Acıbadem Univ. Sağlık Bilim. Derg. 2023; 14 (2): 108-118
https://doi.org/10.31067/acusaglik.1225228

ORIGINAL ARTICLE / ARAŞTIRMA YAZISI

Cell Biology / Hücre Biyolojisi

Mammosphere Formation Assay Optimization in the Characterization of Cancer Stem Cells of the Primary Breast Tumor

Gizem Uslu[1], Zehra Seda Halbutoğulları[2,3], Gülçin Gacar[2], Nihat Zafer Utkan[4], Yusufhan Yazır[2,5]

[1]Department of Biochemistry and Molecular Biology, Institute of Health Sciences, Acibadem Mehmet Ali Aydinlar University, İstanbul, Turkey

[2]Center for Stem Cell and Gene Therapies Research and Practice/ Department of Stem Cell, Institute of Health Sciences, Kocaeli University, Kocaeli, Turkey

[3]Department of Medical Biology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey

[4]Department of General Surgery, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey

[5]Department of Histology and Embryology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey

Gizem USLU
Zehra Seda HALBUTOĞULLARI
Gülçin GACAR
Nihat Zafer UTKAN
Yusufhan YAZIR

Correspondence: Zehra Seda Halbutoğulları Kocaeli Üniversitesi, KOÜ Kök Hücre ve Gen Tedavileri Araştırma ve Uygulama Merkezi (KÖGEM)/ Kök Hücre Anabilim Dalı, Tıbbi Biyoloji Anabilim Dalı, Kocaeli, Türkiye
Phone: +902623034940
E-mail: zehraseda@gmail.com

Received: 31 December 2022
Accepted: 19 February 2023

ABSTRACT

Introduction&Objective: Breast cancer is the most frequently diagnosed cancer and the leading cause of death from cancer among females worldwide. Breast cancer tumors that feature breast cancer stem cells (BCSCs) are known to cause drug resistance and metastasis. Culturing BCSCs from primary tumors as mammospheres is both difficult and costly. This study aims to present an optimized mammosphere formation assay protocol and improve the breast cancer stem cell characterization process by determining the appropriate mammosphere forming method and proper cell density of cancer stem cells isolated from breast tumors.

Material&Method: Cancer stem cells were isolated from breast tumors of two patients with invasive ductal carcinoma (IDC) and culturated. Subsequently, performed specific BCSCs surface markers and ALDH analysis by flow cytometry. Two mammosphere forming methods, i.e., low attachment and agar-coated wells seeded in three different cell concentrations, were compared.

Results: $CD44^+$, $CD24^-$ and $CD133^+$ antibody expressions showed that these cells could be tumor-initiating CSCs. ALDH assay results also indicated that these cells possessed stem cell features. In addition, the results of the mammosphere assay revealed that agar-coated wells at a concentration of 7000 cells/cm^2 had more prominent floating features and viable spheres.

Conclusion: The findings of this study supported the hypothesis that agar-coated culture plates in mammosphere culture would increase the mammosphere formation efficiency (MFE) value and revealed the importance of the number of cells in elucidating the nature of BCSCs.

Keywords: Breast cancer, mammosphere formation assay, cancer stem cell

Primer Meme Tümörü Kanser Kök Hücre Karakterizasyonunda Mamosfer Oluşturma Optimizasyonu

ÖZET

Giriş&Amaç: Meme kanseri, dünya çapında en sık teşhis edilen kanserdir ve kadınlar arasında kansere bağlı ölümlerin önde gelen nedenidir. Meme kanseri kök hücrelerini (MKKH) içeren meme tümörlerinin, ilaç direncine ve metastaza neden olduğu bilinmektedir. Primer tümörlerden MKKH' lerin mamosfer olarak kültürlenmesi hem zor hem de maliyetlidir. Bu çalışma, meme tümörlerinden izole edilen kanser kök hücrelerinden, uygun mamosfer oluşturma tekniğini ve uygun hücre yoğunluğunu belirleyerek optimize edilmiş bir mamosfer oluşturma protokolü sunmayı ve meme kanseri kök hücre karakterizasyon sürecini iyileştirmeyi amaçlamaktadır.

Gereç&Yöntem: İnvaziv duktal karsinomalı iki hastanın meme tümörlerinden kanser kök hücrelerinin izolasyonu ve kültürü gerçekleştirildi. Daha sonra, akım sitometrisi ile spesifik MKKH'leri yüzey belirteçleri saptandı ve ALDH analizi gerçekleştirildi. Düşük tutunma özellikli ve agar kaplanmış kuyucuklara üç farklı konsantrasyonda hücre ekilerek iki mamosfer oluşturma yöntemi karşılaştırıldı.

Bulgular: $CD44^+$, $CD24^-$ ve $CD133^+$ antikor ifadeleri, bu hücrelerin tümör başlatıcı KKH'leri olabileceğini gösterdi. ALDH analizi sonuçları da bu hücrelerin kök hücre özelliklerine sahip olduğunu gösterdi. Ek olarak, mamosfer testinin sonuçları, 7000 hücre/$cm^{2'}$lik bir konsantrasyonda agar kaplı kuyucukların daha belirgin yüzen özelliklere ve canlı sferlere sahip olduğunu ortaya çıkardı.

Sonuç: Bu çalışmanın bulguları, mamosfer kültüründe agar kaplı kültür kaplarının mammosphere oluşum etkinliği (MFE) değerini artıracağı hipotezini desteklemiş ve MKKH' lerin doğasını aydınlatmada hücre sayısının önemini ortaya koymuştur.

Anahtar Kelimeler: Meme kanseri, mammosphere oluşum testi, kanser kök hücresi

Breast cancer (BC) is the most common cancer among women, affecting an average of 2.1 million women each year. According to epidemiological studies, it is the leading cause of cancer-related deaths in women, with a prevalence of 24.5% and a mortality rate of 15.5%. The Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) breast cancer statistical data revealed that 2.3 million and 24.000 females were diagnosed with BC and 685.000 and 7000 females had died from BC worldwide and in Turkey, respectively, in 2020 (1). The morbidity rates associated with BC increase in countries with high human development index, whereas the mortality rates related to BC are higher in developing countries.

BC is a complex and heterogeneous disease involving multiple tumor entities associated with different histological patterns, biological features, and clinical behaviors. At the beginning of the last century, all patients with breast malignancy were treated with one type of treatment. The differences between BC patients in terms of prognosis and the identification of different morphological variants by pathologists over the last 50 years have caused scientists to categorize BC into different variants. The recently published World Health Organization (WHO) tumor classifications cited 20 major and 18 minor variants of BC (2).

