Isolation and characterization of the primary epithelial breast cancer cells and the adjacent normal epithelial cells from Iranian women's breast cancer tumors

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Abstract As an experimental model, most studies rely on established human cancer cell lines; however, some genetical or phenotypical differences exist between these cells and their original tumor. Therefore, primary cells isolated directly from tissue are believed to be more biologically relevant tools for studying human and animal biology. Here, we aimed to isolate primary epithelial cancer and normal cells from breast tumors of Iranian women, for the first time. Thus, we isolated the epithelial and fibroblast cells from biopsy samples of patients with breast cancer based on differential centrifugation followed by culture in selective media. Normal epithelial cells obtained from the tissue biopsy away from the core of the tumor, based on the pathological diagnosis. Flow cytometry analysis indicated the positive

immunoreactivity of the isolated epithelial cells against CD24 and Epithelial Specific Antigen (ESA/EpCAM), while they displayed a concomitant low expression of CD44 and CD49f. In contrast to fibroblasts, the qPCR data indicated the expression of luminal intracellular cytokeratin (Ck18) in both normal and cancer epithelial cells, but there was no expression of myoepithelial/basal markers, CK5 and vimentin. The epithelial cancer cells were reactive to cytokeratin 19 (CK19) antibody, whereas the normal epithelial cells were not. The expression of calmodulin-like protein (*CLP*) was also lower in the cancer epithelial cells than in the normal ones. In conclusion, primary epithelial normal and cancer cells, in addition to the fibroblasts were isolated and characterized from breast tumor of Iranian patients; and *CLP* expression is

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suggested as a susceptibility marker for breast cancer screening.

Keywords Primary breast cell · CD24 · CD44 · EpCAM · Cytokeratin · *CLP*

Introduction

Breast cancer is a major health problem in women worldwide (Coughlin and Ekwueme 2009; Li 2010; Key et al. 2001; Ferlay et al. 2010, 2015), and is the most common diagnosed cancer among women in both developed and developing countries (Coughlin and Ekwueme 2009; Vineis and Wild 2014; Siegel et al. 2016). In Iran, breast cancer is in the first rank of mortality among women's cancers (Movahedi et al. 2012; Taghavi et al. 2012; Jazayeri et al. 2015). The normal human breast gland composed of a branching ductal lobular system is lined by an inner layer of luminal epithelial cells, where an intact basement membrane has separated the outer layer of myoepithelial cells from the interstitial stroma. Various types of breast cancer with different origins are known (Perou et al. 2000; Sorlie et al. 2001; Sørli et al. 2003; Zhao et al. 2004), but it mainly arises from the outgrowth of luminal epithelial cells of the terminal duct lobular units (Taylor-Papadimitriou et al. 1989; Ronnov-Jessen et al. 1996). Thus, human mammary epithelial cells of the lamina lineage are the most important for studies related to breast cancer cell biology (Taylor-Papadimitriou et al. 1989).

The breast cancer cell lines, in vitro, mirror most of the features found in breast tumors. They cluster into basal-like and luminal expression subsets, display heterogeneity, and carry the recurrent genomic and transcriptional abnormalities associated with clinical outcome (Neve et al. 2006; Shipitsin et al. 2007). However, each type of cell line displays increased lineage-restricted profile and enriched for cellular phenotypes that is related to its intratumoral heterogeneity (Keller et al. 2010).

Primary cells are directly isolated from the known and well-identified tumor tissues of human or animals, with naturally occurring tumors or experimental models of induced-cancer. Thus, these cells are physiologically normal and theoretically provide unaltered, “natural” experimental results (Berwald

and Sachs 1963; Bouzakri et al. 2003; Jazayeri et al. 2015). Since, the detailed pathological data of these tumors are available, the characteristics of the culture can simply be compared with those of the original tumor. In addition, because the primary cells retain genetic stability, normal morphology and more complete fundamental cellular functions, they can deliver a biorelevant representation of complex cellular processes, which cannot be achieved using immortalized cell lines. Generally, primary cells retain cellular functions, growth characteristics, cellular markers and signaling pathways more similar to the original tissue when propagated in culture (Berwald and Sachs 1963; Phuchareon et al. 2009; Romano et al. 2009, ZenBio Scientific Publications 2015).

Cells purified from breast tissues are categorized according to the expression of cell surface markers, CD44 and CD24; so that, luminal epithelial cells have been known as CD44⁺/CD24⁺ cells, while basal cells are CD44⁺/CD24[−]. However, a considerable heterogeneity in both CD44 and CD24 expression has been observed in breast tumors (Honeth et al. 2008). Therefore, tumors have also been classified according to expression of either luminal cytokeratins (CK18/19) or basal cytokeratin (CK5), and vimentin (Sorlie et al. 2001).

