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In Vitro Stem Cell Isolation from Human Breast Cancer

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Abstract

Background: Breast cancer (BC) is the most prevalent cancer among women worldwide with significant incidence and death rates. Nowadays, researchers hold that tumor formation, failure in therapy, and disease progression are all related to the presence of a small fraction of cancer cells with self-renewal capability known as "breast cancer stem cells" (BCSCs). Therefore, the study of this cancer cell population can be conducive to eradicating the tumor. The objective of the present study was to survey the existence and in vitro isolation of human BCSCs.

Method: An in vitro research study was conducted under controlled laboratory settings to isolate, enrich, and identify breast cancer stem cells. Briefly, fresh breast tumors were carried to the lab immediately after surgery, followed by mechanical and enzymatic digestion (2 mg/ml collagenase I). Then, digested samples were passed through cell strainers (70 and 40 µm), and obtained cell suspension was cultured under the serum-free medium supplemented with growth factors for 21 days. The expression of cluster of differentiation 44 (CD44) and cluster of differentiation 24 (CD24) surface markers was assessed using immunocytochemistry, and stem cell gene expression was analyzed via RT-PCR.

Results: BCSCs were able to survive in serum-free conditions and form floating spheres in vitro. Cells obtained from mammospheres expressed CD44 as the membranous and cytoplasmic pattern while CD24 expression was negative. Also, octameter-binding transcription factor 4 and SOX2 gene expression was observed in BCSCs.

Conclusion: The presence of stem cells was confirmed in Iranian women BC, and an efficient in vitro mammosphere culture model was used to enrich and propagate BCSCs. In our opinion, this in vitro model could be a suitable method for isolating and enriching BCSCs.

Keywords: Breast neoplasm, Neoplastic stem cells, Isolation, Purification

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Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the major leading cause of cancer death among women worldwide. In 2018, BC accounted for 24.2% of 8.6 million newly diagnosed and 15% of 4.2 million cancer-related deaths

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of the female population all over the world.[1] In Iran, BC is the most common form of women's cancer, comprising 24.6% of all malignancies.[2] The BC rate is globally increasing, especially in developing countries and Iran; a recent study has shown that the mortality rate is growing and the mean age of BC prevalence in Iranian women is 10 years less than their counterparts in developed countries.[3-6] Despite the application of different therapeutic strategies such as chemotherapy and radiation,[7] metastasis remains the main reason for 90% of cancer-related deaths.[8]

Tumor growth and metastasis are mainly attributed to a fraction of tumor cells with stem-like properties known as cancer stem cells (CSCs),[9] which are a small cell population with self-renewal capacity which lead to a heterogeneous lineage of cancer cells that form the tumor mass.[10] Several studies have demonstrated the existence of stem cells within solid tumors and cancer cell lines such as lung cancer,[11] glioblastoma,[12] gastric cancer,[13] prostate cancer,[14] and BC.[15, 16] Al-Hajj et al. first characterized cluster of differentiation 44 (CD44) +/cluster of differentiation 24(CD24)-/ LOW Lin- as BC tumorigenic cells. When injected into NOD/SCID mice, merely 100 cells were able to form a tumor, while alternate phenotypes did not.[16]

Most studies have approved the role of CSC in tumor formation and metastasis, but the CSC concept is still regarded as controversial.[17, 18] There are a couple of major controversies on the origin and evolution of CSC. Primarily, it is proposed that cancer stem cells might originate from different populations of normal stem and progenitor cells transforming and they possibly acquire malignant characteristics or derive from cancer cells that acquire the self-renewal potential. Second, despite the compelling evidence on the presence of cancer stem cells within tumors and their role in tumor progression, some researchers bring up the clonal evolution model instead of CSC theory, in which some cancer cells are susceptible to mutations and entail tumor growth and expansion.[19]

Furthermore, it is more complicated to employ an appropriate technique for identifying and enriching CSCs from clinical samples. The gold standard for assessing CSC self-renewal is in vivo tumorigenicity assessment, but it is necessary to apply an in vitro technique to efficiently enrich CSCs.[20] Furthermore, conducting experiments on primary tumors seems to be the optimum approach to exploring tumor repopulation.[21] The current study aimed to demonstrate the existence of breast cancer stem cells (BCSCs) in Iranian patients' breast tumors and to suggest a standard protocol of in vitro mammosphere culture as a suitable method for enriching CSCs.