Histologically, BC is categorized into four basic subtypes: ductal carcinoma in situ, lobular carcinoma in situ, invasive ductal carcinoma, and invasive lobular carcinoma. This study focused on invasive ductal carcinoma (IDC), the most common breast malignancy without any further subtype, constituting 75-80% of breast carcinomas (3). IDC is a malignant epithelial tumor that can invade surrounding tissues and metastasize to distant organs such as the lung, liver, and brain (4). In terms of the molecular classification of the tumor, BC is categorized into four determined by the positivity of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor 2 (Her2), Ki67 and cytokeratin (CK) 5/6 markers. These subclasses are luminal A [ER (+), PR (+), Her2 (-), Ki67 ≤ 14%], luminal B [ER (+), PR (+), Her2 (-), Ki67> 14%], Her2-2 positive [ER (-), PR (-), Her2 (+), Ki67 (high)], and basal-like/triple-negative [ER (-), PR (-) and Her2 (-)]. Luminal A is the most common molecular BC subtype with a relatively good prognosis. This study focused on the most common BC subtypes and tumors with IDC-Luminal A character in terms of histological-molecular subtypes as a cellular source.

Breast tumors, like other solid tumors, contain a subgroup called breast cancer stem cells (BCSC). BCSCs are aggressive tumor cells responsible for tumor formation, progression, and metastasis. BCSCs delay the response in cancer treatment and cause metastasis and relapse of the disease after treatment (5). Therefore, efforts to develop current cellular therapies target BCSCs.

Tumor recurrence occurs in approximately 30% of the invasive BC cases. The mortality rate in BC cases with tumor recurrence is as high as 90% (6). This high mortality rate is attributed to the fact that BCSCs are resistant to radiotherapy, chemotherapy, and endocrine therapy and can reactivate the tumorogenic potential depending on specific signals while in the dormant state (7). BCSCs were first identified in 2003 by Al Hajj et al. based on cell surface markers (CD44+/CD24-/low) (8). Recent studies have cited aldehyde dehydrogenase 1 (ALDH1), CD133, and CD49 as BCSC markers, given that they were found to be associated with resistance to chemotherapy and radiotherapy (9). CD44 is a cell-matrix adhesion molecule expressed at physiological levels in normal cells, such as embryonic stem cells and stromal cells, and at high levels in cancer cells. When it binds to its ligands, CD44 activates a variety of signaling pathways that lead to cell adhesion, proliferation, migration, and metastasis (10). In addition, CD44 has been shown to be associated with secondary events such as the epithelial-mesenchymal transition (EMT) process, apoptosis resistance, invasion, metastasis, and poor prognosis in many other cancer types such as prostate, stomach, pancreas, colon, and breast cancers (11-13).

ALDH1 catalyzes the oxidation of a large group of toxic aldehydes to carboxylic acids inside the cell. High levels of ALDH1 expression and ALDH1 activity increase the detoxification capacity of BCSCs, creating resistance to cancer treatments such as chemotherapy and radiotherapy. In addition, ALDH has been shown to mediate the angiogenic phenotype in tumor neovascularization by increasing Vascular endothelial growth factor (VEGF) expression in BCSCs (14). ALDH1 has been associated with strong tumorigenicity in both in vivo and in vitro experiments and was recognized as a reliable biomarker for BCSCs (14). Accordingly, BCSCs are characterized by high CD44/CD24 ratios and ALDH expression levels (15). BCSCs also have the ability to form a highly proliferative spheroid, i.e., mammosphere, in non-adherent suspension culture (16). The proliferation and invasiveness of BCSCs are directly proportional to the mammosphere formation ability.

Studies on metastatic cancers, in general, focus on CSCs, which are implicated in multiple drug resistance, radiotherapy resistance, and disease relapse after treatment. Thus, it is essential to develop studies on optimizing cancer stem cell characterization. Accordingly, this study was carried out to develop a method to culture and characterize BCSCs.

MATERIALS and METHOD

Isolation of and culturing of BCSCs from primary tumor tissue

Breast invasive ductal carcinoma tissue samples were obtained from the Kocaeli University Faculty of Medicine Hospital, Department of General Surgery. Tumor tissues of the two patients diagnosed with IDC were removed by mastectomy in ~7-8 mm^3 tissue fragments for cell isolation (Table 1). The tumor tissue was delivered to the laboratory in Dulbecco's modified eagle medium-low glucose (DMEM-LG) supplemented with 5% penicillin/streptomycin (pen/strep) antibiotic and 10% fetal bovine serum (FBS). Tumor tissue was stored in a laminar flow cabinet (Safe Fast Elite 2150, Italy), then taken to a sterile petri dish with a diameter of 90 mm. The blood tissue and blood vessels were harvested from the tissue by washing it twice with 10 ml of Hank's balanced salt solution (HBSS) supplemented with 5% pen/strep antibiotic (Capricorn). Subsequently, the tumor tissue was taken into a new petri dish and divided into small pieces with the help of a scalpel. Four ml of the tumor dissociation enzyme mix (Collagenase/Hyaluronidase, StemCell Tech #07912, Canada) was added to the dissected tumor tissue, and the entire tissue/enzyme mixture in the petri dish was transferred to a 50 ml falcon tube. The tumor tissue/enzyme mixture was incubated overnight in a 37°C shaking water bath (17). Following the incubation, 7 ml of inactivation medium (DMEM-LG with 10% FBS) was added, and the enzymatic reaction was halted. Waste tissue pieces were removed by subjecting the tissue to a cell/tissue suspension through a 70 µm strainer. The cell suspension was centrifuged at 1600 rpm for 5 minutes, and the enzyme was removed by discarding the supernatant solution. Cell pellet BCSC medium [(DMEM/F12 (Gibco), 10% FBS (Gibco), 1% Penicillin/Streptomycin (Capricorn), 1% Glutamax (Gibco), 4 µg/ml Insulin (Sigma), 1 µg/ml Hydrocortisone (Sigma)] was homogenized with 10 ng/mL epithelial growth factor (EGF; Winsent)], inoculated in a T-25 cell culture dish (SPL Biosciences, Korea) and cultured at 37 °C in5% CO_2medium. Cells were propagated by subjecting them to 0.25% Trypsin/0.02% EDTA solution with trypsinization every 4-6 days (~70-80% confluency).

Table 1: Histopathological features and Bloom-Richardson grading of breast tumors of IDC patient1 and 2.