Human calmodulin-like protein, *CLP*, (synonyms: CALML3, calmodulin-related protein NB-1) is a calcium sensor protein with 148 residues, which is very similar in sequence to calmodulin (Hopper 2001; Ferlay et al. 2015). It belongs to S-100 family (Lee et al. 1992) and displays a unique function (Durussel et al. 1993; Edman et al. 1994). CLP is expressed almost exclusively in normally differentiating epithelial cells in some tissues like breast, thyroid, prostate, kidney, and skin (Johanson 2001) and is involved in various cellular processes (Rogers and Strehler 2001). The depression of CLP (or NB-1) expression in four primary breast epithelial cells has been reported for the first time in 1990. Then, its involvement in the differentiation and/or suppression of tumorigenicity was postulated (Yaswen et al. 1990). It was also shown that the human CLP gene is strongly down-regulated upon malignant transformation (Brooks et al. 2009).

Here we isolated both cancerous and normal primary cells from breast tumors and adjacent normal tissues of Iranian women patients with breast cancer, after surgery and biopsy preparation by the surgeon and diagnosis by a pathologist in the Atieh Hospital, to

investigate and characterize them according to cell surface markers and *CLP* expression.

Materials and method

Materials

Penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glutamine, bovine serum albumin (BSA), cholera toxin, hydrocortisone, insulin, epidermal growth factor (EGF), bovine pituitary extract (BPE), diaminobenzidine (DAB), hematoxylin, trypan blue, ethidium bromide, agarose and xylene were purchased from Sigma, Chem. Co. (St. Louis, MO, USA). Ethanol was obtained from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12), Fetal Bovine Serum (FBS) were purchased from Life Technology (Carlsbad, CA, USA). Horse Serum was bought from the Veterinary Medicine Faculty (University of Tehran, Iran). Estrogen receptor (ER), progesterone receptor (PR), receptor tyrosine-protein kinase (ErbB-2 or Her2/neu), cytokeratin 7 (CK7), Ki67, Antigen KI-67 or MKI67 were purchased from Dako (Hamburg, Germany). The biotin-HRP-labeled anti-rabbit IgG as a secondary antibody was purchased from the Kermanshah University of Medical Science. Anti-CD44, CD24, Epithelial Specific Antigen (ESA) and CD49f were obtained from Abcam (Cambridge, United Kingdom). RNA extraction Kit (RiboX) and cDNA synthesis Kit were bought from GeneAll (Seoul, South Korea). The SYBR Green master mix was purchased from Amplicon (Odense M, Denmark). All other materials and reagents were of analytical grade.

Preparation of cell suspensions from primary tumor specimen

Samples of human breast tumors of patients, in the range 40–51 years old, was obtained from consenting patients with a surgeon, according to the Ethics Boards of the Tarbiat Modares University (Tehran, Iran). Histologically, malignant tissues were obtained from the core of the tumors and non-malignant tissue fragments were obtained from areas away the primary lesion in the same mastectomy specimens as was diagnosed by a pathologist. Total numbers of patients

were five with different pathologic features as shown in Table 1. Breast samples were transferred to the laboratory within 20 min of surgery. The tumor biopsy was washed with phosphate buffer saline (PBS) containing 100 U/ml penicillin, 100 µg/ml streptomycin (Pen/Strep), and was cut up with scissors into small pieces. Mincing was done in sterile DMEM/F-12 1:1 (V:V) and Pen/Strep under sterile conditions in the cell culture room (Speirs et al. 1998). Preparation of single cell suspensions of tumor and normal cells was carried out with two different methods as follows:

Enzymatic method

To obtain single cell suspensions, samples of tumor and normal breast tissues were treated according to the methods described previously (Hammond et al. 1984; Speirs et al. 1998). Briefly, the samples were mixed with collagenase III to a final concentration of 0.2 mg/ml in a serum-free medium, DMEM/F-12 and Pen/Strep and incubated at 37 °C overnight. After that, the enzymatically digested tissue was filtered through a 45-µl nylon mesh and washed with DMEM/F-12, then washed twice with PBS.

Table 1 Pathoclinical details of breast tumors used in this study

Clinical parameter	Numbers
Patients	
Total number in study	5
Age (years)	
Mean	43
Range	40–51
Tumor histology	
Duct origin	5
Tumor receptor	
ER	5
PR	5
Her2	2
Ki67	5
Tumor grade	
II	2
III	3
Lymph node status	
Positive	4
Negative	1