Materials and Methods

Patients and tumor samples

The present in vitro research study was performed under controlled laboratory settings to isolate, enrich, and identify breast cancer stem cells in vitro. Primary tumor samples were obtained surgically from six patients (aged 20-53), who signed a written informed consent according to the guideline approved by the Ethics Committee of Kermanshah University of Medical Sciences. Breast tumors were sampled to determine the grade and stage of the tumors. Relevant histopathological assessments were performed by a pathologist. Of all samples, one was lobular carcinoma, two were metastatic BC, and three were ductal carcinoma (Table 1). Patients had no previous treatment in chemotherapy, radiotherapy, or any other therapies.

Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12), fetal bovine serum (FBS), and trypsin were purchased from Sigma-Aldrich Chemical Co (St. Georg-Heyken, Hamburg, Germany). Cell culture flasks were obtained from SPL Life sciences Co. (Gyeonggi-do, South Korea). The RNX-Plus solution was provided by SinaClon BioScience Co. (Tehran, Iran).

Primary cell culture

BC samples were obtained immediately after surgery and transferred to the laboratory in DMEM-F12 medium containing penicillin/streptomycin antibiotics. After washing twice with PBS, tumor samples were mechanically

dissociated into small fragments using a sterile scalpel blade. For enzymatic digestion, tumor fragments were suspended in collagenase I (2 mg/ml) in DMEM-F12 medium and incubated at 37˚C overnight. After the tissue suspension was filtered through 70 and 40 µm cell strainers, single cells were resuspended in DMEM-F12 medium containing 10% FBS and transferred to 75 cm^2 flasks. Cells were cultured for seven days and the medium was changed every two days.

Sphere forming culture

After seven days of primary cell culture, when 70%-80% confluency was reached, cancer cells were detached using EDTA/Trypsin (0.25%) and centrifuged at 2000 rpm for 5 min. Thereafter, the cell pellet was resuspended and single cells were plated at 1,000 cells/ mL in serum-free DMEM-F12 and supplemented with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 5 µg/mL insulin, and 0.4% bovine serum albumin. The cells were grown in these conditions as non-adherent spherical clusters of cells (mammospheres). The study period was 21 days, and the medium was changed every two days. Every seven days, the supernatant was collected, centrifuged at 2000 rpm for 5 min, and the cell pellets were then transferred to glass slides for immunocytochemical assessments.

