Assessment of Cytokeratin-19 Gene Expression in Peripheral Blood of Breast Cancer Patients and Breast Cancer Cell Lines

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ABSTRACT: Detection of cytokeratin-19 (CK19) expression as an epithelial-specific marker in circulating tumor cells (CTCs) of breast cancer patients can be important for diagnostic purposes. Comparison of CK19 expression in breast cancer cell lines can indicate that expression of this marker is different in various breast cancer cell lines based on their category. Thirty-five breast cancer patients were evaluated for detection of CK19 mRNA in their peripheral blood using CK19-specific primers and a nested reverse transcriptase polymerase chain reaction (RT-PCR) technique. CK19 expression levels were detected in MCF7, T47D, SK-BR-3, and MDA-MB-231 cell lines by semiquantitative RT-PCR and Western blot analyses. Statistical analysis of our data indicates that there is no significant difference between CK19 expression and histopathological parameters and some molecular markers, including Ki-67, HER-2, and P53, but there are statistically significant correlations between estrogen receptor ($P = 0.040$) and progesterone receptor ($P = 0.046$) with CK19 expression. CK19 expression was detected in MCF7, T47D, and SK-BR-3 cell lines but not in MDA-MB-231 cell line. More studies are needed to determine the relationship between this marker and other markers in the diagnosis and treatment of breast cancer. On the other hand, the study of different markers using breast cancer cell lines as experimental models of breast cancer could have an impact on improving the health outcomes of patients with breast cancer.

KEYWORDS: breast cancer, cytokeratin-19 (CK19), circulating tumor cells (CTCs), cell line, reverse transcriptase polymerase chain reaction, (RT-PCR), Western blot

Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-related death in women.[1] Circulating tumor cells (CTCs) in the blood can be an important prognostic indicator for breast cancer patients. In both primary and metastatic breast cancer patients, CTCs play an important role in the assessment of treatment efficacy.[2,3] Tumor markers consist of various substances that can be detected in plasma or other body fluids and tissues. A diagnostic tumor marker is one that is used to help in the diagnosis of disease. The use of tumor markers in clinical oncology research can significantly improve our understanding about disease processes.[4] Cytokeratin-19 (CK19) is a specific epithelial cytoskeleton marker that is expressed in high levels in epithelial tumors; specifically, its expression is considerably tissue specific in breast cancer. It can be an appropriate diagnostic marker for detection of tumor cells in the peripheral blood of patients with cancer.[5,6] In several studies, CK19 was used as a marker for the detection of cancer cells in the bone marrow, peripheral blood, and lymph nodes.[6–8] In addition, CK19 marker is considered as an independent prognostic indicator in patients with cancer.[9] Detection of mRNA transcripts for specific epithelial markers using methods based on RNA by reverse-transcriptase polymerase chain reaction (RT-PCR) can result in a high diagnostic sensitivity that can be useful to monitor disease progression.[7]

On the other hand, cell lines have been used as an in vitro model in many aspects of cancer studies. Breast cancer cell lines are effective experimental models for studying the breast epithelial cell biology.[10] Generally, genetic studies on breast cancer are based on cell lines. These cells show expression heterogeneity and genetic disorders similar to primary tumors. In addition, breast cancer cell lines, such as primary tumors, can be classified into basal-like and luminal expression subsets.[11] Since different cell lines express different genes and molecular markers, studying and comparing the marker expressions are very important in these research models of breast cancer.

The aim of this study is to evaluate the expression of CK19 marker in the peripheral blood of breast cancer patients by nested RT-PCR. In this study, the relationship between pathological and biological characteristics of the tumor was also investigated. On the other hand, the expression of CK19 was compared at both RNA and protein levels in various breast cancer cell lines by semiquantitative RT-PCR and Western blot analyses. Furthermore, the biological characteristics of the studied breast cancer cell lines

were evaluated, and the cell lines were classified according to the expression of this marker.

