

Mammospheres from MDA-MB-468 Breast Cancer Cells Exhibits some Cancer Stem Cell traits and displays Drug Resistance to some Conventional Anticancer Drugs

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ABSTRACT

Breast cancer (BC) is now a global epidemic and poses overwhelming impact in humans. It is one of the most commonly diagnosed cancers in females. Even with the advances in treatment, management of patients is still a hurdle as recurrence and drug resistance to conventional cytotoxic drugs still occurs. Cancer stem cells (CSCs), a tiny subset of rare progenitor cells have been identified as a major contributor to chemo-resistance. *In vitro* research, mammospheres mimic tumor progression and enhance the stem cell-like features of cancer cells. This study assessed the drug sensitivity of mammospheres made from the MDA-MB-468 breast cancer cell (BC) line, the cells were cultured in mammosphere medium for 3 weeks and mammospheres generated were harvested on day 7, day 14 and day 21 for further analysis. Some CSC markers (CD 133 and ALDH+) were determined by FASC analysis and data obtained demonstrated that the mammospheres over expressed these stem cell marker when compared to the MDA-MB-468 attached cells. MTT cytotoxicity studies showed that the mammospheres were notably resistant to four chemotherapeutic drugs (cisplatin (CDDP), vincristine (VCR), doxorubicin (DOX), and docetaxel (DOC) respectively) compared to the MDA-MB-468 attached cells. 96-well flat-bottomed microtiter plates with 5000 overnight-cultured cells per well exposed to drugs for 48 or 72 hours. Following 48 hours of treatment with docetaxel (DOC), 72 hours of CDDP, 48 hours of VCR, and 72 hours of DOX, the cells were put through a routine MTT assay data obtained demonstrated that MDA-MB-468 attached cells were more sensitive to these drugs having lower IC-50 while mammospheres were significantly resistant.

Key word: MDA-MB-468 Breast Cancer Cells, Mammosphere, Chemoresistance, Cancer Stem Cell traits.

1.1 INTRODUCTION

Globally, cancer is a demoralizing disease that imposes a serious danger to human life and health. One of the most frequent cancers diagnosed in females, breast cancer (BC) has one of the highest mortality rates in humans and is responsible for about 2.26 million fatalities [1, 2]. Currently, immunotherapy, chemotherapy, radiation, surgery, and targeted therapy are used to treat BC [3]. One of the treatment methods used to limit the growth and progression of tumors is chemotherapy, which involves using cytotoxic medications to target cancer cells. Drug resistance, however, usually develops with prolonged use of chemotherapeutic drugs, contributing to treatment failure [4].

The survival rate of many BC patients has significantly increased due to improvements in clinical diagnosis and treatment plans; nonetheless, metastasis and recurrence continue to be significant obstacles. A tiny subset of progenitor cells known as cancer stem cells (CSCs) has the capacity to differentiate and self-renew [5, 6]. They can also replicate the heterogeneity of solid tumors in immunodeficient mice [7]. For many tumors to form, recur, spread, and become resistant to treatment, these CSCs are essential [8].

Mammospheres are spherical collections of cells in suspension that are created from original tumor or non-attaching cell line cultures. These three-dimensional cell cultures replicate the growth of tumors *in vitro*. They are helpful in researching cancer biology and behavior, typically originating from a CSC, and provide a more accurate *in vitro* model of cancer cell behavior than 2D cell cultures [9]. Breast cancer stem cells (BCSCs) have been linked

to the resistance that BC cells exhibit to certain anticancer treatments, which is further exacerbated by cancer medications [10]. Additionally, BC cells that produce higher ALDH aid in the breakdown of cytotoxic medications [11].

1.2 RATIONALE AND AIMS OF THE STUDY

In this study, the drug sensitivity of mammospheres made from the MDAMB-468 breast cell line was assessed, as was the possibility that these mammospheres express more CSC markers than the attached cells.

2.0 METHODOLOGY

2.1 Cell lines and reagents:

Our source for the MDA-MB-468 cell line was ATCC, Middlesex (United Kingdom). Lonza (Wokingham, UK) supplied the fetal calf serum (FCS), DMEM, and dimethylsulfoxide (DMSO), whereas Sigma (Dorset, UK) supplied the docetaxel, doxorubicin, vincristine, and cisplatin.