Obtained cells from the last step, were then filtered again through sterile 0.2 μm nylon mesh to omit bacterial contaminations. The cells were then washed in DMEM/F-12 and Pen/Strep, and centrifuged at $1000\times g$, the process of resuspension and centrifugation was repeated three times. Two cell populations were isolated using differential centrifugation. The organoid pellet was collected by centrifuging the cell suspension at $40\times g$ for 1 min followed by one wash with DMEM/F-12. The supernatant from the first centrifugation was saved to serve as a source of mammary epithelial cells. Thus, the supernatants were concentrated by centrifugation at $100\times g$ for 2 min and then the epithelial-cell-rich pellet was resuspended and cultured in standard tissue culture UV-treated conical polystyrene flasks. The pellet of the normal epithelial cells was resuspended in DMEM/F-12 supplemented with 7% horse serum or FBS, Pen/Strep, 2 mM glutamine, 10 mM HEPES, 0.075% BSA, 10 ng/ml cholera toxin, 0.5 $\mu\text{g/ml}$ hydrocortisone, 5 $\mu\text{g/ml}$ insulin, 5 ng/ml EGF, and 70 $\mu\text{g/ml}$ BPE, while the pellet of cancer epithelial cells was resuspended in 5% FBS, insulin, and hydrocortisone (Hammond et al. 1984; Speirs et al. 1998). The supernatant of this step contains fibroblast (or stromal) cells that were cultured in the DMEM containing FBS 10%. Therefore, a combination of differential centrifugation followed by culture in selective medium was performed, encouraging epithelial cancer cell proliferation, while not supporting rapid growth of normal and fibroblast cells over extended periods of time.

Mechanical method or explant cultures

The tissue was cut with scalpels into pieces of about 1 mm thickness in fresh dissection medium, seeded into flasks and cultured for about 2 weeks with daily medium changes in DMEM/F-12, supplemented with horse serum or FBS, Pen/Strep, glutamine, HEPES, BSA, cholera toxin, hydrocortisone, insulin, EGF, and BPE as explained in the previous section. During this period, an epithelial outgrowth formed around most explants. Explants were removed by simply shaking them off and washing the flask, and the culture was continued in DMEM/F-12 supplemented as described above. Primary cultures were passaged after several weeks when the circumference of the islands stopped growing. Cells were passaged as clumps of about five to 10 cells rather than as single cells, because this

appeared to aid survival and maintenance of the epithelial phenotype. In order to produce clumps, trypsinization was shortened and performed at room temperature, the cells were scraped off with a cell scraper. Cells were then routinely cultured at 37 °C and passaged every 3–4 days and maintained for approximately 20 passages. As mentioned previously by Gordon et al. (2000), no change in phenotype was observed with careful handling.

Subculture

For the first 24 h, cells from the organoid and epithelial fractions were plated in 75% organoid medium (OM) and 25% DMEM/F-12 containing 10% horse serum/FBS, respectively. The OM consisted of DMEM supplemented with 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 2 mM glutamine, 10 mM HEPES, 0.075% BSA, 10 ng/ml cholera toxin, 0.5 $\mu\text{g/ml}$ hydrocortisone, 5 $\mu\text{g/ml}$ insulin and 5 ng/ml EGF, pH 7.4. After 24 h, the medium was removed and replaced with OM. Cells were maintained in this situation in the whole experimental study.

For the subculture of monolayer cells, cells were incubated with trypsin–EDTA for 2–5 min. The cells were reseeded at an appropriate split ratio (Speirs et al. 1998). The photographs of the isolated cells was captured by Motic Microscope (Wetzlar, Germany).

Immunohistochemistry

Histological sections were prepared from paraffin-embedded biopsy samples collected from each core site of tumor. The histological sections were located on polylysine-coated plus glass slides. After deparaffinization with xylene and ethanol, the tissue sections were autoclaved at 120 °C for 20 min in a citrate buffer (pH 6) for antigen retrieval. After the addition of normal goat serum as a protein blocker on the blank sites of the tissue sections, the tissues were subjected to double labeling. Briefly, the primary antibody against ER, PR, Her2/neu, CK7 and Ki67 markers diluted with PBS (2:100) was added to the tissue sections and incubated for 1 h. Then, the sample was washed with PBS, and anti-rabbit IgG were added. After 30 min the progress of reaction was detected with DAB, as a chromogen. The sections were washed with PBS; and DAB was oxidized in the presence of horseradish peroxidase and H_2O_2 . Finally, hematoxylin staining

was used as the background staining (Shi et al. 1997; Bartlett et al. 2001; Tan et al. 2005; Lehmann-Che et al. 2011). The photographs of the tissue biopsies were captured by an Olympus Microscop (Tokyo, Japan).

Cell characterization

Flow cytometric analysis was carried out on all primary epithelial cell cultures ($n = 2$, where n represents the passage number). Cells were detached from the culture vessel by treatment with PBS/trypsin–EDTA at 37 °C. Cells were counted and then transferred to a 5 ml tube, washed twice with PBS and stained. For each sample, a total of 10,000 cells was analyzed by flow cytometry (Applied Biosystems, Carlsbad, CA, USA). Gates were set such that the negative control contained only 5% positive cells. Antibodies with appropriate dilution were then added and incubated for 30 min on ice, and then the cells were washed twice with PBS.

The expression of a panel of epithelial cell markers was distinctly evaluated on cells obtained from normal and cancerous pieces. The antibodies used were anti-epithelial-specific antigen (ESA), anti-CD44, anti-CD24 and anti-CD49f directly conjugated with phycoerythrin. After incubation, cells were washed once a time with Hank's balanced salt solution (HBSS) and were resuspended in HBSS supplemented with 5% FBS.