Materials and Methods

Cell culture. Human breast cancer cell lines (MCF7, MDA-MB-231, SK-BR-3, and T47D purchased from National Cell Bank, Pasteur Institute of Iran) were used to study the expression of CK19. The MCF7, MDA-MB-231, and SK-BR-3 cell lines were derived from mammary gland adenocarcinoma, and the T47D cell line was derived from ductal carcinoma. A cervical cancer cell line (HeLa) was used as a negative control. All cell lines were cultured in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Invitrogen) and antibiotics. These cells were maintained at 37°C in 5% CO_2. The cells were grown to 70–80% confluence, and they were washed with phosphate-buffered saline (PBS). Then, the cells were collected after treatment with trypsin and EDTA. The cells were counted with a hemocytometer and pelleted by centrifugation at 2000 rpm (Sigma) for 14 minutes.

Patients and healthy controls. Blood samples from 35 breast cancer patients (age range, 32–74 years) at different stages (I–IV) were collected (Table 1). Thirty-five samples from healthy donors were used as control. Sample collections were done according to the permission from the National Ethical Committee from Pasteur Institute of Iran. Patients had not received any systemic treatment (eg, radiotherapy or chemotherapy). The required information about the patients and the histopathological characteristics of the tumors were recorded from the patients' files with consent forms signed by the patients. This research was conducted in accordance with the principles of the Declaration of Helsinki.

RNA isolation. RNA isolation from blood samples was done with an RNX-Plus kit (SinaClon BioScience Co.). First, 700 µL of RNX-Plus solution was added to 300 µL of blood and then vortexed for 5–10 seconds and incubated at room temperature for 5 minutes. Then, 300 µL of chloroform was added with shaking for 15 seconds, and then, it was incubated on ice for 5 minutes. The mixture was centrifuged at 12,000 rpm at 4°C for 15 minutes. After transferring the aqueous to a new RNase-free 1.5-mL tube and adding 500 µL isopropanol, samples were gently mixed and incubated on ice for 15 minutes, and the mixture was centrifuged at 12,000 rpm at 4°C for 15 minutes. Then, 1 ml ethanol (75%) was added to the pellet and vortexed for 2 minutes and centrifuged at 7500 rpm at 4°C for 8 minutes. After discarding the supernatant and drying the pellet at room temperature for few minutes, the RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water.

Total RNA from cultured cells was isolated using the Fast Pure RNA kit (TaKaRa), according to the manufacturer's protocol. The RNA concentration was measured by absorbance at 260 nm (A260). RNA purity was assessed by the ratio of absorbance at 260 and 280 nm (A260/A280).

Table 1. Patient characteristics.

VARIABLE	NUMBER OF PATIENTS	%
Pathology size		
<1 cm	7	20
1–2 cm	23	65.70
>2 cm	5	14.30
Clinical stage		
I	4	11.43
II	22	62.85
III	6	17.15
IV	3	8.57
Clinical grade		
G1	3	8.57
G2	17	48.57
G3	11	31.43
ND	4	11.43
Lymph node status		
N0	14	40
N1	16	45.71
N2	1	2.86
N3	1	2.86
NX	3	8.57
Histology		
Invasive ductal carcinoma	31	88.58
Invasive lobular carcinoma	2	5.71
Breast cancer	2	5.71
Age		
<50 years	18	51.42
≥50 years	17	48.58
Molecular markers		
ER		
Positive	20	57.14
Negative	8	22.86
ND	7	20
PR		
Positive	18	51.43
Negative	10	28.57
ND	7	20
P53		
Positive	11	31.43
Negative	14	40
ND	10	28.57
HER-2		
Positive	12	34.30
Negative	16	45.70
ND	7	20
Ki-67		
Positive	21	60
Negative	2	5.7
ND	12	34.3
Distant metastasis		
Metastasis	2	5.7
Without metastasis	33	94.3

Reverse transcription. Total RNA from blood samples and cell lines were reverse transcribed using AccuPower® RT/PCR PreMix kit (Bioneer). The reaction was performed in a volume of 20 μL using random hexamer primers, following the protocol provided by the manufacturer. We used 10 μL of the isolated mRNA as a template for cDNA synthesis. The cDNA synthesis was done at 42°C for 60 minutes and at 94°C for 5 minutes. The cDNA was stored at −80°C. Integrity of RNA was confirmed by amplifying the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene using the GAPDH primers[12] shown in Table 2.

