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Diagnostic of Cytokeratin-19 Gene Expression in Iranian Breast Cancer Patients

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ABSTRACT

Sentinel lymph node (SLN) biopsy is currently the recommended procedure for axillary staging in clinically node-negative early breast cancer at diagnosis. The present study aimed to identify Cytokeratin-19 (CK19) gene profiles that accurately predicted the outcome of breast cancer patients. Fifty tumor samples from breast cancer patients were analyzed for the expression of the CK19 gene using quantitative PCR. Also, normal breast tissues (N = 50) were taken from the same patients that had undergone partial or total mastectomy. This gene signature was confirmed based on tumor's stage, grade, and estrogen receptor (ER) status, using conditional logistic regression. Based on these findings, the negative reported lymph nodes for metastasis had micrometastasis in significant values. There was a significant difference between normal and cancer samples in CK19 expression. In this sentinel node evaluation, the relationship of this gene with tumor characteristics needs to be established and discussed finding a clear role for this gene in tumor outcome.

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Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-related mortality in women (1). Circulating tumor cells (CTCs) in the blood can be an important prognostic indicator for breast cancer patients (2). In both primary and metastatic breast cancer patients, tumor markers consist of various molecules that can be detected in plasma or other body fluids and tissues (3). A diagnostic tumor marker can be used to help in the diagnosis of a disease. The use of tumor markers in clinical oncology research can significantly improve our understanding of disease processes (4). Cytokeratin-19 (CK19) is a specific marker of the epithelial cell cytoskeleton expressed in high levels in epithelial tumors; specifically, its expression is highly tissue-specific in breast cancer. It can be a suitable diagnostic marker for the 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 (7,8).

CK19, one of the three main keratins besides CK8 and CK18 expressed in the simple and stratified epithelium and various carcinomas including breast cancer (9), is cleaved by caspase 3, and the soluble fragments are released and detected in cancer patients (10). It can therefore be speculated that CK19 not only is a marker for epithelial tumor cells but also may have some biologically relevant functions in early metastatic spread. In view of the hypothesis on the role of cancer stem cells in metastatic spread (6), CK19 has been notably suggested as a potential breast stem/progenitor cell marker (11-14). Thus, it can be speculated that CK19-positive tumor cells might be an important subset of breast cancer cells.

In addition, the CK19 marker is considered an independent prognostic indicator in patients with cancer (15). 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 (16).

Breast cancer cell lines are effective experimental models for studying breast epithelial cell biology (17). In general, genetic studies on breast cancer are based on cell lines. These cells show expression heterogeneity and genetic disorders like primary tumors. In addition, breast

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cancer cell lines, such as primary tumors, can be classified into basal-like and luminal expression subsets (18). Since different cell lines express distinct genes and molecular markers, studying and comparing the marker expression is very important in these research models of breast cancer.

This study aimed to evaluate the expression of the CK19 marker in the peripheral blood of breast cancer patients by nested RT-PCR. In this study, the relationship between pathological and biological traits of the tumor has also been investigated. Furthermore, the expression of CK19 has been compared at both RNA and protein levels in various breast cancer cell lines by semiquantitative RT-PCR and Western blot analyses. The biological characteristics of the studied breast cancer cell lines were evaluated, and the cell lines have been classified according to the expression of this marker. Finally, the functional role of the CK19 gene profile and its underlying mechanism have been explored in the sentinel lymph node.

Materials and Methods

General information

Breast cancer specimens from 50 women (mean age of 40.2 ± 65.55 years) who had undergone surgery at the Tehran University-affiliated hospital and Shohadaye Tajrish-based referral and teaching hospital affiliated to Shahid Beheshti University of Medical Sciences were collected. Also, normal breast tissues (N = 50) were taken from the same patients that had undergone partial or total mastectomy. Data for all patients were collected for analysis. All of them were diagnosed with breast cancer without metastasis and tested by IHC staining.

Patients were assigned on the basis of national/international breast cancer protocols and the study was approved, according to local law and regulations, by the Institutional Review Boards of each participating referral hospital. Written informed consent was requested from patients and a questionnaire have been administered.

Total RNA Isolation and cDNA Synthesis

Genomic DNA and total RNA from each sample were extracted using a QIAamp DNA mini Kit (Qiagen, Germantown, MD) and an RNeasy mini kit (Qiagen, Germantown, MD) used according to the manufacturer's instructions. The extracted genomic DNA and total RNA were quantified and confirmed for OD 260/280 values between 1.8 and 2.2 and OD 260/230 values greater than 1.