IDC Case 1 (Age:57)	IDC Case 2 (Age:42)
ER: 100% positive	ER: %80 positive
PR: 95% positive	PR: %60 positive
Ki67: 11% positive	Ki67: %10 positive
Bloom–Richardson Grading System	
Tubule formation: 2	Tubule formation: 2
Nuclear pleomorphism:2	Nuclear pleomorphism:1
Mitotic index: 2 (12/10 BBA)	Mitotic index: 1 (2/10 BBA)
Total score: 6, Grade: II	Total score: 4, Grade: I

Flow cytometry

BCSCs were harvested from both patients after passaging the cells with trypsin enzyme and counted following enzyme inactivation. Consequently, 2×10^5 cells were analyzed for each marker. After the cells were washed with the washing solution, a specific fluorescent fluoresce in isothiocyanate (FITC) and phycoerythrin (PE) – conjugate to the determined surface markers, i.e., CD44, CD73, CD90, CD105, CD13, CD29, CD140b, CD24, anti-HLA DR, anti-cytokeratin, CD45, CD34, CD15, and CD14 human monoclonal antibodies were incubated at room temperature at dark for 45 minutes. Subsequently, the washing solution was added again, and the resulting solution was centrifuged at 300×g for 5 minutes. The washing process was completed by discarding the supernatant. The cells were homogenized again using 350 µl washing solution for analysis in the FACS Calibur (BD Biosciences) flow cytometry device. The relevant analyses were performed using the Cell Quest software package (BD Biosciences).

ALDH assay

The ALDH enzyme activity levels of the isolated BCSCs obtained from the two patients were analyzed using the ALDH assay kit (StemCell Technologies-Aldefluor TM Assay Kit, U.S.) per the manufacturer's instructions. Accordingly, BCSCs were harvested using the trypsin enzyme and resuspended in single cells. Simultaneously, human leukocytes, i.e., white blood cells (WBCs), were obtained from peripheral blood and included in the ALDH test as the reference material. 3×10^6 cells reserved for ALDH analysis were centrifuged at 250×g for 5 minutes. The supernatant solution was discarded, and the cells were homogenized using 6 mL of test buffer. Flow cytometry tubes were prepared for propidium iodide (PI), 7-actinaminomycin-D (7-AAD), diethylaminobenzaldehyde (DEAB), verapamil, CD133 with ALDH parameters. The prepared cell suspension was distributed to each tube in portions of 500µl (5×10^5 cells).

5µl of DEAB reagent was added to the tube and mixed thoroughly. 2.5 µl of Aldefluor substrate was added to the tubes except for PI and 7-AAD tubes and mixed thoroughly. All tubes were incubated at 37 °C in the dark for 45 minutes. Subsequently, the cells were centrifuged at 250×g for 5 minutes. The supernatant solution was discarded and homogenized again with 300 µl of test buffer. 5 µl of CD133, PI, and 7-AAD probes were added to CD133, PI, and 7-AAD tubes, thoroughly mixed, incubated at 4°C in the dark for 20 minutes, then centrifuged at 250×g for 5 minutes. The supernatant solution was discarded, and the cells were homogenized once more using 500 µl of Aldefluor test buffer for analysis in the FACS Calibur (BD Biosciences) flow cytometry device. The relevant analyses were performed using the Cell Quest software package (BD Biosciences). The ALDH activity of BCSCs was evaluated in comparison with negative control DEAB and verapamil samples.

Mammosphere formation assay/mammosphere culturing and passaging

The mammosphere formation analysis featured the suspension cultures of isolated BCSCs obtained from both cases. The ability of these BCSCs to form a mammosphere on the agar-coated and low-attachment surfaces was comparatively examined. Prior to the experiment, 6-well plates (Falcon, U.S.) on which cells were seeded were covered with 3% agar (Fluka) containing 1:1 mammosphere medium.

The mammosphere medium used in the study was enriched and customized with additional add-ons, unlike the mammosphere medium contents described in the literature. Mammosphere medium consisted of DMEM/F12 supplemented with 1% pen/strep (Capricorn), 1% Glutamax (Gibco), 20 ng/ml EGF, basic fibroblast growth factor (bFGF), 1x B27, 4 µg/ml heparin (Sigma), 1 µg/ml hydrocortisone (Sigma), and 4 µg/ml insulin (Sigma).

In order to optimize the cell concentration or to determine the appropriate cell concentration in the mammosphere formation analysis, three groups with the following cell concentrations were created: 3×10^3 cells/cm^2, 5×10^3 cells/cm^2, and 7×10^3 cells/cm^2. Cells were cultured with a mammosphere medium for seven days by seeding in agar-coated and low-attachment 6-well flasks in accordance with the determined concentrations. Generation I mammospheres were examined on the 7th day of culture by imaging with a phase-contrast microscope, the mammosphere counts were counted, and the mammosphere formation efficiency (MFE) was calculated using the following formula: *"MFE (%) = (# of mammospheres per well) / (# of cells seeded per well) x 100".*

Passaging mammospheres

On the 7th day of culture, generation I mammospheres were counted, and each well was transferred to a 15 ml conical tube. Mammospheres that remained on the surface were washed with the phosphate-buffered saline (PBS) solution, transferred to the tubes, and centrifuged at 115×g for 10 minutes. The supernatant solution was discarded. The pellet was re-suspended with 500 µl of TrypLE enzyme and incubated at 37°C for 3 minutes. The enzyme was neutralized with 500 µl of FBS. After centrifugation at 500×g for 5 minutes, the supernatant solution was discarded, and the pellet was re-suspended with 100 µl of mammosphere medium. It was pipetted up and down several times and passed three times through a 25 G syringe to form a single-cell suspension. After the cell count was done, inoculation was performed on agar and low-attachment six well plates at concentrations of 3×10^3 cells/cm^2, 5×10^3 cells/cm^2, and 7×10^3 cells/cm^2. It was cultured with a mammosphere medium for seven days, and a mammosphere count was done on the 7th day. MFE formulation was calculated as in generation I (18).

Statistical analysis

SPSS 10.0 (Statistical Package for the Social Sciences, version 10.0, Chicago, IL, U.S., 1999) software package was used to perform all statistical analyses. Research data were tested with the paired t-test. Newman–Keuls method was used for multiple analyses. Each experimental group consisted of at least three replicates. The difference between the experimental and control groups was deemed significant and highly significant in cases where the probability (p) statistics were ≤ 0.05 and <0.01, respectively. The MFE value of Generation I and II was calculated using the following formula: *"MFE (%) = (# of mammospheres per well) / (# of cells seeded per well) x 100".*

RESULTS

Isolation and culturing of BCSCs from primary tumor tissue

BCSCs isolated from the breast tumor tissue were cultured adherently, and their morphology was regularly examined under a phase-contrast microscope (Figure 1). BCSCs did not show morphological signs of aging or differentiation during culturing. In fact, it was observed that they proliferated quite rapidly, even during additional passages.