Semi-quantitative RT-Polymerase chain reaction. Polymerase chain reaction (PCR) was performed using specific primers for the CKl9 gene that was designed from the previously published sequences.[6] Two pairs of primers were used for the first and second rounds of nested PCR (Table 2). The first round of nested PCR was performed in a 50-μL reaction mixture comprised 10 μL cDNA product, 0.5 units of Taq DNA polymerase, 1 μL dNTP (10 mM), 100 ng each of 5′ and 3′ sequence-specific primers, 1.5 mM $MgCl_2$, 5 μL 10 × PCR buffer, and DEPC-distilled water (DEPC-DW). Thirty-five cycles of PCR amplification for the first round of CK19 amplification using outer primers were used. The PCR conditions were as follows: 94°C for 60 seconds, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes in a DNA thermal cycler. The second round using nested PCR primers with 10 μL of the first round of PCR product was performed in the same conditions as the first round of PCR.

Semiquantitative RT-PCR using another specific primer of the CKl9 gene[13] (Table 2) was performed for the breast cancer cell lines. PCR was performed in a 25-μL reaction mixture composed of 8 μL cDNA product, 0.5 units of Taq DNApolymerase, 1 μL dNTP (10 mM), 50 ng of each of 5′ and 3′ sequence-specific primers, 1.5 mM $MgCl_2$, 2.5 μL 10 × PCR buffer, and DEPC-treated water. PCR conditions were as follows: 40 cycles of 94°C for 15 seconds and 60°C for 1 minute.

Table 2. Sequences for PCR primers.

TARGET GENE	5′-SEQUENCE-3′
First round of Nested-PCR	
CK19 A	5′-AAGCTAACCATGCAGAACCTCAACGACCGC-3′
CK19 A′	5′-TTATTGGCAGGTCAGGAGAAGAGCC-3′
Second round of Nested-PCR	
CK19 B	5′-TCCCGCGACTACAGCCACTACTACACGACC-3′
CK19 B′	5′-CGCGACTTGATGTCCATGAGCCGCTGCTAC-3′
CK19 C	5′-GAAGAACCATGAGGAGGAAATCA-3′
CK19 C′	5′-ACCTCATATTGGCTTCGCATGT-3′
GAPDH F	5′-ACATCGCTCAGACACCATGG-3′
GAPDH R	5′-GTAGTTGAGGTCAATGAAGGG-3′

Table 3. CK19 expression in samples detected by nested RT-PCR.

SAMPLES	NUMBER	CK19 EXPRESSION	
		NUMBER	(%)
Healthy control	35	0	0
Stage I patients	4	0	0
Stage II patients	22	9	40
Stage III patients	6	4	66.6
Stage IV patients	2	1	50
Not defined	1	0	0

The final products were separated in a 2% agarose gel containing ethidium bromide and were visualized under UV light in a gel documentation system. The percentage results of CK19 expression in the cells by nested RT-PCR are shown in Table 3.

Western blot analysis. CK19 protein expression was assessed in the cell lines (MCF7, MDA-MB-231, SK-BR-3, and T47D). Cell protein lysates were separated on SDS-PAGE (12%) and transferred to nitrocellulose membrane. First, the cells were washed twice with 1 × PBS. In order to prepare the samples for loading on SDS-PAGE (12%), the cells were mixed with Laemmli buffer and were heated at 97°C for 20 minutes, and then, they were loaded onto a 12% polyacrylamide gel. After transferring the proteins to nitrocellulose membrane, the membrane was blocked with a blocking buffer (Skim milk, 1 × PBS plus 1%Tween 20) for 24 hours at 4°C. After three times washing the membrane with TPBS (Tween-PBS) solution (1 × PBS plus 1%Tween 20), it was incubated for 2 hours with the Mouse Monoclonal Anti-Human CK19 (1:1000, AbD Serotec) and again washed three times with the TPBS solution, followed by 1 hour incubation with an anti-mouse IgG-peroxidase-conjugated secondary antibody (1:6000, Sigma). Then, the protein bands were visualized with 3, 3′-diaminobenzidine (DAB) staining. Detection of β-actin protein was performed as a control gene.