Determination of cell viability

To promote cell attachment, epithelial cells were plated at 2×10^4 cells per well in 24-well plates in 75% OM, 25% DMEM containing 10% horse serum for 24 h, before transferring to OM for the remainder of the experiment. Because of the difference in doubling time, normal cells were counted every day; but, cancer cells were counted every 2 days. The culture medium was changed every 2–3 days (Speirs et al. 1998).

Cell viability was determined by the ability of cells to exclude 0.4% trypan blue (TB) dye. An equal volume of TB solution (0.1% w/v), PBS, and cell slurry were combined and allowed to sit for a few minutes at room temperature. The sample was loaded onto a hemocytometer and the cells scored as living or dead based on the uptake of dye. At least 500 cells per sample were counted (Samsel et al. 2004).

Once the isolated normal and cancer epithelial cells reached 80–90% of confluence, they were counted in a Neubauer camera at 1:2 dilutions with TB dye exclusion. The cells were plated at a density of 2000 cells per well in a 96 well plate to determine the proliferation parameters by the MTT assay, by using dimethyl thiazolyl diphenyl tetrazolium salt (Freshney 1986).

Quantitative real-time PCR (qPCR) of epithelial/mesenchymal markers

The qPCR reaction mixture was prepared as follows: 4 µl of diluted cDNA, 5 pmol of forward primer (1 µl), 5 pmol of reverse primer (1 µl), 10 µl of 2× SYBR Green Master Mix and 8 µl water. Sixteen µl of reaction mixture was filled in the 0.2 ml volume microtube and a 4 µl volume of cDNA was added as PCR template. Real-time PCR was carried out in ABI 7500 Real-time PCR instrument.

The microtubes were closed, centrifuged and placed into a cycling rotor. A four-step experimental run protocol was used: (1) denaturation program or initial step (15 min at 95 °C); (2) amplification and quantification program repeated 40 times (15 s at 95 °C followed by 1 min at 58 °C); (3) melting curve program (66–90 °C under similar condition); (4) cooling program down to 40 °C. The qPCR assay was performed with HPRT as a housekeeping gene using specific primer (Table 2) and considered as internal control, thus the results of this step showed as ΔC_T (Eq. 1), where C_T is a cycle threshold and is defined as the number of qPCR cycles at which the fluorescence signal rises above the threshold value and is inversely proportional to the amount of template present in the reaction. Then, $\Delta\Delta C_T$ was calculated according to the data obtained from the cancerous cells in comparison with the normal cells of the same sample. The fold change of the expression of the gene of interest in cancer epithelial cells (RQ) was then calculated by Eq. (3), according to following formula (Schmittgen and Livak 2008):

$$\Delta C_T = C_{T \text{ Target gene}} - C_{T \text{ HPRT}} \quad (1)$$

$$\Delta\Delta C_T = \Delta C_{T \text{ Cancer Cell}} - \Delta C_{T \text{ Normal Cell}} \quad (2)$$

$$RQ = 2^{-\Delta\Delta C_T} \quad (3)$$

Table 2 The name, DNA sequence, length and melting temperature (T_m) of all the designed and used forward (F) and reverse (R) primers in this study

Name of primer	Primer sequence	Length (bp)	T_m (°C)
HPRT	F: 5'-CCTGGCGTCGTGATTAGTG-3'	125	58.3
	R: 5'-TCAGTCCTGTCCATAATTAGTCC-3'		57.1
Vimentin	F: 5'-CCAGCTAACCAACGACAAA-3'	158	55.76
	R: 5'-GAGACGCATTGTCAACAT-3'		53.05
KIM 18	F: 5'-ATCTTCGCAAATACTGTGG-3'	172	53.06
	R: 5'-GTCGTGTGATATTGGTGT-3'		51.96
KIM 5	F: 5'-GGCGAGGAATGCAGACTCAG-3'	171	60.81
	R: 5'-GTAGCTTCCACTGCTACCTCC-3'		59.86
KIM 19	F: 5'-CCACTACTACACGACCATC-3'	137	54.45
	R: 5'-CCTGTTCCGTCTCAAAC-3'		54.12
CLP	F: 5'AAGGACACGGACAACGAGGA3'	211	60.5
	R: 5'ACACCAGCACACGGACAA3'		56

In addition, an aliquot of the final PCR product (15 μ l) and DNA ladder (SMOBIO DM2300 ladder) was run on 1.5–2% agarose gel and stained with ethidium bromide.

Growth kinetics analysis

To calculate the doubling time (DT), cells were harvested, counted, and replated when they reached 70% confluence. DT was calculated using the following equation (Eq. 4) (Mennan et al. 2013):

$$DT = (t2 - t1) \ln(2) / \ln(n2 - n1) \quad (4)$$

where $n1$ is the cell number at plating, $n2$ is the cell number at harvesting, $t2$ and $t1$ are the number of days in culture.

