin (100 ng/ml) treated Single Cell Clones.

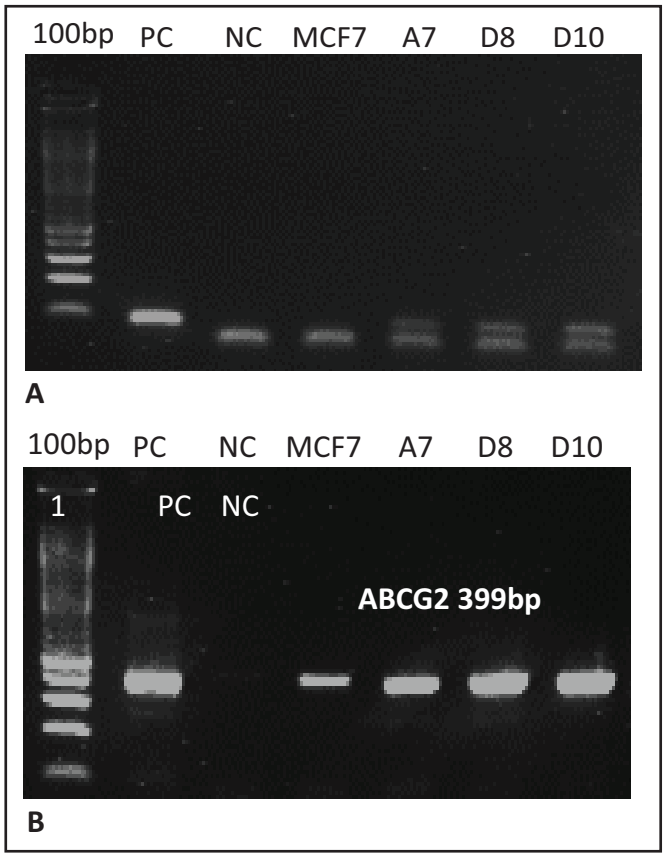


**Fig. (6):** Gene expression analysis of Stem Cell markers *i.e.* CD44, 83 bp **(A)** and ABCG2, 399 bp **(B)** using semi quantitative RT- PCR.

relapse [7]. Therefore, the enrichment of CSCs from the tumor is indispensable for understanding cancer biology and achieving an effective therapeutic response by applying appropriate therapeutic strategies targeting CSCs. In this study, we have established an *in vitro* model of CSCs by growing single-cell clones in a serum-free medium, synchronizing them to serum-deprived conditions followed by treatment with the anticancer drug doxorubicin. We exploited the drug-resistance property of cancer stem cells reported to be present in almost all established cancer cell lines and primary tumors [17, 18]. It seems that in response to doxorubicin treatment cycles, most of the MCF-7 cells died while a small population managed to grow after being synchronized to serum-deprived conditions and in ultra-low attachment plates. Serum deprivation in ULA plates causes SCCs to grow in spheres rendering them undifferentiated phenotype without adhering to the surface of the culture plates. This shows that the single cell clones acquired cancer stem cells-like phenotype as the differentiated cells grow only when they are attached to a surface in media containing serum *in vitro*. Furthermore, the SCCs acquired drug resistance as evident from the increased expression of breast cancer resistance protein (ABCG2) as compared to the parental MCF cells. The formation of mammospheres in these conditions is another property of stem-like cells that grow in an undifferentiated phenotype. When these spheres were dissociated into single cells and sub-cultured in media containing 5% serum, all clones grow successfully like differentiated cells attached to the surface of 6-well plates (data not shown).

The results of RT-PCR confirmed the expression of CSC markers in these mammospheres *i.e.* CD44 and ABCG2 (**Fig. 6A and 6B**), which is consistent with previous studies that CD44 and ABCG2 are markers for breast cancer stem cells [19] whereas ABCG2, breast cancer resistant protein, is actively involved in drug-resistance and EMT. The mammospheres obtained in this study appeared circular in shape, and smoothly outlined with tightly connected cells (**Fig. 5**). Thus, compared with other approaches to obtain CSCs *i.e.* reprogramming [20], growth in hypoxia [21], and suspension culture [22], this is an easy and cost-effective way to enrich breast cancer stem cells for *in vitro* studies.

## CONCLUSION

We report the successful establishment of a breast cancer stem cell-like cell model cell line using serum deprived-growth factor enriched culture conditions *in vitro*. This cell line exhibits stem cell-like properties which can serve as an excellent model to unravel mechanisms of breast cancer recurrence attributed to CSCs. Further characterization of the established clones using immunological and molecular methods will help in understanding the mechanism of drug resistance, EMT, and tumorigenesis and declaring them an ideal model to design therapeutic strategies targeting stem cells.

## ETHICAL APPROVAL

This study was approved by the Ethical Research Committee (ERC) of Aga Khan University (1200-Rec/ERC-09).

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA

Not applicable.

## FUNDING STATEMENT

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR'S CONTRIBUTION

ENL conceived the idea, designed and supervised the study and interpreted the data, ZAN performed all experiments, analyzed and interpreted the results, and prepared and critically reviewed the manuscript, FN prepared and reviewed the manuscript. All authors have read the manuscript and approved for publication.

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