Statistical analysis. The χ^2 test and Student's t-test were performed to evaluate expressions of CK19 molecular marker in patients as a tumor characteristic. Data analyses were performed using SPSS software version 21, with $P_{value} < 0.05$ being considered as significant.

Results

CK19 gene expression in breast cancer patients by nested RT-PCR. In this study, blood samples were taken from 35 patients at various stages (I–IV) of breast cancer and from 35 healthy blood donors. Nested PCR amplification was detected by a 745bp agarose gel band in 14 patients. The results of the assessment of GAPDH and CK19 gene expression in breast cancer patients are shown in Figure 1.

As shown in Table 3, 14 samples of breast cancer patients (40%) were CK19 positive but it was not expressed in any of the healthy donor samples. We investigate correlations
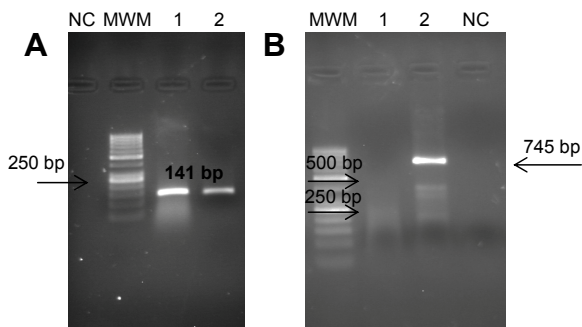
Figure 1. mRNA detection of GAPDH internal control (**A**) and mRNA of CK19 (**B**) in breast cancer patients. Lane 1; patient sample 1, lane 2; patient sample 2, the sizes of the respective PCR products are shown. **Abbreviations:** NC, negative control; MWM, DNA standard molecular weight marker.

between the expression of CK19 and some histopathological parameters. There was no statistically significant correlation with histopathological parameters such as the lymph node status, pathological size, histological type, and histological grade. Furthermore, the correlations between the expression of CK19 and some molecular markers including Ki-67,

P53, HER-2, ER (estrogen receptor) and PR (progesterone receptor) were also investigated. No correlation was observed among Ki-67, HER-2, and P53 expression with this marker. However, statistically significant correlations between ER ($P = 0.050$) and PR ($P = 0.046$) with CK19 were observed during our analysis.