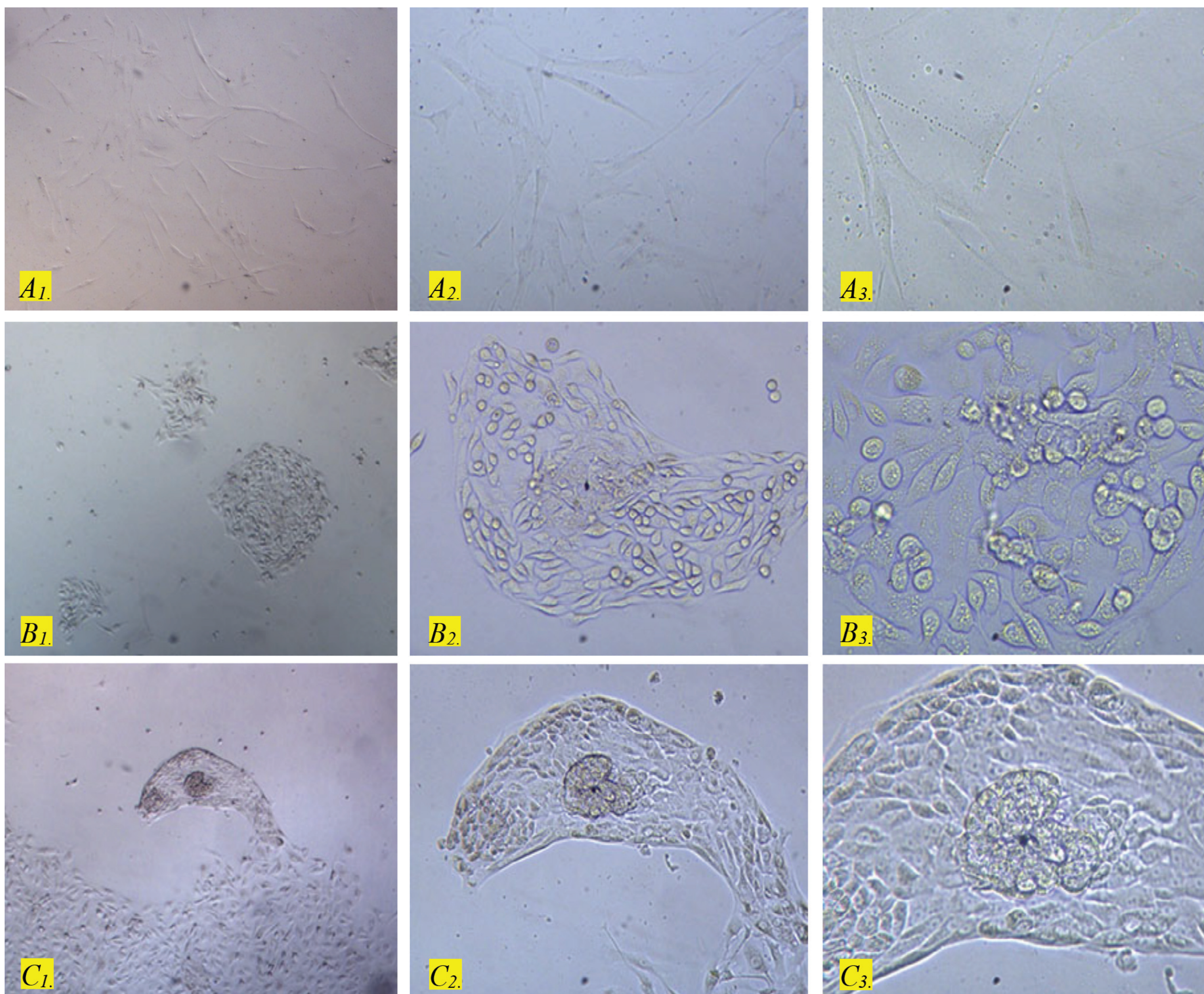

Figure 1. A1-A3: Morphological characteristics of breast cancer cells after seven days of culture with DMEDM- F12, supplemented 10% FBS (Invert microscope), B1-B3 after seven days, and C1-C3 after 14 days of culture in serum-free DMEDM-F12, supplemented with growth factors.

DMEDM- F12: Dulbecco's modified eagle medium/nutrient mixture F-12; FBS: Fetal bovine serum

Table 1. The age of the patients and types of BC according to the pathology diagnosis

Patient code	Age (year)	Type of tumor
BCR1	20	Lobular carcinoma
BCB4	37	Ductal carcinoma
BCT3	39	Metastatic breast cancer
BCB1	44	Ductal carcinoma
BCB3	47	Metastatic breast cancer
BCB2	53	Ductal carcinoma

BC: Breast cancer

Immunocytochemistry

For immunocytochemical assessment, floating mammospheres were harvested from the supernatant on days 7, 14, and 21 and centrifuged at 2000 rpm for 5 minutes; afterward, the cell pellet was resuspended in DMEM-F12 medium and transferred to L-lysin coated glass slides. Next, the slides were dried for 4 hours at room temperature. The cells were fixed with 4% paraformaldehyde for 20 min to perform antigen retrieval, and fixed cells were treated with Tris-EDTA buffer (PH=9, 95˚C) for 30 min. Subsequently, endogenous peroxidase was removed using 3% H_2O_2 for 15 min in the dark, and the cells were then stained with primary antibodies: anti-CD24 and anti-CD44 (1:200, Biolegend); immunodetection was carried out using the Peroxidase/DAB detection system (Dako). Finally, hematoxylin staining, dehydration with ascending graded alcohol, and clearing with xylem were performed, and the slides were mounted for assessment with the light microscope.

Reverse transcriptase polymerase chain reaction (PCR) (RT-PCR)

Total RNA was extracted using the RNX-Plus RNA isolation kit according to the manufacturer's instructions. Gene amplification was applied using a PCR master mix kit (Cinnagen), and cDNA was prepared using a cDNA synthesis kit manual (Takara). The following forward and reverse primers were used for

SOX2, 5′-GGGAAATGGGAGGGGTGCAAAA GAGG-3′
and 5′- TTGCGTGAGTGTGGATGGGATTGGTG -3′;
for OCT4, 5′-ACATCAAAGCTCTGCAGAAAGA ACT-3′
and 5′-CTGAATACCTTCCCAAATAGAACCC-3′.