2.2 Primary mammosphere culture

Mammosphere culture was carried out with few modifications utilizing Dontu et al. [12] method. In a flask, MDA-MB-468 cells with 80% confluence were trypsinized, collected, and re-suspended with DMEM/F-12 supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 30% F12 (Sigma), 2% B27 (Invitrogen, Paisley, UK), 20 ng/ml basic fibroblast growth factor (FGFb) (Invitrogen, Paisley, UK), and 20 ng/ml epidermal growth factor EGF (Sigma, Dorset, UK). The plates were additionally supplemented with 0.5% methylcellulose (R&D Systems, UK) to avoid cell clumping. Six-well tissue culture plates covered with Poly-2-hydroxyethylmethacrylate (Sigma, Dorset, UK) were seeded with the cells at a density of 1×10^4 cells/ml.

To keep the culture going, 2 ml of new mammosphere medium was given to each well every two days without removing the old media. For seven days, the plates were maintained at 37 °C and 5% CO₂. Mammospheres, which were non-adherent compact spheroids with a diameter of 50 to 100 µm, developed after 7 days. After centrifugation (200 x g), the mammospheres were physically broken up by passing through a 25G needle six times. They were then enzymatically degenerated for five minutes at 37°C in a trypsin/DMEM (1:1) solution. In ultra-low attachment six-well plates, single-cell suspensions of the initial culture of mammosphere of MDA-MB-468 cells were reseeded in triplicate under non-adherent conditions on days 7, 14, and 21 correspondingly at a limiting dilution of 1×10^4 cells per well.

2.3 For *In vitro* Cytotoxicity Assay

96-well flat-bottomed microtiter plates with 5000 overnight-cultured cells per well were exposed to drugs for 48 or 72 hours. Following 48 hours of treatment with docetaxel (PAC), 72 hours of cisplatin (CDDP), 48 hours of vincristine (VCR), and 72 hours of doxorubicin (DOX), the cells were put through a routine MTT assay.

2.4 Flow cytometry

The cells were trypsinized, rinsed with staining buffer (three percent FBS, PBS, and 0.03% sodium azide), counted, and then resuspended in staining buffer + 10% FBS for ten minutes at 4°C.

2.4.1 Detection of ALDH Positive Population

To determine which population tested positive for aldehyde dehydrogenase (ALDH), procedures were carried out in accordance with the supplier's instructions using the ALDEFLUOR kit (StemCell Tech., Durham, NC, USA). Using an assay buffer and an ALDH substrate, the cells (2.5×10^5) were stained for 30 minutes at 37°C before being analyzed. For the negative control, diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, was utilized.

2.4.2 Flow Cytometric Analysis of CD 133

The adherent was put into a 25G needle after trypsinization. For 20 minutes, the cells (2.5×10^5) were treated with a CD 133 antibody (BD Pharmingen, Oxford, UK) at 4°C. Unbound antibodies were removed using 2% fetal calf serum (FCS) HBSS (Sigma), and the cells (10,000 events) were examined on a BD FacsCalibur as soon as possible after staining.

2.5 STATISTICAL ANALYSIS

Data was gathered and displayed using means \pm standard deviations (SD). SPSS 22.0.0.0 was also used to perform analysis of variance (ANOVA) (SPSS Inc., USA). Statistical significance was assumed for the variances when the p-value was less than 0.05. At least three duplicate runs of each experiment were carried out.

3.0 RESULTS

3.1 Morphological features:

Figure 1 shows 3 generations of mammosphere extracted on days 7, 14, and 21 as well as adhering cells (MDA-MB-468 cells) under light microscopy. Though initially irregular in shape, the spheres that had formed after three weeks displayed more regular spherical and 3-dimensional shapes.