Semi-quantitative RT-PCR analysis of CK19 expression in breast cancer cell lines. We analyzed CK19 expression in the breast cancer cell lines including SK-BR-3 (HER-2-positive cell line), MDA-MB-231 (Normal-like/Claudin-low cell line), MCF7, and T47D (Luminal cell lines). Different expressions of CK19 in three subgroups of breast cancer cell lines were confirmed by semiquantitative RT-PCR using GAPDH as control. CK19 was expressed in MCF7, SK-BR-3, and T47D cell lines (Fig. 2). However, CK19 expression was not detected in MDA-MB-231 cell line by RT-PCR. For semiquantitative RT-PCR evaluation of CK19 expression, the relative expression of CK19 was compared with GAPDH as internal controls. The Lab Works software was used to measure the amount of amplified products. Comparison and relative quantification of CK19 expression was performed by semiquantitative RT-PCR analysis in CK19-positive breast cancer cell lines (MCF7, SK-BR-3, and
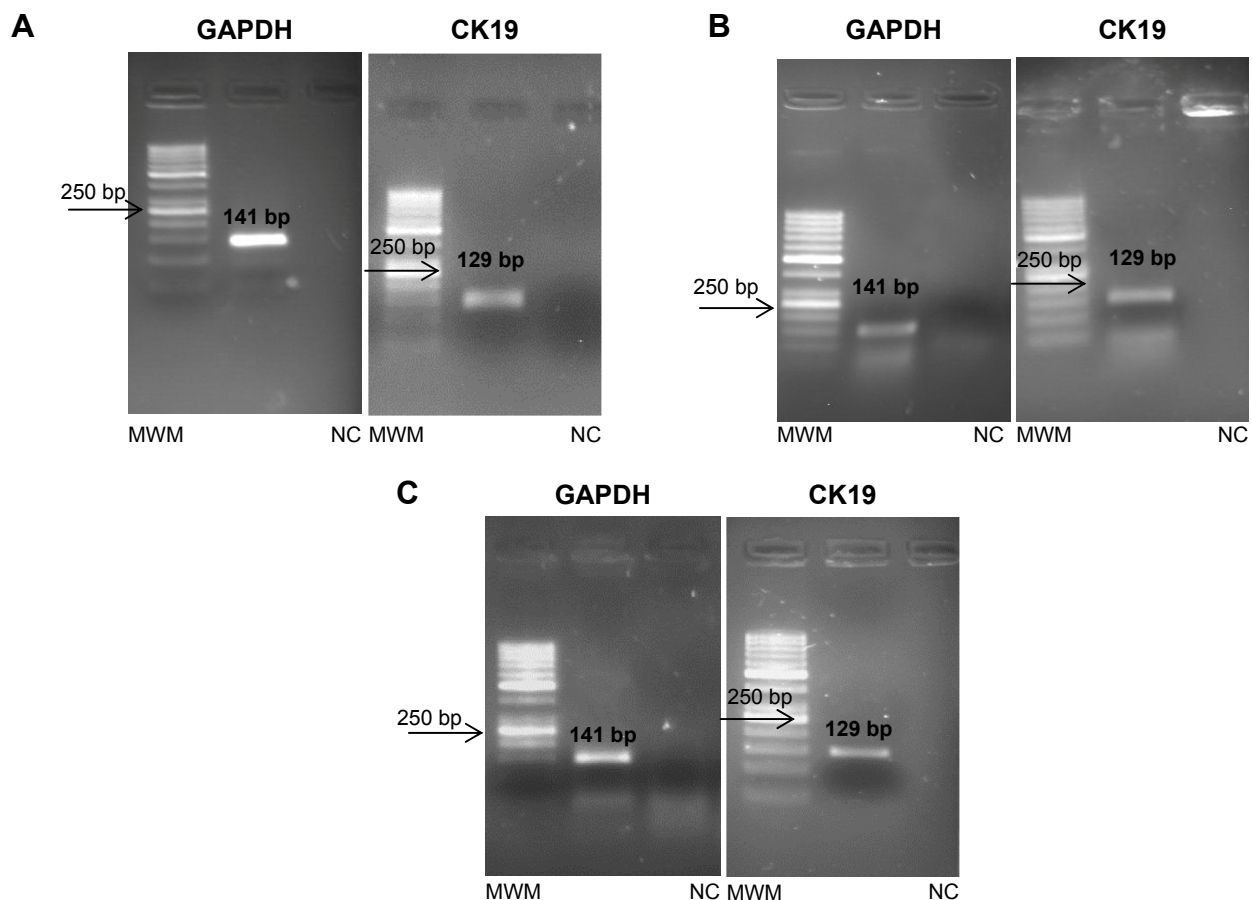

Figure 2. CK19 expression detected by RT-PCR in MCF7 (**A**), T47D (**B**) and SKBR3 (**C**) cell lines, respectively. GAPDH is reported as internal RNA control. The sizes of the respective PCR products are shown.
Abbreviations: MWM, standard DNA molecular weight (50 bp marker); NC, negative control.
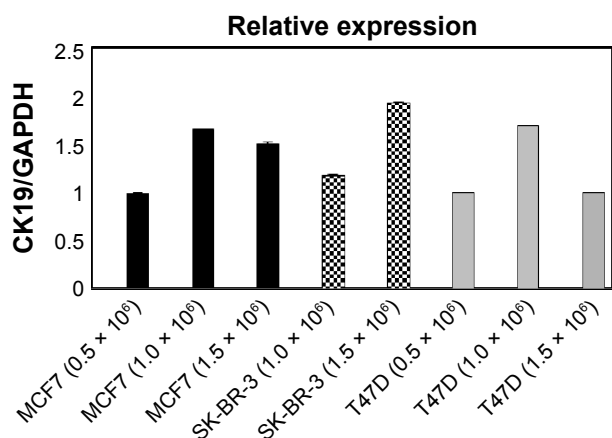
Relative expression