Whole cell RNA isolation from sentinel lymph nodes of breast cancer patients was performed using 1 mL of reagent added to 50 µg of SLN specimen and homogenized. After incubation from 5 min at room temperature, 0.2 mL of chloroform was added and the mixture was homogenized and centrifuged at 12000rpm for 15 min at 4 ℃. RNA was precipitated, retained and added with isopropyl alcohol. The upper aqueous phase was removed, and centrifuged at 12000 rpm for 10 min at 4℃; the further pellet was rinsed twice with 1 mL of ethanol (75%). The RNA was re-suspended in DEPS water to a concentration of 0.5µg/µL.

Real-time quantitative polymerase chain reaction

The amount of 0.5µg of oligo DT and 16µL RNase free water was added to 5µg of the whole RNA and incubated for 10 min. RNA extraction was performed using the Qiagen RNeasy Plus Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Total RNA concentration was measured spectrophotometrically and equal concentrations were added to each well of a 96-well PCR plate. RT-qPCR was performed using the BioRad iTaq reagent in a Bio-Rad CFX96 (Bio-Rad, Hercules, CA). The primers for SYBR Green real-time PCR were designed specifically for each checkpoint gene and for the *ACTB* gene (*β-actin*) as an internal control. The assays were repeated in their entirety for each measurement. Reverse transcription was carried out with the Super Script First-Strand Synthesis System for RT-PCR. The following procedure was based on the manufacturer's instructions using total RNA (5 µg), random hexamers (3 µl, 50 ng/µl), 10 mM dNTP mix (1 µl) and DEPC H_2O (10 µl). Samples were incubated at 65°C for 5 min and then put on ice for at least 1 min. The reaction mixture consisted of 10x RT buffer (2µl), 25 mM $MgCl_2$ (4 µl), 0.1 M DTT (2 µl) and RNAase (1 µl). The reaction mixture was added to the RNA/primer mixture, mixed briefly, and then placed at room temperature for 2 min. Then 1 L (50 units) of SuperScript II RT was added to each tube, mixed and incubated at 25°C for 10 min. Tubes were further incubated at 42°C for 50 min, heat-inactivated at 70°C for 15 min, and chilled on ice. RNase H (1 µL) was added and incubated at 37°C for 20 min. The reaction stored the 1st strand cDNA at -20°C until use for real-time PCR.

CK19 (Forward): CACCAGCCGGACTGAAGAAT,
CK19 (Reverse): GCAGGTCAGTAACCTCGGAC,
ACTB (Forward): AGAGAAGTGGGGTGGCTTTT,
ACTB (Reverse): GCCGAGGACTTTGATTGCAC.
Product length: 86 bp

Data analysis was performed with the manufacturer-supplied software.

Statistical analysis

A comparison of the results between the treated group and the corresponding control was carried out by SPSS. 20 software with t-test and Pearson chi-square. All comparisons were considered significant at $p< 0.05$.

Results

The expression level of CK19 from tumor tissues increased significantly (P=0.21) compared to controls. Also, the expression level of metastatic lymph nodes increased significantly.

Sentinel lymph node samples were removed after surgery from the breast of women with breast cancer. The average age of patients was 51.1 years. The results of pathology tests in the samples were negative, which means that the sentinel lymph node was free of micrometastases. The second group of samples was obtained from healthy

Table 1. Extracted RNA qualification based on case and control group.

Sample	206 nm	280/260	µg/mL
Tumor	0.801	1.418	16.2
Tumor	1.193	1.453	2386
Tumor	1.143	1.449	2286
Margine	0.868	1.479	1736
Margine	1.241	1.472	2482
Margine	1.667	1.591	3334

women without the disease, and the third group of samples contained metastatic tissues. The *ACTB* gene was the normalizing gene and its expression was examined in all groups along with test genes (Table 1).

In the current study, there was a significant relationship between the expression of *CK19* and grade of tumor (p=0.000) and stage (p=0.016), but not with ER status (p=0.186). As previously illustrated, two groups of samples were obtained: 50 tumor micrometastases and 50 adjacent normal breast cancer tissues. Also, 20 macrometastatic breast cancers as positive control and 50 samples from normal mastectomy breast tissues with no evidence of malignancy were included in this study. The β-actin gene was used as an internal control for RT-PCR performance. With regard to tumor pathological features, the stage of tumor was determined according to AJCC-02-TNM international criteria for classifying, where the samples of the present study were in stages 1 and 2, whereas for the grade of tumor, they were grouped as I, II and III according to pathological standards. The ER status was reported as positive and negative. Moreover, the extent of expression of each gene was evaluated with reduction or increase compared to β-actin expression level. In the evaluation of ER gene expression, about 56% (27 samples) were ER-negative and 44% (23 samples) (Table 2,3).