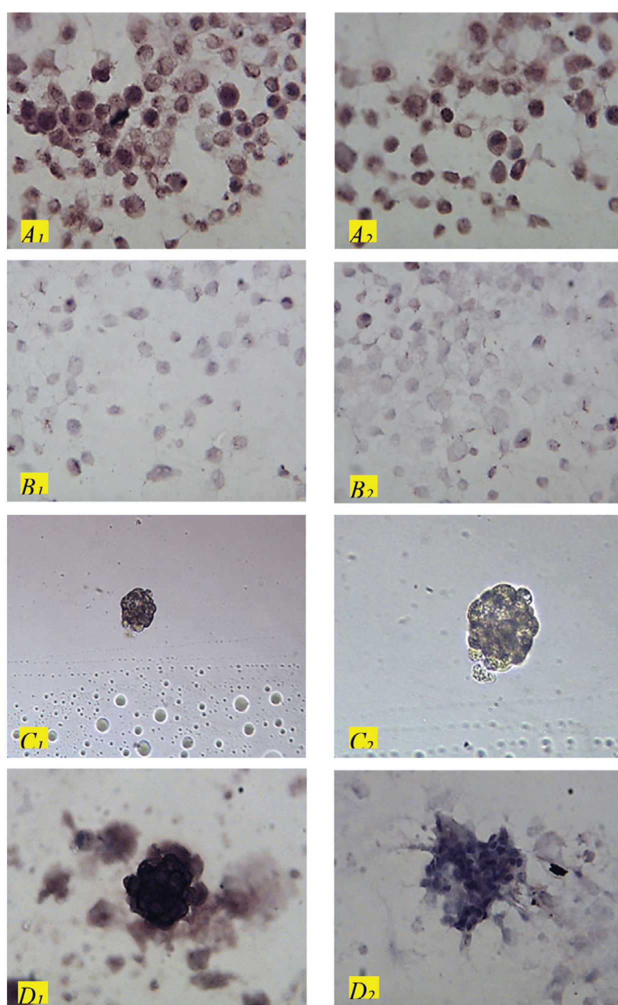

Figure 2. A1-A2: Immunocytochemical analysis of mammosphere-derived single cells for CD44 expression. The majority of cells expressed CD44 (brown color), B1-B2: for CD24 expression. The majority of cells did not express CD24, C1-C2: morphological characteristics of floating mammospheres at 21 days of culture with serum-free DMEDM- F12, supplemented with growth factors. Floating mammospheres are visible from day 8, but they grow in size on day 21 (Invert microscope), D1-D2: Immunocytochemical analysis of mammospheres for CD44 and CD24. Mammospheres strongly expressed CD44 (left) whereas they did not express CD24 (right) (Light microscope).

CD44: Cluster of differentiation 44; CD24: Cluster of differentiation 24; DMEDM-F12: Dulbecco's modified eggle medium/nutrient mixture F-12

Standard PCR reaction was used by Eppendorf Master Cycler Pro system with an initial 95°C, 5 min denaturing step followed by 30 cycles of 95°C (30 s), 56°C and 58°C (30 s), 72°C (30 s) then 5 min at 72°C for the second extension. Finally, the samples were loaded on 1% agarose gel and visualized on a UV detector system (Quantum ST4, USA).

Results

Sample collection and the establishment of primary culture

The pathological diagnoses demonstrated that all samples were in grade III, in which cancer cells were different from normal ones in appearance and they were fast-growing. The BC stage was defined based on the tumor size, lymph node involvement, and metastasis into other body parts. Accordingly, ductal and lobular carcinomas were characterized as stage III, and metastatic BC was specified as stage IV. We first prepared a primary culture of cancer cells obtained from BC to form a monolayer. In monolayer culture conditions, cancer cells were expanded fibroblast-like and attached to the bottom of a flask (Figure 1A1-A3). After seven days, the cells were harvested and cultured with serum-free DMEM/F12 containing complementary factors (EGF, bFGF, Insulin, and BSA) for 21 days. During this period, only a few cancer cells were able to form floating spheres. Furthermore, cancer cells underwent significant morphological changes which were divided into three time periods: first, second, and third.