Figure 3. Relative comparison of CK19 expression by semi-quantitative RT-PCR analysis in three breast cancer cell lines with three cell numbers (0.5×10^6, 1×10^6, and 1.5×10^6). GAPDH was used as an internal control for total RNA. Duplicate amplifications of CK19 and GAPDH for each cell line were examined to determine the relative levels of CK19 expression. Values are shown as mean ± SD. The diagram represents the highest expression of CK19 level normalized over cell number. The ratios of CK19 to GAPDH in each cell line are considered.

T47D). Comparison of CK19 relative expression in breast cancer cell lines indicates that CK19 expression in SK-BR-3, MCF7, and T47D cell lines can be observed (Fig. 3). Our results show that there are differences between CK19 expression levels of luminal, claudin-low, and HER-2-positive breast cancer cell lines.

CK19 protein expression in breast cancer cell lines. Expression of CK19 protein was investigated in breast cancer cell lines MCF7, T47D, SK-BR-3, and MDA-MB-231 by Western blot analysis using monoclonal antibody specific for CK19. Horseradish peroxidase-conjugated secondary antibody was detected by 3, 3′-DAB chemical staining. In this
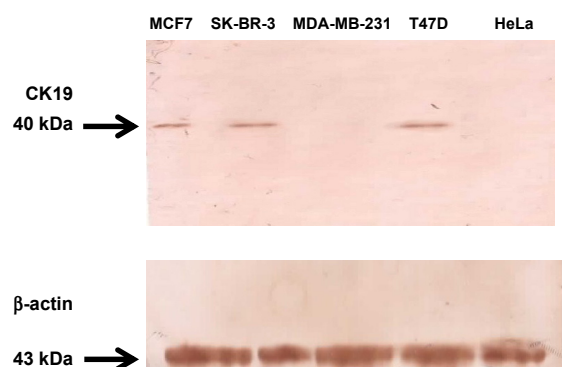

Figure 4. Western blot analysis of CK19 expression in breast cancer cell lines. Mouse Monoclonal Anti Human CK19 as primary antibody and Anti Mouse IgG-Peroxidase conjugated as secondary antibody were used. Three cell lines, MCF7, T47D, SK-BR-3 show CK19-positive band at 40 kDa. MDA-MB-231 does not show CK19 protein expression. β-actin used as internal control. Protein extraction of HeLa cell line was used as a negative control.

study, HeLa cell line (cervical cancer cell line) was used as a negative control. Expression of CK19 protein was detected in three breast cancer cell lines, including MCF7, T47D, and SK-BR-3, but not inMDA-MB-231 cell line (Fig. 4).

Discussion

In this study, we investigated CK19 expression, as a specific marker in breast cancer patients and breast cancer cell lines. In several studies, CK19 as a marker for epithelial cells has been used in the diagnosis of CTC in peripheral blood of patients with breast cancer.[5,14,15] CK19 expression often changed in epithelial cancer, and especially, it has been widely used as a marker for the diagnosis of breast epithelial cells.[16,17] CK19 mRNA indicates the presence of CTCs in some cancers, especially breast cancer.[18]

In this study, RT-PCR-based assays were used including nested RT-PCR for detection of CK19 mRNA in CTC detection of breast cancer patients and semiquantitative RT-PCR for evaluation of CK19 in breast cancer cell lines. In several studies, RT-PCR-based assays have been performed for CK19 detection as an epithelial marker. The results of studies showed that the major advantage of RT-PCR methods over other methods is its high sensitivity.[19–21]

We detected CK19-mRNA in 40% of breast cancer patients. CK19 expression was positive in the blood cells of breast cancer patients at various stages, II–IV, but not in the patients at stage I and healthy controls. Stathopoulou et al[22] showed that the detection of CK19-positive cells in the peripheral blood of patients with operable breast cancer (stages I and II) before the initiation of any adjuvant treatment is an independent predictive and prognostic factor. In our study, the expression of CK19 was mainly shown in breast cancer patients at stages II and III. It is proposed that CK19 expression increases with increasing stage of the disease.[22]

In this study, statistical analysis was performed using SPSS between CK19 expression and some molecular markers (ER and PR). The association of CK19 gene expression with ER and with PR was statistically significant with $P_{\text{value}} = 0.040$ and $P_{\text{value}} = 0.046$, respectively.