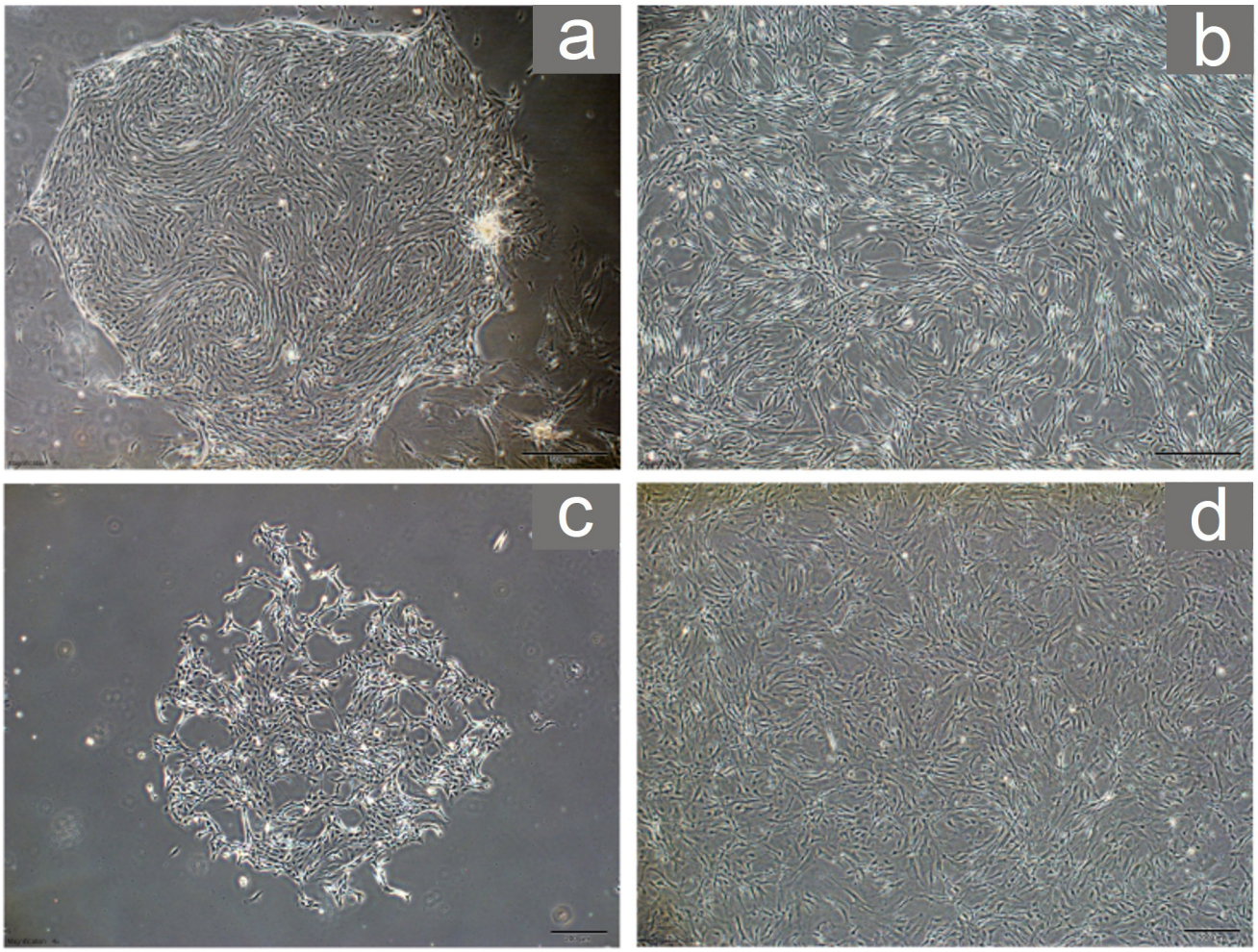

Figure 1. *P0 (a) and P3 (b) light microscope images of the BSC obtained from case 1. P0 (c) and P3 (d) light microscope images of the BSC obtained from case 2. Barr; 500µm.*

Flow cytometry

As the isolated BCSCs of cases 1 and 2 reached passage 3 (Figure 4.3), stem cell markers, i.e., CD73, CD90, CD105, CD13, CD29, and CD140b, showed high positivity, in addition to exhibiting CD44+/CD24- breast cancer stem cell phenotype. Hematopoietic and epithelial cell markers such as human leukocyte antigen-D related (HLA-DR), cytokeratin, CD45, CD 34, CD15, and CD14 were also negative (Figure 2).

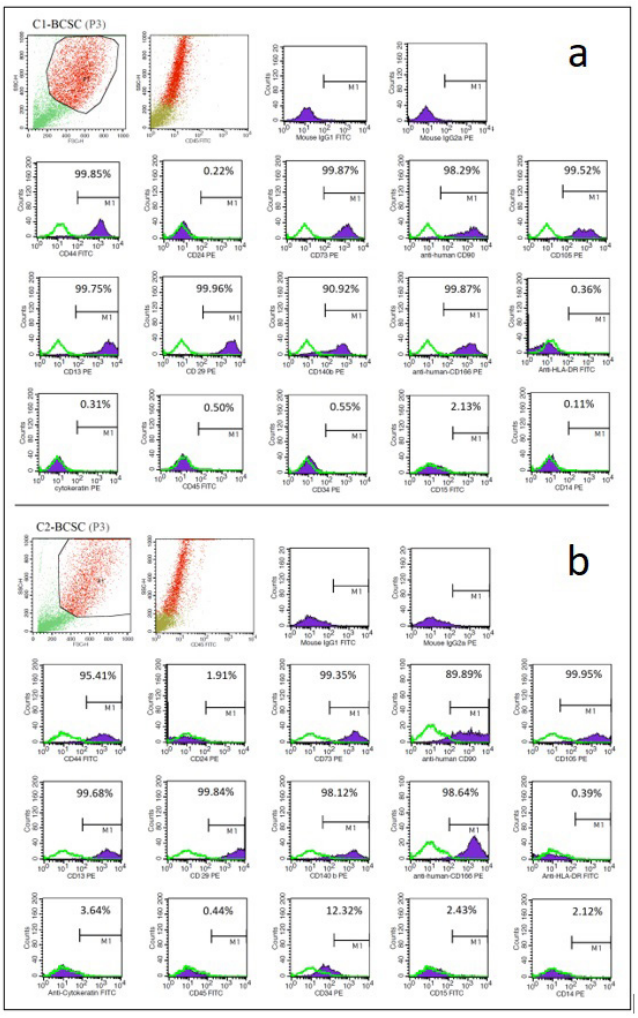

Figure 2. *Flow cytometry analyses of the BCSCs of case 1 (a) and case 2 (b)*

ALDH assay

ALDH enzyme activity was positive, over 99% in all double staining performed within the scope of the ALDH test. CD133 expression was 61.74% in the BCSC of case 1 and 89.13% in the BCSC of case 2. The 7-AAD and PI values were less in the BCSC of case 1 than in the BCSC of case 2, and below ~15% in the BCSCs of both cases. The ALDH test revealed CD133 positivity suggesting that the isolated tumor cells have a cancer stem cell phenotype.

