Detection of CK19 mRNA in the blood of breast cancer Female Egyptian patients and its relation to established prognostic parameters

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Abstract: Purpose Breast cancer is a leading cause of cancer-related deaths in women Worldwide. The clinical course of this disease is highly variable and clinicians continuously search for prognostic parameters that can accurately predict prognosis. peripheral blood cytokeratin-19 (CK-19) mRNA-positive cells and its correlation with well established prognostic factors including pathologic parameters, hormonal status and biologic marker; HER 2/ neu in breast female Egyptian cancer patients was studied . **Patients and Methods** A total of 60 peripheral blood specimens were collected for study. Patients were forty newly diagnosed breast cancer and 10 patients with benign breast lesions .The10 apparently healthy donors and patients with benign lesions were used as the control group. They were analyzed for the presence of CK-19 mRNA-positive cells using nested reverse transcription polymerase chain reaction assay (RT-PCR). Immunohistochemical staining for HER 2/neu, estrogen and progesterone receptors were carried out for all cases. The association with known prognostic factors and the effect of CK-19 mRNA-positive cells on patients' prognosis was investigated. **Results** CK-19 mRNA-positive cells were detected in the blood of 14 patients (35%) of the 40 patients. There was statistically significant association between the presence of CK19 mRNA-positive cells and the patients' tumor size and histologic grade of the tumor, stage of disease and the involved lymph nodes P =<0.001, 0.006 , 0.007and0.005 respectively. CK-19 mRNA-positive cell detection also showed high significance with HER2 expression receptor (p=0.001). None of the patients with CK 19 positive cells expressed dual ER & PR positivity being both negative and either negative. There was no statistically significantly association between the detection of CK19 mRNA-positive cells and patient's age, menstrual status or pathologic type. **Conclusion:** Peripheral-blood CK-19 mRNA-positive cells might constitute a biologically active subset of breast cancer patients with high tumor burden and bad prognosis.

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1. Introduction

Breast cancer ranks first among cancers affecting woman throughout the world and its marked impact is not restricted to Western industrialized societies **[1]**. Carcinoma of the breast is the most prevalent type of cancer among Egyptian women and constitutes 29% of National cancer Institute cases. Median age at presentation is one decade younger than countries of Europe and North America and most patients are premenopausal. Tumors are relatively advanced at presentation **[2].**

Biological aggressiveness of breast cancer which is encountered in Egypt is more than in the West and is explained partly by the predominant premenopausal patients and partly by the late presentation of patients at an advanced stage **[3, 4].**

El-*Bolkainy et al.,* [5] reported clinical (T) categories in NCI series to be as follows: T1 (1.2%), T2 (30%) and T3 (26.4%), due to lack of screening programs and the lack of public awareness of the importance of early detection of breast cancer among Egyptian population, so most of Egyptian patients lack investigations for early detection of breast carcinoma.

Breast cancer is considered a systemic disease because early dissemination may occur even in patients with small tumors [**6].** Micro-metastases, which are undetectable by the classic images and laboratory studies, can contribute to disease relapse [**7].** Therefore, their identification in patients with early breast cancer may have substantial effect on determining prognosis and individualizing treatment for those patients [**8**].

Since cancer cells are very heterogeneous, different cancers express different markers and even cells from the same tumor may not be identical so different assays have been developed for detection of tumor cells in the peripheral blood of patients with various malignancies [**9].** . Reverse transcription polymerase chain reaction (RT-PCR) amplification technique can identify cell-specific mRNA and detect up to one tumor cell in 10^7 normal peripheral blood or bone marrow mononuclear cells [**10],** which is at

least 10 times more sensitive than immunohistochemistry **[11].**

Cytokeratin 19(CK 19) is one of the main keratins expressed in simple or stratified epithelium and considered a general marker of epithelial cancers including breast cancer. It is cleaved by Caspase 3 and the soluble fragments are released and detected in cancer patients .It is one of the markers used for RT-PCR detection of circulating tumor cells (CTCs) in breast cancer patients and it is correlated to an unfavorable prognosis [**12, 13]** and seems to be the most sensitive and reliable tumor marker in both patients with operable and metastatic breast cancer **[14, 15].**

In the present study, we aimed at studying the prognostic significance of CK 19m RNA in peripheral blood of newly diagnosed, non metastasizing Egyptian females cases of breast carcinoma with relation to CK19 and the other well established prognostic factors including pathologic parameters, hormonal status and the biologic marker HER /2 neu.

2. Patients and Methods

A total of 60 peripheral blood specimens were collected for study. Patients were forty newly diagnosed breast cancer cases and 10 patients with benign breast lesions who presented to the outpatient clinic at the National Cancer Institute, Cairo University during the time period from December 2009 till June 2010. Ten apparently healthy donors were also included. The donors and patients with benign lesions were used as the control group. Our few cases can be justified by the time limit and by our conservative culture; our female patients are not willing to contribute with samples to research or acknowledge the presence of problems in their breast

Written informed consent was obtained before enrollment into the study.