In the first week, the cells gathered to form cell clusters. Moreover, cell shape was more epithelial-like and cell expansion decreased, but no mammosphere was observed (Figure 1B1-B3). On days 8-14 of the culture period, cell aggregates increased, reflecting the augmented rate of cell proliferation. We further observed the formation of small and attached mammospheres (Figure 1C1-C3). During days 15-21, the proliferation of cells increased significantly and both floating single cells and mammospheres were detected. The number and size of the floating

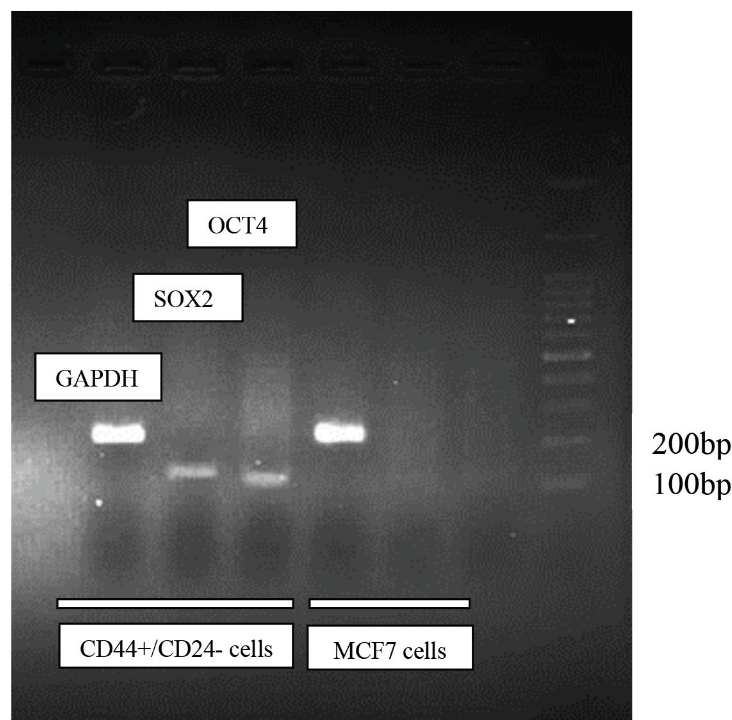

Figure 3. RT-PCR analysis of SOX2 and OCT4 expression for CD44+/ CD24- cancer cells (left) and MCF7 cell line (right). GAPDH was amplified in both cell types while SOX2 and OCT4 were only expressed in CD44+/ CD24- cancer cells.

RT-PCR: Reverse transcriptase polymerase chain reaction; SOX2: SRY (sex determining region Y)-box 2; OCT4: Octamer-binding transcription factor 4; CD44: Cluster of differentiation 44; CD24: Cluster of differentiation 24

mammospheres also increased (figure 2C1-C2).

CD24 and CD44 expression

Analyzing the expression of surface markers CD24 and CD44 using immunocytochemistry of single cells obtained from mammospheres showed that these cells expressed CD44 significantly. The expression of CD44 appeared following the immunodetection reaction. Although CD44 expression was dominantly membranous, the majority of cells expressed both membranous and cytoplasmic patterns (Figure 2A1-A2:), and a few cells displayed either membranous or cytoplasmic expression. CD44 expression was seen as dotty or uniform distribution. In the front face of CD44 expression, the majority of cells did not express CD24 (Figure 2B1-B2). These cells were detected with clear cytoplasms, and there were no signs of immunodetection reaction within cells. Only a small number of cells expressed CD24 both membranous and cytoplasmic patterns. Also, the expression of CD44 and CD24 was evaluated in the mammospheres (Figure 2D1-D2).

SOX2 and OCT4 expression

SOX2 and OCT4 are the two most important transcriptional factors maintaining the pluripotency state of embryonic stem cells. As a control, we compared SOX2 and OCT4 expression in CD44+/CD24- cancer cells with those of cells cultured from the MCF7 cell line. GAPDH expression indicated gene amplification in two-cell types. RT-PCR analysis of SOX2 and OCT4 showed that both genes were expressed in CD44+/CD24- cancer cells (Figure 3), but MCF7 cells did not express these genes.