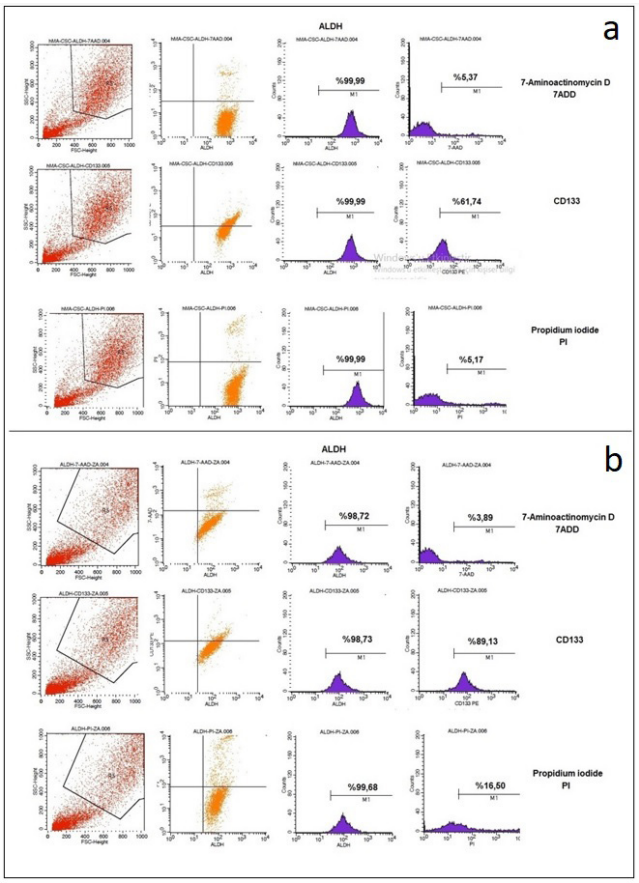

Figure 3. *ALDH analyses by flow cytometry of the BCSCs of case 1 (a) and case 2 (b). ALDH double stained with 7ADD, PI, and CD133.*

Optimization of the mammosphere culture system

Primary tumor cells obtained from both cases featured mammosphere formation in the serum-free spheroid culture system.

MFE values of the BCSCs were calculated according to the mammosphere count, and mammosphere size measurement was performed in generation I and generation II (Figures 4 and 5).

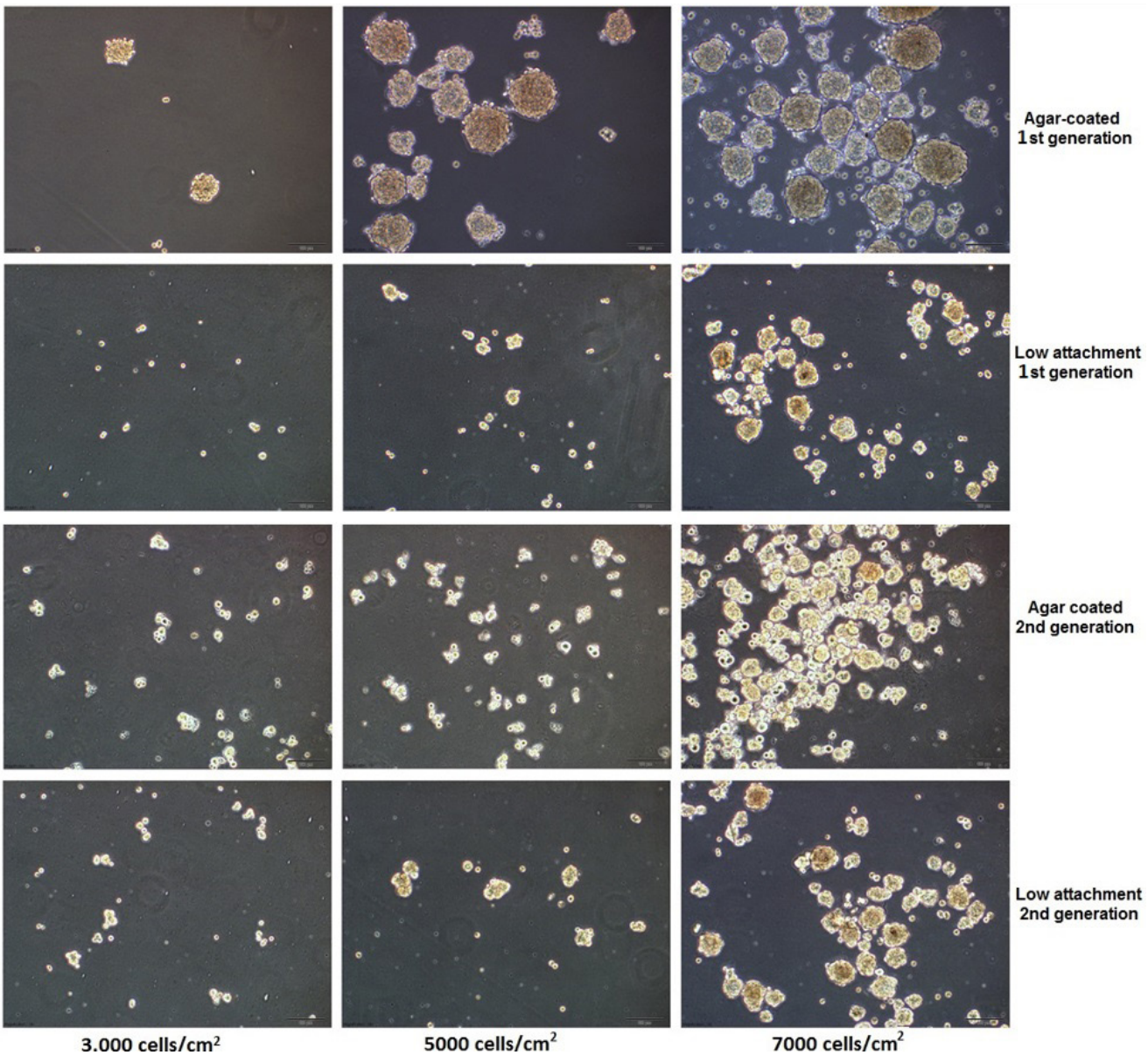

Figure 4. *Morphology of mammospheres from the BCSCs of case 1 seeded on different cell concentrations, agar-coated, and low attachment wells. Barr; 100µm*