Peripheral blood samples of (8ml in EDTA) were collected. All blood samples were obtained at the middle of vein puncture after the first 5 mL of blood were discarded. This precaution was undertaken to avoid contamination of the blood sample with epithelial cells from the skin during sample collection.

All cancer patients were subjected to chest x-ray, liver and bone scan to exclude metastasis.

RNA Extraction:

Peripheral blood mononuclear cells (PBMCs) were obtained by gradient density centrifugation using Ficoll-Hypaque 1077 (Sigma) at 1,200 g for 30 minutes at 4°C. The interface cells were removed, washed twice with 50 mL of sterile PBS (pH 7.3), pelleted, and resuspended in 1 mL of PBS. The cells were pelleted again at 1,200 g for 2 minutes. Cell pellets were kept at -80°C till RNA extraction.

Total RNA isolation was performed using RNA QIAamp RNA Blood Mini Kit Catalog no. 5230 according to the manufacturer's instructions. All preparations and handling steps were done under RNAse-free conditions. RNA quantity and integrity were checked immediately prior to reverse transcription

RT-PCR Assay:

Reverse transcription of RNA was carried out using the GeneAmpGold RNA PCR Reagent Kit (P/N 4308206 Applied Biosystems) using 3-4 µg of RNA according to the manufacturer's instructions.

The CK-19 gene expression was evaluated by nested PCR as described by **Datta *et al.* [16].** The sequences of primers used were (synthesized by Gencet, Paris, France):

First round PCR:

(P1; forward): 5'AAGCTAACCATGCAGAACCTC AACGAC CGC 3'

(P2; reverse); 5'TTATTGGCAGGTCAGGAGAAGA GCC 3'

Second round PCR (nested):

(P3; forward); TCCCGCGACTACAGCCACTACTA CACGACC

(P4; reverse); CGCGACTTGATGTCCATGAGCCG CTGGTAC

These primers extend across at least an intron, so an eventual DNA contamination would not pose a significant problem.

Beta-actin: was used as a house keeping gene to indicate the presence of intact RNA and successful first-strand cDNA preparation. The primers' sequences used were: CATCCTGTCGGCAATGCCAGG (forward A1) and CTTCTTGGGCATGGAGTCCTG (reverse A2).

The corresponding sizes of PCR products were 745 base pairs for the CK-19 nested PCR and 540 base pairs for B-actin. The second round PCR was carried out using the nested primers and 0.5 µL of the first round product. The cycling conditions for the first round PCR of CK-19 were: one cycle at 95°C for 10 minute followed by 40 cycles at 94°C for 1 minute, 58°C for 3 minutes and a final extension at 72°C for 10 minutes. The conditions for the second round PCR were: one cycle at 95°C for 10 minutes, followed by 40 cycles at 94°C for 1 minute, 64°C for 3 minutes, and a final extension at 72°C for 10 minutes. The cycling conditions for beta-actin were: one cycle at 95°C for 10 minutes,, followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds and a final extension at 72°C for 4 minutes. Ten microliters of all PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide

Cell lines:

The human mammary carcinoma cell line MCF-7, which expresses the CK-19 gene was obtained from the NCI, Tissue Culture Department. Cells grown in monolayer were harvested, counted and viability was assessed by trypan blue dye exclusion and stored at -70 °C.

To improve sensitivity and specificity of CK-19 mRNA Detection in Peripheral Blood, the following was done:

RNA extracted from MCF-7 was amplified using the set of primers described above. The MCF-7 cell line was consistently positive. Moreover, no amplification product could be detected by nested RT-PCR performed on RNA from the chosen cell line in the absence of the RT enzyme, which demonstrates that any contaminating DNA derived from the processed pseudo- gene would not amplify using the above-mentioned pair of primers subsequently; the sensitivity of CK-19 mRNA detection was evaluated by nested RT-PCR analysis. For this purpose, MCF-7 cells were mixed with PBMCs from healthy blood donors in a cell ratio ranging from 1:10 to $1:10^6$ cells, which mimics the clinical setting for detection of mammary cells in patients' peripheral blood. Representative results of a positive nested RT-PCR are shown in Fig 1, demonstrating 745-base pair whereas the 540-base pair corresponding to the beta-actin gene. This assay was capable of detecting one MCF-7 cell among 106 normal hematopoietic cells as described by 7-**Stathopoulou *et al.* [13]**. All of samples from control patients and healthy blood donors were positive for beta-actin which indicates the presence of intact RNA and successful first-strand cDNA preparation. These results indicate that the detection of CK-19 mRNA in the peripheral blood is highly associated with breast cancer, despite the fact that a small number of false-positive results (usually < 5%) may be obtained in healthy female blood.