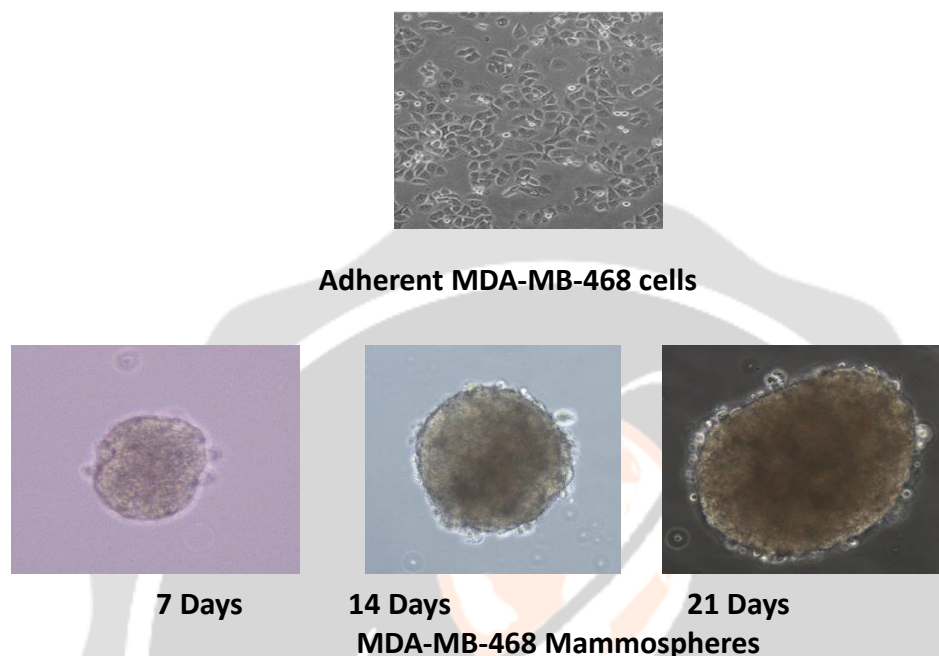


Figure 1: Morphology of adherent MDA-MB-468 cells and mammospheres at 7, 14, and 21 days of culture ($\times 400$)

3.2 MTT Cytotoxicity Assay

At Days 7 and 21, MTT assay was used to evaluate the cytotoxicity of many common anticancer medications, including Cisplatin, Doxorubicin, Vincristine, and Docetaxel, on MDA-MB-468 attached cells and mammospheres. When compared to mammospheres from Days 7 and 21, the cell viability curve (Figure 2) of MDA-MB-468 attached cells was more sensitive to the anticancer medications, according to data from MTT cytotoxicity. As shown in Table 1, it was discovered that the mammospheres from day 21 were considerably resistant to every anticancer medication tested.

Table 1: IC-50 Value from cell viability curve of anticancer drugs (Cisplatin, Doxorubicin, Vincristine and Docetaxel) in MDA-MB-468 Attached cells (Control), Mammospheres formed from MDA-MB-468 cells at 7 Days and 21 Days.

Anticancer Drug/ IC-50	MDA-MB ATT	Mammosphere Day 7	Mammosphere Day 21	P-value
Vincristine (nM)	6.30	19.00	104.71	0.0001*
Doxorubicin (nM)	64.11	308.19	1026.01	0.0000*
Cisplatin (μ M)	256.09	990.38	4000.33	0.0000*
Docetaxel (nM)	5.26	26.35	100.29	0.0000*