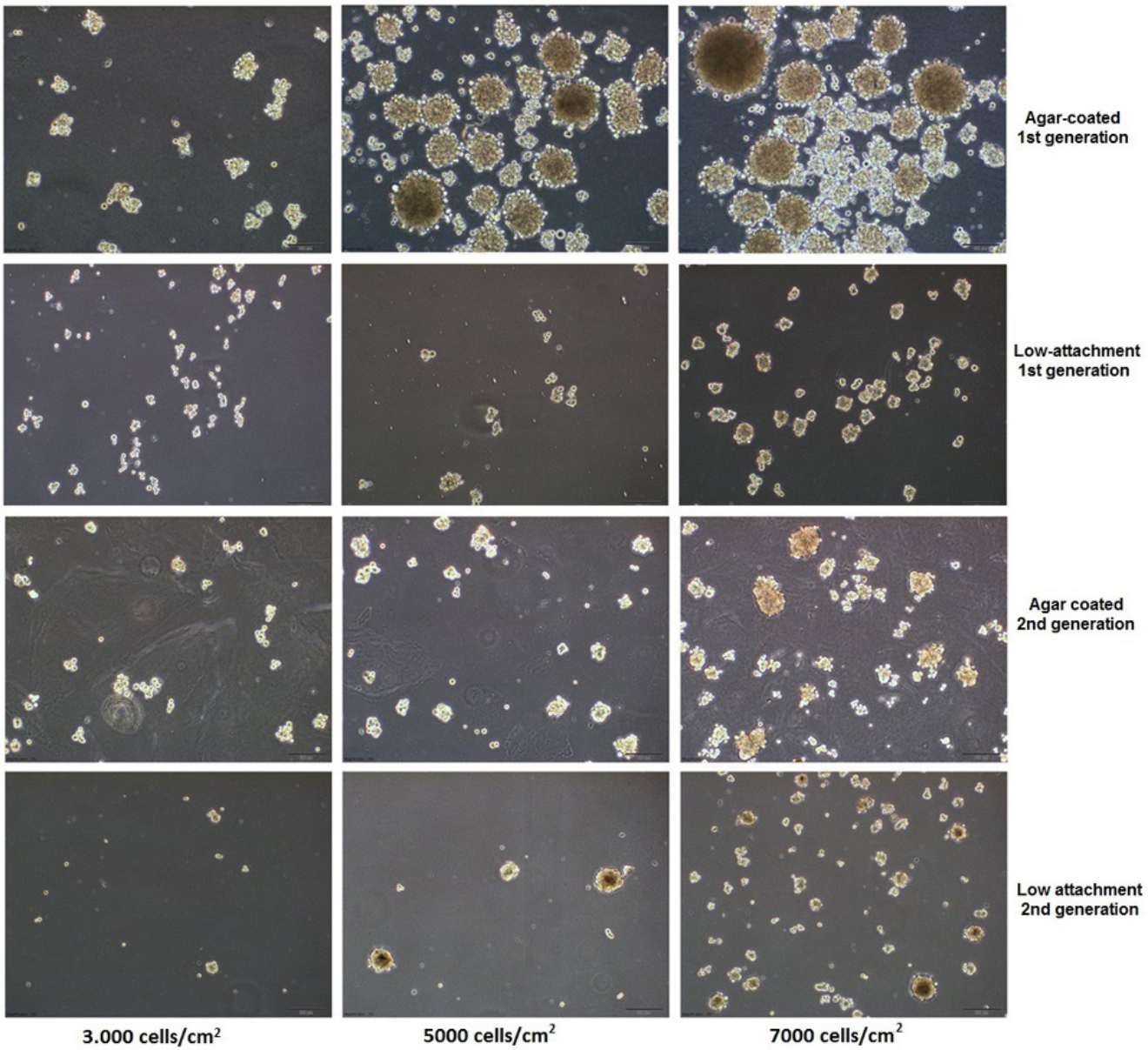

Figure 5. *Morphology of mammospheres from the BCSCs of case 2 seeded on different cell concentrations, agar coated, and low attachment wells. Barr; 100µm*

The cell concentration of 7,000 cells/cm^2 was found to be more effective in evaluating the MFE of primary breast tumor cells compared to the other cell concentrations of 3,000/cm^2 and 5,000/cm^2. The number of cells planted is a crucial factor in forming the mammosphere. The MFE value of the cells planted at a cell concentration of 7000 cells/cm^2 was found to be significantly higher than at other cell concentrations ($p< 0.05$ and $p<0.01$) (Figure 6, Figure 7).

In terms of the mammosphere number, size, and quality, it was observed that the agar-coated spheroid culture system was relatively advantageous over the low attachment spheroid culture system. The results of the mammosphere experiment indicated that the BCSCs of case 1 had a significantly higher ability to form spheroids in the two isolated cell lines compared to the BCSCs of case 2. In the agar coating mamosphere formation; Case I MFE value is 2.1 times higher in Case II MFE value in Generation 1, while it is 3 times higher in Generation II (7,000 cells/cm2) ($p<0.001$).

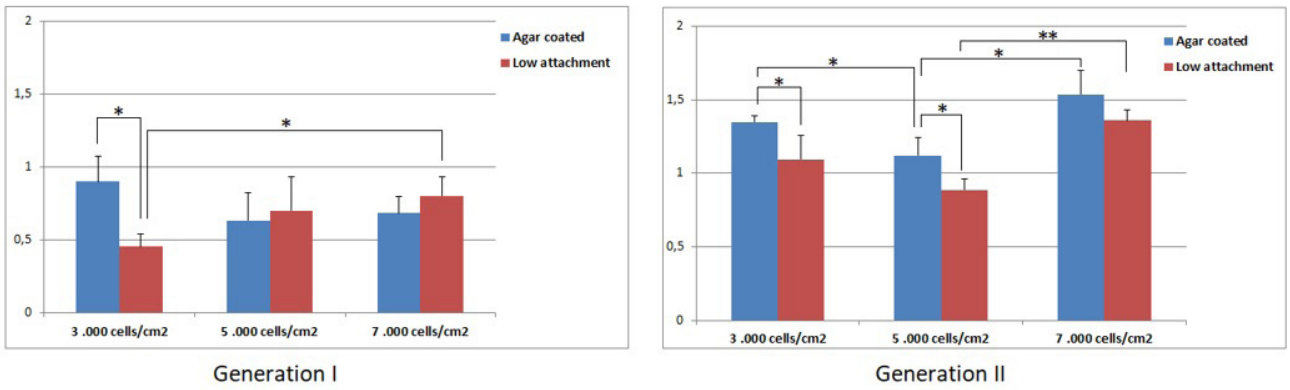

Figure 6. *MFE of mammospheres obtained from the BSCS of case 1. Data were mean±SD, *p<0.05, **p<0.01*