Results

As Table 1 shows, samples from a total of five patients were analyzed in this study and both epithelial (Fig. 1a, b) and fibroblast cells (Fig. 1c) was separated from the tumor tissues. Normal epithelial cells (Fig. 1b) were isolated from the tissue away from the core of the tumor of the same tissue sample. Cell pellets, enriched in epithelial cells displayed a characteristic cobblestone and polygonal morphology that is completely different from fibroblast cells, with typical spindle-like morphology. The latter form

parallel arrays upon reaching confluence, a feature typical of fibroblasts.

The results indicated no differences between the two methods used for isolation of the epithelial cells, because of using the same cocktail medium in both methods. However, the isolation yield was more in the enzymatic than the mechanical method. Cell density was another substantial factor in the growth of the isolated cells. In the mechanical method, cell density was low and breast cancer cells did not grow well, while in the enzymatic method, cancer cells were seeded at the appropriate density (usually 10^6 cells per 35-mm plate), which led to the significant growth rate. Thus, we used the enzymatic method in all experiments.

Cell growth

In the earlier stage of culture, both the normal and cancerous cells proliferated as adherent patches in the culture flasks. As Fig. 1a, b show, in long-term culture, isolated epithelial cells proliferated in monolayer and reached complete confluence. Figure 2a, b illustrate the DT of epithelial cells derived from different tumors and adjacent normal tissues, as determined by TB. Figure 2c, d show the DT of the mentioned cells as determined by MTT assay. Normal cells after 7–8 days and cancer cells after about 10–12 days began to reach a plateau. Cell viability assay by both methods of TB exclusion and MTT

Fig. 1 Primary cell cultures of human mammary cell. **a** Cancer epithelial cells; **b** normal epithelial cells; and **c** fibroblast cells. These monolayer cells were isolated from the same tumor tissue. Scale bar for all figures: 100 µm

assay ranged from 85% at beginning of the experiment, up to 99% at the end.

Immunophenotypic characterization of breast cancer cell cultures by flow cytometry

Cells isolated from breast tissues were categorized according to the presence of the specific cell surface markers to distinguish the origin of the isolated cells as epithelial or mesenchymal stem cell. Figure 3a–d show the results of the flow cytometric detection of CD24, CD44, EpCAM and CD49f as the surface markers of cells, using antibodies against each marker. The results indicate the presence of CD24 and EpCAM in the surface of all epithelial cells, while these cells lack CD44 and CD49f at the surface. These data confirm the nature of the isolated cells, both cancer and normal, as epithelial cells.

Characterization of epithelial/mesenchymal cell markers by qPCR

To determine and confirm that whether cultured cells expressed the epithelial or mesenchymal phenotype markers, we have also investigated the expression of various cytokeratins (CKs) and vimentin by qPCR in both normal ($n = 5$) and cancer cells ($n = 5$) isolated from the tumors. CK18 was considered as epithelial marker; while, CK5 and vimentin were studied as the myoepithelial/mesenchymal markers. Figure 4a shows the presence of CK18 in the isolated epithelial cancer cells of all patients (p1 to p5). But, Fig. 4b, c indicate that CK5 and vimentin, respectively, were not expressed in the isolated epithelial cancer cells. As a control, the lack of expression of CK18 in the isolated fibroblast cells (F) is shown in Fig. 4a and expression of CK5 and vimentin in these cells is shown in Fig. 4b, c. The pattern of expression of these genes was similar in the normal epithelial cells (data not shown). Table 3 shows the C_T values of these genes in both normal and cancer epithelial cells.

The magnitude of expression of CK19 gene against HPRT, as the housekeeping gene, was calculated according to Eq. (1) and expressed as ΔC_T . Then, $\Delta\Delta C_T$ was calculated as explained in the methods using Eq. (2) and the data obtained for both cancer and normal cells of the same tissue sample. After that, according to Eq. (3), RQ (fold changes in expression) was obtained and used as a measure of alteration in

Fig. 2 Doubling time of the isolated normal and cancer epithelial cells derived from tumors and adjacent normal tissues of five patients. The doubling time for each cell was obtained from the slope of the linear part of each curve. **a** Cancer epithelial cells as determined by trypan blue (TB). **b** Normal epithelial cells as determined by TB. **c** Cancer epithelial cells as determined by MTT assay. **d** Normal epithelial cells as determined by MTT assay. *N* normal, *c* cancerous. (Color figure online)

Fig. 3 The flow cytometric detection of the cell surface markers of the normal and cancerous epithelial cells. **a** CD24; **b** CD44; **c** EpCAM; and **d** CD49f in normal cells. **e** CD24; **f** CD44; **g** EpCAM; and **h** CD49f in cancer cells

Fig. 4 Electrophoresis of the real-time PCR products of CK18 (a), CK5 (b), and vimentin (c) and CLP in the isolated epithelial cells of five patient samples (P1 to P5) and the isolated fibroblasts (F)

gene expression. Table 4 shows the mean $\pm$ standard deviation (SD) of the data obtained for CK19 in five samples. It indicates that CK19 was highly expressed in cancer epithelial cells, while its expression was still lower than that in the normal epithelial cells of the same sample.