The RT-PCR reaction was performed on all cancer and tumor samples as described earlier and the whole data were analysed statistically.

Discussion

The present investigation is a continuation of our previous studies (19-21). Breast cancer is the most common malignancy in women, accounting for 627,000 deaths worldwide in 2018 (22). Lymph node involvement is one of the most important prognostic factors in breast cancer (23). The determination of CK19 mRNA copy number can predict the presence of micro- or macro-metastases in the SNL (24). The data provided in different studies resulted in a ranking of tumors according to their CK19 expression across a large variety of tumor types including breast cancer (25,26).

Sentinel lymph node (SLN) biopsy is currently the standard approach for clinically node-negative breast cancers. Axillary lymph node dissection is reserved for patients with ≥3 positive lymph nodes on SLN biopsy (27,28). Women without SLN metastases should not receive axillary lymph node dissection. In addition, axillary lymph node dissection should be avoided in patients with 1–2 positive SLNs when whole-breast irradiation (WBI) therapy is planned (27,28). Randomized trials have shown that SLN biopsy is not inferior to axillary lymph node dissection in patients with 1–2 positive SLNs. However, the radiation therapy volumes in these trials varied from standard whole-breast irradiation (WBI) to high-tangential WBI and WBI plus regional nodal irradiation. The axillary nodal burden is one of the important indicators of breast cancer. It is well established that patients with positive nodes benefit from regional nodal irradiation after axillary dissection.

In the present investigation, analysis expression of tumor tissue, as well as marginal and metastatic specimens, has been done. A major strength of our model is that it is based on pathological features available in common clinical practice. The hypothesis presented in the article was supported by the experimental results of this investigation.

The expression level of CK19 from tumor tissue compared to control was significant. Also, the expression level of metastatic lymph nodes showed a significant increase in expression.

CK19, a characteristic intermediate filament of epithelial cells and their malignant counterparts, is one of the most frequently studied markers for micro-metastasis. CK19 mRNA expression in peripheral blood has been associated with poor patient outcomes (29,30), and CK19 has been detected in the bone marrow and tumor cells from breast cancer patients via immunoassay (31) OSNA-CK19 has proven useful to detect sentinel lymph node involvement in breast cancer patients (32-34). CK19 is a marker expressed by several solid tumors of epithelial origin, but not by healthy lymphatic tissue, moreover, cytokeratin 19 (CK19) is a fast, objective, automated, and reproducible way, raising a general interest, to explore its utility for lymphatic metastasis identification in different malignancies (35). Our study developed a nomogram to predict non-sentinel lymph node metastasis for breast cancer patients with a positive axillary sentinel lymph node. Nomogram that uses 6 risk factors is commonly available to accurately estimate the likelihood of non-sentinel lymph node (nSLN) metastasis for the individual patient, which might be helpful for radiation oncologists to make a decision on regional nodal irradiation to identify the risk factors of nSLN metastasis in breast cancer patients with 1~2 positive axillary sentinel lymph node and construct an accurate prediction model (36). In relation to other cancers, it is necessary to study and research in this regard (37-41).

Conclusion

While additional validation studies are needed, the present investigation showed that CK19 can be detected

Table 2. Sample design based on case and control group.

Case Group	Control Group	Metastasis Group
50 tissues	50 tissues	30 tissues
Sentinel Lymph Node	Normal Tumor Margin	Sentinel Lymph Node
No metastasis	No other cancers	Metastatic

Table 3. Comparison of sentinel lymph node and normal breast tissue for CK19 gene expression.

Target gene	Reaction efficiency	Expression	Std. error	95% C.I.	P(HI)	Result
CK19 tumor	0.98	3.81	3.38±0.0467	1.814 to 2.286	0.00179	UP
CK19 marginal zone	0.98	1.46	1.33±0.1023			
CK19 lymph node	0.97	6.33	3.88±0.0577			UP

in peripheral blood samples of breast cancer patients, and can predict SLN status before surgery. Further, the CK19 copy number was strongly correlated with the number of metastasis-positive LNs. The inclusion of this tumor marker within already-existing predictive models, which are currently primarily based on clinicopathologic data, would enhance the predictive accuracy of these models in determining LN status in breast cancer patients even before surgery.

Author contributions

AHP and NM wrote the original manuscript; AAK and HHM analysed the data, designed tables and scientific illustrations; MRT and ZM checked the associated database and raw data; MM edited the manuscript; AM and MI supervised and revised the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

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