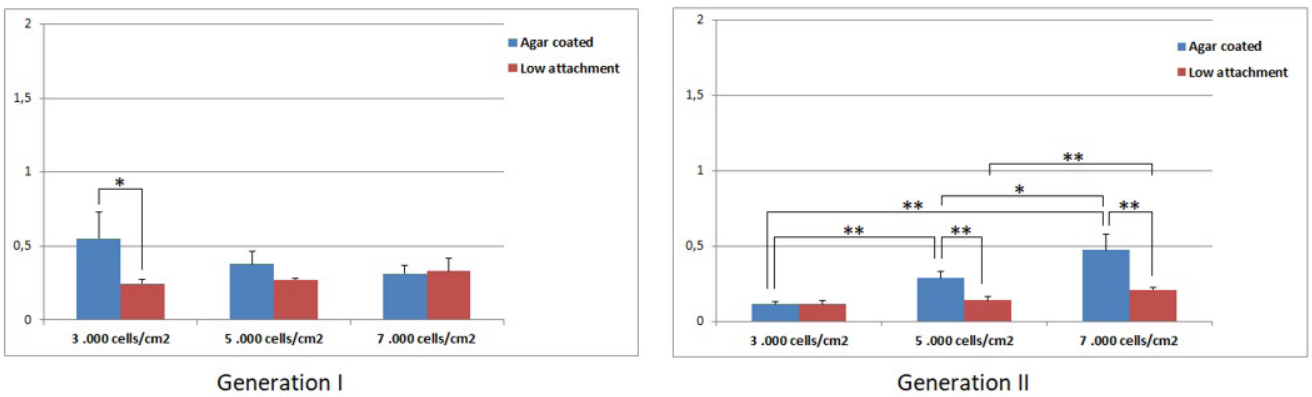

Figure 7. *MFE of mammospheres obtained from the BSCS of case 2. Data were mean±SD, *p<0.05, **p<0.01.*

DISCUSSION

As in many solid tumors, high ALDH activity in breast tumors is considered an indicator of aggressive and metastatic tumors. Increased ALDH activity in vitro in colony formation, migration, and invasion is associated with in vivo metastasis (19). A study on breast cancer cell lines demonstrated that ALDH-positive cell lines had high invasion capacities (20).

The characterization analysis revealed that the cells isolated from the primary tumor tissue within the scope of this study had cancer stem cell phenotype. BCSCs, the tumorigenic subpopulation of breast tumors, were mainly identified by CD44 positivity and CD24 negativity ($CD44^+/CD24^-$) (8). The results of the flow cytometry analyses indicated that the cells isolated from tumor tissues had $CD44^+/CD24^-$ phenotype, CD61 positivity, another basic marker, and high ALDH enzyme activity (21). In addition, the high positivity of other stem cell markers, i.e., CD133, CD166, and CD29, suggested that the isolated primary tumor cells had the characteristics of cancer stem cells. CD133 is a transmembrane glycoprotein expressed in healthy somatic progenitor/stem cells and cancer stem cells, the metastatic precursor cells of solid tumors (22). Due to its more restricted expression compared to other CSC markers, such as CD44 and ALDH, CD133 has long been considered one of the most rigorous indicators of malignant precursors in different solid tumors, including breast cancer (22). In a study featuring MDA-MB-231, MDA-MB-468, and triple-negative high ALDH, $CD44^+$ phenotype cell lines, CD133 was found to be associated with enhanced malignant/metastatic behavior in both in vitro and in vivo experiments (23). In addition to being a sharp CSC marker, CD133 is a valuable prognostic marker as it is positively correlated with high tumor grade, distant metastasis, and poor overall survival (24).

The high rate of cancer stem cell markers, mammosphere forming capacity, and ALDH positivity indicated that cancer stem cells, a subpopulation in the tumor, were successfully isolated in this study. As Zhang et al. stated, there are four basic methods used in cancer stem cell identification and isolation (28), the most common being the methods that feature the separation of surface markers or combination of biomarkers, followed by the one that features side population cell isolation with Hoescst 33342 dye, the aldeflour method, and mammosphere formation. Other methods other than cell separation are isolation methods based on cancer stem cell properties. The efficiency of creating two generations of mammosphere after isolation was demonstrated in this study, in addition to $CD44^+$ and $CD24^{-/low}$, surface markers, and ALDH positivity. Furthermore, the fact that the cells featured self-renewal and proliferation activity in the advancing passages indicated that these cells were, in fact, cancer stem cells. It is known that cell lines such as MCF-7 and MDA, which are frequently used in studies, are heterogeneous. Therefore, $CD44^+$ and $CD24^-$ cells have been selected over these cell lines in many recent studies (29). Given the surface marker results and cancer stem cell properties obtained after isolation, it was the primary cell line that featured cancer cell characteristics comparable to the in vivo environment in this study.

Mammosphere-forming efficiency varies depending on the cancer cell lines and cells isolated in primary culture (30). As a matter of fact, the results obtained using human-derived breast tumors from two cases in this study revealed high cell proliferation in both cases with varying mammosphere-forming abilities.

CONCLUSION

In conclusion, the cell lines obtained from the tumor tissues of two cases had high CD44 positivity, CD24 negativity, and ALDH rates. Mammosphere formation was significantly higher in the BCSC of case 1 than in the BCSC of case 2, this difference can be associated with histopathological features of cancer tissue and Bloom-Richardson grading of breast tumors.

CD44 positivity, CD133 positivity, CD24 negativity, ALDH positivity, and high mammosphere forming abilities indicated that the cancer stem cells were isolated. In addition, the MFE value of the cells was significantly higher in the agar-coated culture plates than in the low-attachment culture plates. The mammosphere experiment demonstrated that 7000 cell/cm^2 concentration was more suitable for mammosphere assay.

DECLARATIONS

Funding

This study is supported by Kocaeli University, Scientific Research Unit under the grant number 2018/112.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This study was approved by the Ethics Committee of Kocaeli University (approval number: KU GOKAEK 2018/4.24). Informed consents, approved by the institutional ethics committee, were obtained from each patient.

Author contributions

G.U. and Z.S.H. conceived the project. Z.S.H. and Y.Y. supervised the project. analysis. G.U. and Z.S.H. performed BCSCs isolation and cell culture. G.G. performed flow cytometer. G.U. and Z.S.H. performed MFE analysis. N.Z.U. performed the surgeries and contributed to tumor tissue sample collection. G.U. and Z.S.H. contributed to writing and revising the draft. All authors read and approved the final manuscript.