Expression of CLP gene

Calmodulin-like protein gene (*CLP*) expression was investigated in the isolated cells by qPCR and is presented in Table 4. It indicates the expression of this tumor suppressor gene was lower in the cancer cells than the normal cells.

Immunohistochemistry of cancer biopsies

The hormone profile of each cancerous tissue was detected by immunohistochemistry of the specific receptors. Figure 5a–e show the immunohistochemical results of the cancer cells that were reactive with the specific antibodies against each receptor. The

Table 3 The C_T values for different genes in the samples. The values are presented as mean $\pm$ SD. The HPRT C_T values are presented in Table 4

Gene	CK18	CK5	VIM
Cancer	17.06 $\pm$ 0.19	33.76 $\pm$ 0.27	33.71 $\pm$ 0.49
Normal	18.68 $\pm$ 0.22	32.13 $\pm$ 0.39	32.48 $\pm$ 0.33

Table 4 The qPCR values calculated according to the Eqs. (1)–(3) for different genes in the samples

Gene	C_T	ΔC_T	$\Delta\Delta C_T$	RQ
C-CK19	26.19 $\pm$ 0.14	4.85 $\pm$ 0.47	−3.77	13.61
N-CK19	30.26 $\pm$ 0.55	8.62 $\pm$ 0.59		
C-CLP	31.36 $\pm$ 0.45	10.02 $\pm$ 0.53	3.01	0.12
N-CLP	28.65 $\pm$ 0.31	8.62 $\pm$ 0.68		
C-HPRT	21.33 $\pm$ 0.22	0.00 $\pm$ 0.36		
N-HPRT	21.63 $\pm$ 0.32	0.00 $\pm$ 0.59		

The values presented are mean $\pm$ SD

N normal, C cancer

results obtained from the five biopsy samples are summarized in Table 1, as the percentage of the presence of each receptor in the cancer cells.

Discussion

In the present study, we have isolated both cancer and normal epithelial cells as well as the fibroblast cells from the tumor biopsies of five Iranian women breast cancer patients. The isolated cells were immunophenotyped for four surface markers and characterized as epithelial or mesenchymal phenotypes.

Primary culture of epithelial cells derived from such tumors has been a goal of many laboratories to increase our understanding of human mammary gland biology (Speirs et al. 1996; Ethier et al. 2000). In contrast to immortalized cell lines that grow in the

Fig. 5 The histopathological detection of surface receptors on cells isolated from sample biopsies. **a** An ER positive cell; **b** a PR positive cell; **c** a Her2 positive cell; **d** a Ki67 positive cell; **e** a CK7 positive cell. Scale bar for all Figures: 20 μ m

medium containing serum, proteins, hormones, lipids, and other necessary factors for growth, to the culture medium of primary cells no serum should be added to avoid the differentiation and growth of contaminating cells. In addition, it needs some especial supplements e.g. growth factors and proliferation factors. Therefore, the high cost of culture medium is a disadvantage for studies using primary cells. However, a major advantage of the primary cells is that, these cells do not display some potential risks inherent when using immortalized cell lines (Shah 2015). Furthermore, in contrast to the variability associated with individuals, which lead to the donor-specific characteristics of primary cells, they can be appropriate as a starting point for targeted therapy research and genomic analysis of a specific disease state (Jazayeri et al. 2015; Shah 2015). In a similar way, cell culture established directly from patient tumors and its adjacent normal tissue are not only important models for examining and manipulating the potentially relevant molecular and cellular processes underlying malignant breast disease, but also are relevant models for studying and developing new strategies of cancer therapy and drug development (Harris et al. 1992; Henderson 1993; Henson and Tarone 1994).

On the basis of our knowledge, this is the first time that such isolation is reported in Iranian patients, which enable us to prepare the cancer and normal cell lines expressing specific cell surface markers of Iranian women and to compare with other cell lines. These cells could be better candidates for study of targeted therapy and personalized medicine in the future.

Untransformed cells in cell culture generally display greater strength of adhesion and more flattened morphology than their transformed counterparts (Martz et al. 1974). This fact that is clearly seen in Fig. 1a, b.

Tumor biopsies were characterized by using immunohistochemistry of hormone receptors (e.g., ER and PR). The results indicated the presence of ER, PR and Ki67 in all cancer biopsies, but Her/neu2 was only detected in 40% of them (Table 1). The isolation of epithelial cell-enriched preparations was done by using a differential centrifugation and a specific cocktail for luminal epithelial cell growth. Then, the epithelial fraction was characterized using the specific surface markers.

Breast tumor samples contain both normal and malignant cells. One of the major stumbling blocks in

this type of work is using a selective growth medium and methods to focus on enriching and developing cancer cell populations with a minimum number of normal epithelial and fibroblast (stromal) cells. In a medium containing many different factors, as is important for the expansion, epithelial breast cancer cells proliferate rapidly, *in vitro* (Speirs et al. 1996; Ethier et al. 2000).