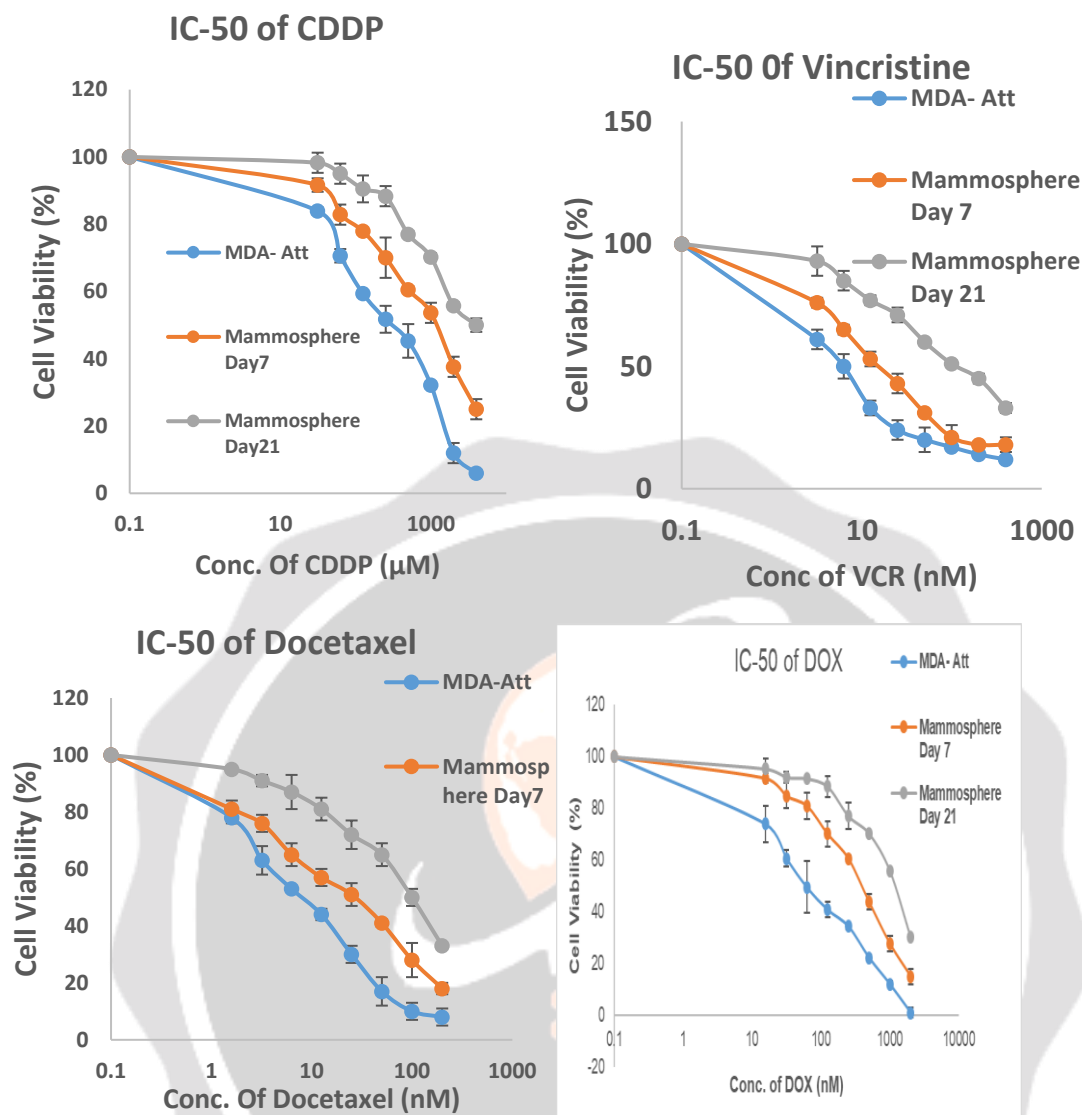


Figure 2: Representative Drug Concentration Response Curves of MDA-MB-468 Attached cells and Mammospheres formed from MDA-MB-468 cells at 7 Days and 21 Days to Cisplatin, Doxorubicin, Vincristine and Docetaxel.

3.3 Flow cytometry Analysis

3.3.1 Detection of ALDH Positive Population and CD 133 Expression

The mammosphere (days 7 and 21) expressed more ALDH+ cells than the associated MDA-MB-468 cells before being treated with Diethylaminobenzaldehyde (DEAB), according to FASC data (Figure 3). Figure 4 shows that the mammosphere (days 7 and 21) overexpressed the CD 133 stem cell marker.