REFERENCES

1 Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Ca-a Cancer Journal for Clinicians. 2021;71:209-49. DOI:10.3322/caac.21660

2 Ellis I, Cornelisse C, Schnitt S, et al. Invasive breast carcinomas. In: Tavassoli FA., Devilee P., eds. WHO Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs. 3 ed: IARC Press; 2003.

3 Li CI, Uribe DJ and Daling JR. Clinical characteristics of different histologic types of breast cancer. Brit J Cancer. 2005;93:1046-52. DOI:10.1038/sj.bjc.6602787

4 Chen XF, Zhang CY, Guo DJ, et al. Distant metastasis and prognostic factors in patients with invasive ductal carcinoma of the breast. Eur J Clin Invest. 2022;52. DOI:10.1111/eci.13704

5 Lawson JC, Blatch GL and Edkins AL. Cancer stem cells in breast cancer and metastasis. Breast Cancer Res Tr. 2009;118:241-54. DOI:10.1007/s10549-009-0524-9

6 Gwynne WD, Shakeel MS, Girgis-Gabardo A, et al. The Role of Serotonin in Breast Cancer Stem Cells. Molecules. 2021;26. DOI:10.3390/molecules26113171

7 Saha T and Lukong KE. Breast Cancer Stem-Like Cells in Drug Resistance: A Review of Mechanisms and Novel Therapeutic Strategies to Overcome Drug Resistance. Front Oncol. 2022;12. DOI:10.3389/fonc.2022.856974

8 Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. P Natl Acad Sci USA. 2003;100:3983-8. DOI:10.1073/pnas.0530291100

9 Crabtree JS and Miele L. Breast Cancer Stem Cells. Biomedicines. 2018;6:77. DOI:10.3390/biomedicines6030077

10 Ponta H, Sherman L and Herrlich PA. CD44: From adhesion molecules to signalling regulators. Nat Rev Mol Cell Bio. 2003;4:33-45. DOI:10.1038/nrm1004

11 Blick T, Hugo H, Widodo E, et al. Epithelial Mesenchymal Transition Traits in Human Breast Cancer Cell Lines Parallel the CD44(hi/) CD24(lo/-) Stem Cell Phenotype in Human Breast Cancer. J Mammary Gland Biol. 2010;15:235-52. DOI:10.1007/s10911-010-9175-z

12 Patrawala L, Calhoun T, Schneider-Broussard R, et al. Highly purified CD44(+) prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. Oncogene. 2006;25:1696-708. DOI:10.1038/sj.onc.1209327

13 Hou WY, Kong LW, Hou ZP, et al. CD44 is a prognostic biomarker and correlated with immune infiltrates in gastric cancer. Bmc Med Genomics. 2022;15. DOI:10.1186/s12920-022-01383-w

14 Ciccone V, Terzuoli E, Donnini S, et al. Stemness marker ALDH1A1 promotes tumor angiogenesis via retinoic acid/HIF-1/VEGF signalling in MCF-7 breast cancer cells. J Exp Clin Canc Res. 2018;37. DOI:10.1186/s13046-018-0975-0

15 De Beca FF, Caetano P, Gerhard R, et al. Cancer stem cells markers CD44, CD24 and ALDH1 in breast cancer special histological types. J Clin Pathol. 2013;66:187-91. DOI:10.1136/jclinpath-2012-201169

16 Grimshaw MJ, Cooper L, Papazisis K, et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res. 2008;10. DOI:10.1186/bcr2106

17 Shi AP, Fan ZM, Ma KW, et al. Isolation and. characterization of adult mammary stem cells from breast cancer-adjacent tissues. Oncol Lett. 2017;14:2894-902. DOI:10.3892/ol.2017.6485

18 Lombardo Y, De Giorgio A, Coombes CR, et al. Mammosphere Formation Assay from Human Breast Cancer Tissues and Cell Lines. Jove-J Vis Exp. 2015. DOI:10.3791/52671

19 Rodriguez-Torres M and Allan AL. Aldehyde dehydrogenase as a marker and functional mediator of metastasis in solid tumors. Clin Exp Metastas. 2016;33:97-113. DOI:10.1007/s10585-015-9755-9

20 Charafe-Jauffret E, Ginestier C, Iovino F, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res. 2009;69:1302-13. DOI:10.1158/0008-5472.CAN-08-2741

21 Shao J, Fan W, Ma BA, et al. Breast cancer stem cells expressing different stem cell markers exhibit distinct biological characteristics. Mol Med Rep. 2016;14:4991-8. DOI:10.3892/mmr.2016.5899

22 Lorico A and Rappa G. Phenotypic Heterogeneity of Breast Cancer Stem Cells. J Oncol. 2011;2011. DOI:10.1155/2011/135039

23 Croker AK, Goodale D, Chu J, et al. High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J Cell Mol Med. 2009;13:2236-52. DOI:10.1111/j.1582-4934.2008.00455.x

24 Brugnoli F, Grassilli S, Al-Qassab Y, et al. CD133 in Breast Cancer Cells: More than a Stem Cell Marker. J Oncol. 2019;2019. DOI:10.1155/2019/7512632

25 Darvishi B, Boroumandieh S, Majidzadeh-A K, et al. The role of activated leukocyte cell adhesion molecule (ALCAM) in cancer progression, invasion, metastasis and recurrence: A novel cancer stem cell marker and tumor-specific prognostic marker. Exp Mol Pathol. 2020;115. DOI:10.1016/j.yexmp.2020.104443

26 Xiao M, Wang X, Yan M, et al. A systematic evaluation for the potential translation of CD166-related expression as a cancer biomarker. Expert Rev Mol Diagn. 2016;16:925-32. DOI:10.1080/14737159.2016.1211932

27 Lu H, Clauser KR, Tam WL, et al. A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. Nat Cell Biol. 2014;16:1105-17. DOI:10.1038/ncb3041

28 Zhang X, Powell K and Li L. Breast Cancer Stem Cells: Biomarkers, Identification and Isolation Methods, Regulating Mechanisms, Cellular Origin, and Beyond. Cancers (Basel). 2020;12. DOI:10.3390/cancers12123765

29 Sun H, Jia J, Wang X, et al. CD44+/CD24- breast cancer cells isolated from MCF-7 cultures exhibit enhanced angiogenic properties. Clin Transl Oncol. 2013;15:46-54. DOI:10.1007/s12094-012-0891-2

30 Wang R, Lv Q, Meng W, et al. Comparison of mammosphere formation from breast cancer cell lines and primary breast tumors. J Thorac Dis. 2014;6:829-37. DOI:10.3978/j.issn.2072-1439.2014.03.38