Other factors that we have found to be important include: 1) the cell density should be optimized for breast cancer cell growth; 2) the slow proliferation rate of breast cancer cells when transferred to culture. The second challenge is dependent on the lack of several positive or negative factors affecting the cell growth *in vitro* in comparison with the *in vivo* condition. For example, lack of different hormones and growth factors in the culture medium, which are produced by adjacent normal and fibroblast cells in the *in vivo* condition. There are some other factors that are secreted by normal cells that negatively influence the proliferation of breast cancer cells in breast tumor. The combination of all of these factors causes a difficulty in primary breast cancer cell culture and their long-term growth *in vitro* (Band and Sager 1989; Ethier et al. 2000). To overcome these problems we added the desirable concentrations of cholera toxin, hydrocortisone, insulin and EGF and etc. dissolved in HEPES, to the culture medium.

Mammary epithelial cells could be detected from other cells by CD24 expression; and populations highly expressing CD24 are more precisely distinguished as luminal epithelial cells (Sleeman et al. 2005). CD24 is a mucin-type glycosyl phosphatidylinositol-linked cell surface molecule that has been introduced as a ligand of P-selectin (CD62P) (Kristiansen et al. 2004). Human P-selectin, a Ca^{2+} -dependent endogenous lectin, is expressed in endothelial cells, in addition to platelets (Aigner et al. 1997; Liu et al. 2010). Interaction between the P-selectin of vascular endothelial cells and CD24 on the surface of carcinoma cells might be important for metastatic tumor progression. The process of hematogenous metastasis formation requires the following steps. (1) Separation of tumor cell(s) from the primary tumor tissue; (2) Invasion of the vasculature, (3) Adsorption and adhesion of the cell(s) to target organs by binding of CD24 of tumor cell to P-selectin of vessel endothelial cell. Therefore, CD24-positive tumor cells might possess an increased propensity to this type of

cell adhesion that precedes the growth of a new metastasis (Kristiansen et al. 2004; Lim 2005; Bircan et al. 2006); in addition to that, CD24 has been introduced as a marker of malignancy, with a poor prognosis efficiency (Lim 2005).

CD44 presents a proteoglycan-rich surface protein that is involved in numerous signaling mechanisms and contributes to processes such as cell adhesion, migration and invasion (Lesley et al. 1992). Characterization of a distinct population of highly tumorigenic breast cancer cells revealed CD44 expression (Al-Hajj et al. 2003; Liu et al. 2007). Previous studies have shown that the cells with $\text{CD44}^-/\text{CD24}^+$ phenotypes are luminal epithelial cells, but the cells with $\text{CD44}^+/\text{CD24}^-$ phenotypes are basal cells (Shipitsin et al. 2007); however, a considerable heterogeneity in expression of both CD44 and CD24 was reported in breast tumors (Honeth et al. 2008). Therefore, the prevalence of $\text{CD44}^{\text{low}}\text{CD24}^+$ phenotype has been accepted for luminal epithelial breast cancer cells (Leccia et al. 2012). Our results, using flow cytometry technique indicated the same phenotype of breast cancer cells in Iranian women.

The results presented here indicated the presence of the epithelial cell adhesion molecule, EpCAM/CD326, on the surface of all isolated epithelial cells. EpCAM was originally found as an epithelial cell specific surface antigen in colon carcinoma (Herlyn et al. 1979). This glycoprotein is expressed at basolateral membrane in normal polarized epithelia and mediates the Ca^{2+} -independent hemophilic cell–cell adhesions (Winter et al. 2003; Gosens et al. 2007). However, its overexpression was observed in different solid malignancies including colon, gastric, prostate, ovarian, lung, and breast cancer (Spizzo et al. 2006; Went et al. 2006). In breast cancer, EpCAM has been shown to be hyperglycosylated to a 40–42 kDa isoform (Pauli et al. 2003; Munz et al. 2007) and its higher degree of expression was reported in less differentiated tumors (Schmidt et al. 2008). In addition, both of the isolated cancer and normal cells from breasts highly expressed EpCAM; so that, 98% of the luminal epithelial cells were EpCAM positive and this was introduced as a key marker of this type of cells (Leccia et al. 2012).

CD49f is an integrin molecule ($\alpha 6$ integrin). Human mesenchymal stem cells can express CD49f (Cariati et al. 2008). Our flow cytometry data indicated that this marker was only expressed in 0.2% of the isolated

cancer and normal cells. Thus, the above mentioned data on the characterization of the isolated cells as the epithelial one was confirmed.