On the other hand, in this study, CK19 expression was evaluated in some breast cancer cell lines in two levels of RNA and protein. Human breast cancer cell lines have been used as a rich source for evaluation of molecular and cellular characterization of cancer pathogenesis. Due to the differences in breast cancer cell lines, specific features such as rate of proliferation, drug resistance, and invasiveness can be useful in comparing of the genes and proteins that contribute to the pathogenic phenotypes.[23]

Several studies on the analysis of gene expression and molecular markers have shown that the breast cancer cell lines have maintained the molecular characteristics that are usually found in clinical breast tumors. Neve et al[11] showed that these cell lines reflect most of the important genomic and transcriptional disorders in primary tumors of the breast. Assessment

of the functions of these genes in cell lines reflects how they are involved in the pathophysiology of the breast cancer.[11,24]

Molecular analysis using DNA microarrays showed that according to the investigation of ER, PR, and HER2 gene expression, breast cancer can be classified into five subtypes: luminal A, luminal B, HER2, basal-like, and normal. This classification of breast tumors can be used as a prognostic marker considering that each subtype has a different molecular characteristics and response to therapy.[25,26]

In our study, CK19 expression was detected in MCF7 and T47D cell lines. MCF7 and T47D cell lines belong to the luminal subgroup of breast cancer, luminal subtypes are ER positive. On the other hand, it has been shown that CK19 gene is upregulated by estrogen. According to Choi et al[26] in their study on CK19 gene in MCF7 cells, some of the estrogen response elements are located in the gene and the ER-complex estrogen connection with these elements involves the activation and overexpression of CK19.[24,26] SK-BR-3 cell lines have also been shown to express CK19 marker. This cell line belongs to HER2 subgroup of breast cancer. The study performed by Zhang et al[16] using semiquantitative RT-PCR analysis, Western blotting, and reverse-phase protein arrays indicated that CK19 was expressed in HER-2/*neu*-positive breast tumors.[16]

In this study, the expression of CK19 was not detected in MDA-MB-231 cell line. In several studies, this cell line belongs to the Normal like/Claudin low subgroup of breast cancer.[27–29] Claudin low subgroup has been known as triple-negative (ER, PR, and HER2 negative) breast cancers. This subgroup showed low expression of claudin 3, 4, and 7 that contribute in epithelial cell tight junctions. The MDA-MB-231 cell line expresses high levels of vimentin as a marker of mesenchymal phenotype. Inhibition of the expression of CK19 and overexpression of vimentin have been shown in breast cancer cell lines that are capable of invasion including MDA-MB-231.[1,29,30]

Our results indicate that the expression of CK19 is different in various breast cancer cell lines. It can be concluded that among breast cancer cell lines, there are variations in the expression of many genes and proteins based on their classification. Since, breast cancer cell lines reflect many features of breast tumors, analysis of the expression of specific markers on the cell lines can be used to show a relationship between the expression of these markers in breast cancer cells and in cancer treatment procedures. Moreover, the study of different markers using breast cancer cell lines as experimental models of breast cancer can have the impact on improving health outcomes for patients with breast cancer.

Abbreviations

CK19, Cytokeratin-19; CTC, circulating tumor cells; RT-PCR, reverse-transcriptase polymerase chain reaction; DEPC, diethyl pyrocarbonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; DEPC-DW, DEPC-distilled water; TPBS, Tween-PBS; DAB, 3, 3′-diaminobenzidine; ER, estrogen receptor; PR, progesterone receptor.

Author Contributions

The authors declare to have participated in the drafting of this paper as specified below: SK, NK, SB, and MO contributed to writing the protocol and the article; SK, NK, and ZO: Performed the cell culture and RT-PCR experiments. All authors reviewed and approved of the final manuscript.

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