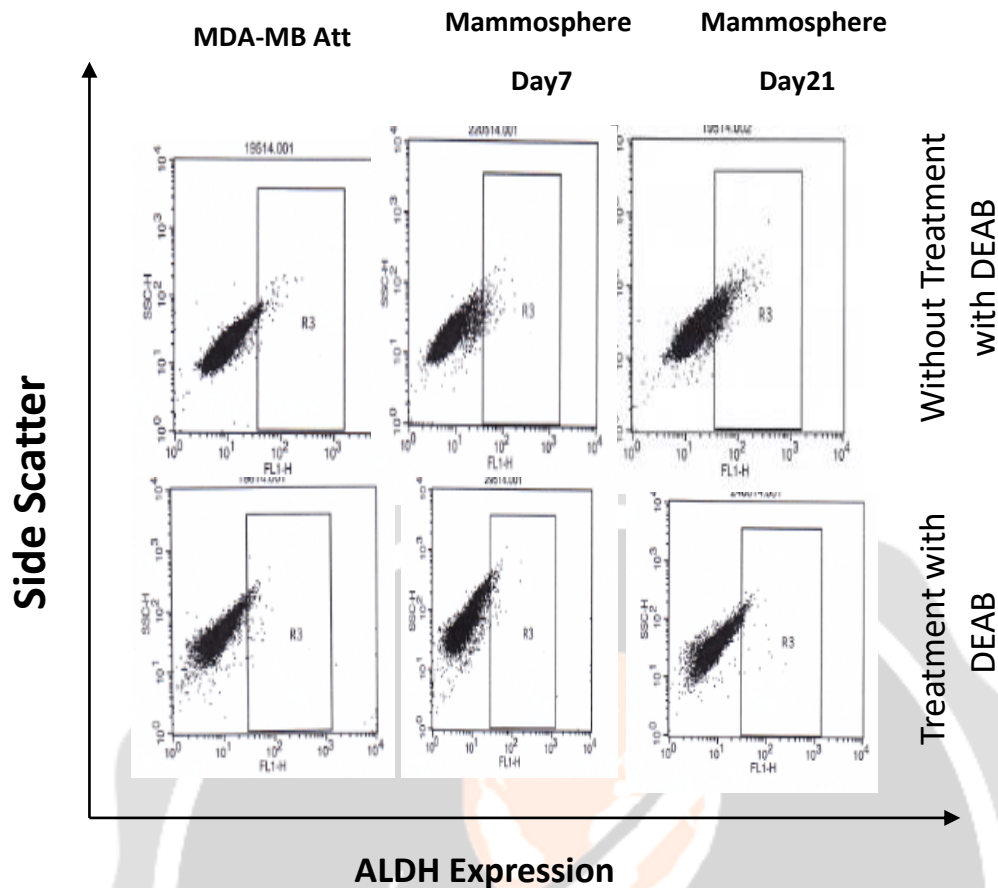


Figure 3: Representative FASCS plots of ALDH⁺ expression with and without DEAB as determined by the ALDEFLUOR test in MDA-MB-468 attached cells (Control), Mammospheres formed from MDA-MB-468 cells at 7 Days and 21 Days.

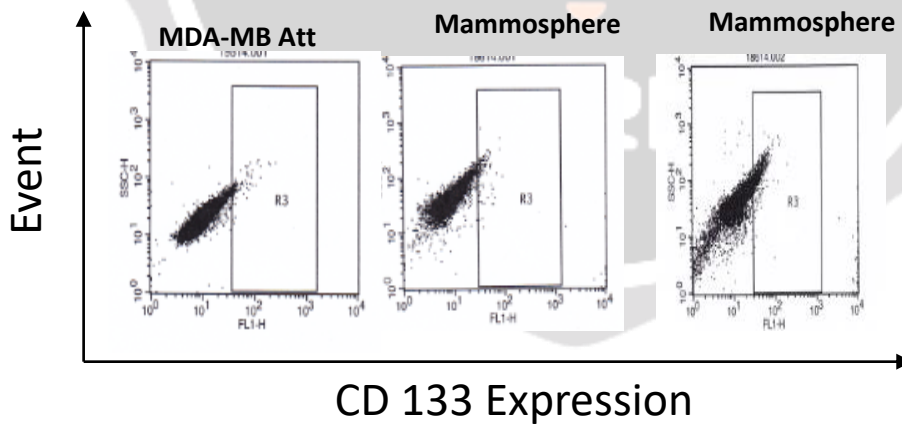


Figure 4: Representative FASCS plots of CD 133 expression in MDA-MB-468 Attached cells, Mammospheres formed from MDA-MB-468 cells at 7 Days and 21 Days.

4.0 DISCUSSION

Treatment with chemotherapy a therapy strategy for cancer management is the use of cytotoxic medications to target cancer cells. Resistance to these chemotherapeutic medications has, however, often been linked to prolonged usage. One of the main problems with BC treatment and management is drug resistance, which occurs

when the disease becomes resistant to standard anticancer medications [4]. A number of mechanisms, including drug efflux and inactivation, the inauguration of bypass signaling or survival pathways, the initiation of epithelial-mesenchymal transition (EMT), and stem cell-like characteristics, have been linked to the induction of drug resistance [13].

Mammospheres, three-dimensional cell cultures cultivated in non-adherent medium replicates the growth of tumors *in vitro*, enhance the characteristics of CSCs. Mammospheres from the MDA-MB-468 cell line were created in this investigation using attached cells (figure 1). Analyzing the mammosphere collected on days 7 and 21, FASC data showed that the cells displayed more CSC markers than the attached cells, including ALDH+ cells (figure 3) and CD133 (figure 4). The chemoresistance may have been caused by the mammospheres' (Day 21) increased expression of CSC markers, which suggested that the cells exhibited a stem-like phenotype.