Breast cancers are broadly classified histopathologically by the expression of either luminal cytokeratins (CK18/19) or myoepithelial/basal cytokeratins (CK5) and vimentin (Sørli et al. 2001). The categorization in subtypes is critical for the prediction of clinical outcome and treatment response. We used qPCR to evaluate the expression of these markers in all isolated epithelial breast cancer cells. Although the overall levels of marker expression were different, all breast cancer cells analyzed in this study did not express CK5 and vimentin (C_T values > 30). CK18 was generally expressed in both of the isolated normal and cancer epithelial cells (C_T values < 30), but a lower level of CK19 mRNA was observed in the normal than the cancer epithelial cells.

Cell proliferation requires calmodulin, a protein that regulates calmodulin-dependent enzymes involved in signal transduction pathway of eukaryotic cells (Edman et al. 1994). The CLP gene (*CALML3*) is the first member of the human calmodulin-like gene family to be chromosomally sublocalized. It is localized near the telomeric end of the short arm of chromosome 10, which is possibly the reason for its high expression in epithelial cells, including normal breast epithelium (Yaswen et al. 1990; Berchtold et al. 1993; Rogers et al. 1999). Since, the CLP gene is localized in CpG islands, the tissue-specific methylation may regulate its expression pattern (Key et al. 2001). Calcium has been known as a key regulator of cellular differentiation in epithelial cells of the breast and skin (Gamel and Vogel 2001; Rosner and Gore 2001), and CLP is a calcium-sensor protein related in amino acid sequence to calmodulin, but it is not an isoform of this protein (Rhyner et al. 1992; Brooks et al. 2013). It contains four “EF-hand” motifs for binding to Ca^{2+} ; however, its overall Ca^{2+} binding affinity is about ten-fold lower than that of calmodulin (Rhyner et al. 1992). In contrast to the similarity of CLP with calmodulin in sequence, Ca^{2+} binding, and regulating specific target proteins, it has a different role in cell growth and differentiation (Rhyner et al. 1992). The role of CLP as a tumor suppressor has been reported previously (Rogers et al. 1999; Brooks et al. 2009).

An increase in the expression of CLP has been reported during keratinocyte differentiation and it has

been suggested that CLP may play a role in terminal differentiation of oral keratinocytes or may be involved in maintaining the differentiated state (Alberg and Singh 2001). As an affirmative reason, reduction of CLP gene expression has been reported in different oral mucosa tissue types representing the various stages of carcinoma (Brooks et al. 2013). The down-regulation of the human CLP gene upon malignant transformation in breast and prostate tumors derived cells has been shown (Yaswen et al. 1990). In a study comparing normal breast cells obtained from mammoplasty specimens with cancer tumor specimens, high level of *CLP* expression has been found in normal breast epithelium, but significant down-regulation of CPL has been observed in 79–88% of invasive ductal carcinoma and lobular carcinoma specimens (Rogers et al. 1999). It was hypothesized that CLP may help to maintain the differentiation state in epithelial cells; and down-regulation of CLP is accompanied with malignant transformation of this type of cells (Rogers et al. 1999). Our qPCR data on the expression of this gene demonstrated a loss of expression in the isolated cancer cells ($C_T > 30$ and $RQ > 1$) in comparison with the normal one ($C_T < 30$ and $RQ < 1$). This difference in expression is repeated in all of our patient’s samples with different histological profiles. Therefore, down-regulation of *CLP* may directly correlate with turning off the differentiation of normal epithelial cells that lead to tumorigenesis. Alternatively, *CLP* down-regulation in tumors could merely be a secondary effect of a tumor suppressor at an earlier stage of cell de-differentiation or reprogramming back to progenitor cells.

Considering our data on down-regulation of CLP in epithelial breast cancer cells, its expression should be studied in a large population of breast cancer patients, at different stages, and its usefulness as a prognosis factor or an early stage breast cancer marker should be investigated. In addition, the mechanism(s) involved in *CLP* down-regulation in breast cancer epithelial cells should be studies in the near future.

Conclusion

This study reports the isolation of epithelial breast cancer cells from tumor tissue of Iranian women for the first time. The tumor tissues were characterized for specific markers of clinical-pathological features.

Then, we investigated some surface markers of mammary tumor-derived cells characterizing clinical-pathological features of these cells. The long term culture of epithelial cells from tumor and normal biopsies, with polygonal-cell morphology, exhibit EpCAM⁺, as a surface marker. These cells also express CK18 (in both normal and cancer epithelial cells) and CK19 (in normal epithelial cell) as the intracellular markers. These patterns are associated with CD44^{low}/CD24⁺ characteristics. In addition, all of these epithelial cells were negative in respect to expression of CK5 and vimentin genes (by real-time PCR); and CD49f protein (by flow cytometry), indicating that they are epithelial cells and not mesenchymal stem cell. In addition to immune-characterization and cytokeratins expression, morphological evaluation revealed the purity of epithelial cells derived from the primary tumor tissues with no significant contamination of other cell types. Our results confirm down-regulation of *CLP* in breast cancer cells in comparison with the normal ones. Since it has been introduced as a tumor suppressor, its inhibition increases the propensity of cancer cells to proliferate rapidly.

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