Discussion

We investigated the presence of CD44+/CD24- cells in primary breast tumors. For this purpose, after preparing single cells from tumor fragments, two culture methods were employed to enrich BCSCs. First, the primary culture of BC cells was prepared to obtain a monolayer of flattened cells. Then, a mammosphere forming culture method was used to enrich CSCs.[18, 19] Under this culture condition, the CSCs were able to survive and form floating mammospheres that exhibited CD44+/CD24- phenotype. Similar to our study, Ponti et al. (2005) reported that a high percentage of cancer cells with self-renewal ability and propagation potential had CD44+/CD24- phenotype.[20] Additionally, Van Phuc et al. (2010) demonstrated that CD44+/CD24- cells were able to propagate in vitro and generate floating mammospheres with tumorigenic potential in vivo.[21] On the contrary, Wang et al. (2014) showed that only one-fifth of mammosphere derived cells were CD44+/ CD24-.[22]

Stem cell theory has been proposed for more than a century, but recent achievements have secured the requested experimental framework for proving this theory.[23] Similarly, what is suggested in the field of CSCs is the application of proper instruments to verify the existence and evaluate the biological characteristics of this population.[24] CSCs are defined as a small fraction of intra-tumor cells with self-renewal capability that could generate a heterogeneous population of tumor cells.[25] Over the recent decade, several experiments have focused on the isolation of BCSCs based on the expression of surface markers CD44+/ CD24-.

The other important aspect of CSC isolation is the expression pattern for surface markers, an issue that is disregarded in similar studies. Based on our results, BCSCs underwent an aberrant expression of surface marker CD44 in the cytoplasm. Honeth et al. (2008) reported that CD44 was expressed in membranous, cytoplasmic, and membranous/cytoplasmic patterns.[26] Although there is no fixed explanation for the difference in CD44 patterns, we believe that the cytoplasmic expression of surface markers may be ascribed to the overexpressed proteins or any disturbance in the membranous distribution of surface marker CD44; some have also suggested that the cytoplasmic expression of CD44 may be attributed to its cytoplasmic domain.[27]

In addition to evaluating the surface markers, we analyzed the SOX2 and OCT4 gene expression of CD44+/ CD24- cells. SOX2 is a transcriptional factor that maintains the pluripotent state of embryonic stem cells. Several studies have shown the relationship between SOX2 expression and

different cancers, such as lung cancer,[28] meningioma,[29] gastric cancer,[30] and prostate cancer.[31] Furthermore, Rodriguez-Pinilla (2007) observed SOX2 expression in 43% of basal-like breast tumors.[32] OCT4 is another transcriptional factor that, as described previously, plays an important role in embryo development and maintenance of the pluripotent state. It has been demonstrated that OCT4 promotes the epithelial-mesenchymal transition of BCSCs and is associated with poor prognosis in patients.[22]

Leis et al. (2012) assessed the expressions of both SOX2 and OCT4. They reported that SOX2 was expressed in breast tumors and mammospheres, and SOX2, alone, was sufficient for mammosphere formation; however, they did not observe OCT4 expression.[33] Similarly, a study conducted by Wang et al (2012) showed a high expression of SOX2 in BCSCs.[34] Using immunoblotting analysis, Ponti et al. (2005) reported the expression of OCT4 in BCSCs,[20] which is in line with Van Phuc et al. (2010) who used the real-time PCR method to evaluate OCT4 gene expression.[21]

Conclusion

In the present study, we verified the presence of BCSCs in Iranian women's breast tumors through the use of an efficient in vitro model to enrich and propagate BCSCs. Under serum-free culture conditions, BCSCs can survive and form floating mammospheres. Afterward, we analyzed CD44+/CD24- stem cell phenotype of single cells obtained from mammospheres along with SOX2 and OCT4 gene expression. Our results showed that in vitro mammosphere culture could be a suitable method for isolating BCSCs, which can pave the way for the identification of CSCs biology. Although the in vitro results related to the identification of BCSCs were satisfying, the present study had some limitations as it did not assess cancer stem cells within the primary tumor and did not confirm the presence of CSC in primary tumors using the immunocytochemistry method.

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Conflict of Interest

None declared.

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