High levels of CSC markers, such as CD 133, were found to dramatically increase a cell's resistance to chemotherapeutic treatments in earlier research [14, 15]. Figure 2 displays the cell viability curve of the attached MDA-MB-468 cells and mammospheres on Days 7 and 21. Data from cytotoxic analysis showed that the mammospheres were considerably resistant to docetaxel, doxorubicin, vincristine, and cisplatin. The mammospheres were shown to be significantly resistant to the anticancer medications examined, according to the IC-50 from the MTT cytotoxic data (Table 1). Results from this study are consistent with previous research [16, 17, 18].

5.0 CONCLUSION

MDA-MB-468 BC cell lines in suspension were successfully used to create mammospheres. These cells were thought to be contributing to drug resistance to chemotherapeutic treatments since they overexpressed specific CSC markers such as ALDH+ and CD 133 and displayed some CSC characteristics.

6. REFERENCES

1. Fahad UM (2019). Breast cancer: current perspectives on the disease status. *AdvExp Med Biol.*;1152:51–64. https://doi.org/10.1007/978-3-030-20301-6_4.
2. Wilkinson L, Gathani T (2022). Understanding breast cancer as a global health concern. *Br J Radiol.*;95(1130):20211033.
3. Dong X, Bai X, Ni J, et al. (2020) Exosomes and breast cancer drug resistance. *Cell Death Dis.*;11(11):987.
4. Koual M, Tomkiewicz C, Cano-Sancho G, et al. (2020). Environmental chemicals, breast cancer progression and drug resistance. *Environ Health.*;19(1):117.
5. Dalerba P, Cho RW and Clarke MF (2007): Cancer stem cells: models and concepts. *Annu Rev Med.* 58:267–284.
6. Visvader JE and Lindeman GJ (2008): Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer.* 8:755–768.
7. Sarry JE, Murphy K, Perry R, Sanchez PV, Secreto A, Keefer C, Swider CR, Strzelecki AC, Cavelier C, Récher C, et al (2011). Human acute myelogenous leukemia stem cells are rare and heterogeneous when assayed in NOD/SCID/IL2R γ c-deficient mice. *J Clin Invest.* 121:384–395.
8. Coker, A. K. and Allan A. L (2008). “Cancer stem cells: Implications for the progression and treatment of metastatic disease.” *J Cell Mol Med* 12: 374-390
9. Molyneux G, Regan J, Smalley MJ. (2007). Mammary stem cells and breast cancer. *Cell Mol Life Sci.*;64(24):3248–3260. doi:10.1007/s00018-007-7391-5
10. Gottesman, M. M. (2002). Mechanisms of cancer drug resistance. *Annu Rev Med* 53: 615-627.
11. Moreb, J. S. (2008) “Aldehyde dehydrogenase as a marker for stem cells.” *Curr Stem Cell Res Ther* 3 (4):237-246
12. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, (2003). *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev.*;17:1253–1270
13. Szczygieł M, Markiewicz M, Szafraniec MJ, et al. (2022). Systemic mobilization of breast cancer resistance protein in response to oncogenic stress. *Cancers.*
14. Chen, Y. C, Hsu, H. S., Chen, Y. W., Tsai, T. H., How, C. K., Wang, C. Y., Hung, S. C., Chang, Y. L., Tsai, M. L., Lee, Y. Y., Ku, H. H and Chiou, S. H (2008) “Oct-4 expression maintained cancer stem-like properties in lung cancer-derived CD133- positive cells.” *PLoS ONE* 3(7): 2637.
15. Zhang, Q., Shi, S., Yen, Y., Brown, J, Ta, J. Q. and Le, A. D. (2009). “A subpopulation of CD133(+) cancer stemlike cells characterized in human oral squamous cell carcinoma confer resistance to chemotherapy”. *Cancer Lett* 289 (2): 151–160.

16. Tawari Erebi Patricia (2024). Formation of a Gemcitabine (dFdC) Acquired Resistant BT 549 Triple Negative Breast Cancer Cells. *World Journal of Biology Pharmacy and Health Sciences*, 20(01), 289–295
17. Tawari-Ikeh E. P and Kasia E.B (2020). Acquired Resistance Induces Cross- and Pan-resistance to Some Chemotherapeutic Drugs in Breast Cancer Cell Lines. *International Journal of Scientific Research and Engineering Development*, 3 (2) Mar- Apr : pp 225-268.
18. Videira, M., Reis, R. L., and Brito, M. A. (2014). Deconstructing breast cancer cell biology and the mechanisms of multidrug resistance. *Biochim. Biophys. Acta* 1846 (2), 312–325. doi:10.1016/j.bbcan.2014. 07.011