Histopathologic evaluation

All cases undergone modified radical mastectomy or lumpectomy with lymphadenectomy. Tumor grading was evaluated according to Nottingham combined histologic grade (Elston-Ellis modification of the Scarff Bloom Richardson grading system) **[17].** Tumor staging was evaluated according to American Joint Committee on Cancer (**AJCC**) **[18].**

Three Positively charged slides were prepared from representative tumor block of each case and stained with primary monoclonal antibodies against estrogen receptors (Dako, mouse monoclonal, clone 1D5, ready to use), progesterone receptors (Dako, mouse monoclonal, clone PgR 636, ready to use) and HER2/neu (Dako, rabbit polyclonal, dilution 1:250). According to the manufacturer's instructions

ER and PR status were evaluated according to Allred scoring system considering only positive nuclear staining **[19].**

HER-2 immunostainig results was estimated according to HER2/neu scoring system used to evaluate Hercep Test **[20]**

3. Results:

The characteristics of 40 newly diagnosed breast cancer patients enrolled in the study are listed in **Table 1** . The patients' median age was 49 years (range, 29 to 80 years). Thirty cases with invasive duct carcinoma. Most of cancer patients were postmenopausal constituting 57.5%.None of the studied cases demonstrated any evidence of metastatic disease at time of diagnosis.

According to **American Joint Committee on Cancer (AJCC) [9],** most of the studied patients were of; stage II constituting 60 %, T2 category constituting 47.5% of all cases. Most of cases of invasive duct carcinomas **(Figure 1)** were of grade 2 representing 80% of all case. Most of node positive cases were of N2 category constituting 52% of all studied cases. As regards hormonal status, 22/40 (55%) were positive for estrogen receptor (**Figure 2**) of which 10 cases were negative to progesterone receptors, while 19/40 (47.5%) were positive to progesterone receptor of which 7 cases were negative to estrogen receptor. Dual positivity for both estrogen and progesterone receptors were encountered in 12(30%) cases, while dual negativity was obtained in 11(27.5%) cases. HER-2/neu immunohistochemical results revealed 25% to be positive (score 3) (**Figure 3**).

None of the studied cases demonstrated any evidence of metastatic disease at time of diagnosis.

Results of RT-PCR amplification of CK19 mRNA:

CK19 was found positive in the peripheral blood of fourteen (35%), of the 40 breast cancer patients studied. Positive cases had a median age of 52.2 years **(Figure 4)**, while only one healthy control case exhibited CK-19 positive. The majority of tumors (85.7%) were of invasive duct carcinoma. As regards tumor size, CK 19 positive cases included most of the tumors belonging to T2 & T3 categories (57.9% & 60%) respectively, and none of the T1 category. All patients with grade III tumors had also CK 19 positive cells in their peripheral blood. Only one case with node negative breast cancer was positive for CK 19, while 52% of node positive cases were positive. As regards tumor stage, none of patients with the stage I tumor expressed CK 19 positive cells in peripheral blood. None of the patients with CK 19 positive cells expressed dual ER

& PR positivity being both negative or either negative.

As regards HER-2/neu, all patients with positive reaction to HER-2/neu (score 3) also had CK 19 positive cells in their peripheral blood, while none of the HER-2/neu negative cases exhibited detectable CK 19 positive cells.

Relation between CK19 mRNA+ cases and clinicopathologic parameters, hormonal status and HER -2/neu expression [Table 2]:

Statistically significant association was recorded between CK19 mRNA-positive cells and tumor size (p<0.001), histologic grade (p =0.006), stage of disease (p =0.007), lymph node status (p =0.005) and hormonal status (p =0.003).

There was no statistically significant association between the detection of CK19 mRNA-positive cells and patient's age, menstrual status or pathologic type.

There was a highly significant relation between HER-2 expression and CK-19 mRNA-Positivity recording p <0.001. Interestingly, all the patients with no detectable CK19mRNA- cells were also negative for HER2 by immunohistochemistry and vice versa.

4. Discussion:

Breast cancer is a leading cause of cancer-related deaths in women Worldwide. The clinical course of this disease is highly variable and clinicians continuously search for prognostic parameters that can accurately predict prognosis, and indicate a suitable adjuvant therapy for each patient **[1]**. In developing countries as Egypt, poverty, illiteracy and limited resources for health care are extra challenges facing both patients and medical care providers.

The National Cancer Institute, Cairo University, Egypt is responsible for supplying medical care free of charge to patients who are mainly poor and with little education. We are aiming at choosing the best and most economic managements to serve as many patients as possible and achieve international cure rates.

The TNM system is incapable of identifying women who, although they have an early-stage breast cancer, may be at high risk of relapse and death. This is due to the early dissemination of malignant cells from the original tumor through hematogenous and/or lymphatic pathways and the failure of the adjuvant treatment to eliminate them **[21]**. Notably knowing that solid tumors usually contain multiple clones and it is possible that only a small subset of cancer cells of the primary tumor have the biologic characteristics to become disseminated tumor cells. Therefore, the likelihood of finding disseminated cancer cells may not necessarily parallel the primary tumor load, nor can it be predicted by the well-known risk factors **[13].**

Especially for breast cancer, CK19 is stably and abundantly expressed on epithelial breast tumors but not on mesenchymal haemopoietic cells and has been successfully used for the detection of breast cancer cells in the bone marrow, lymph nodes and peripheral blood **[22].**

Up to our knowledge, limited studies demonstrated CK-19 expression by nested PCR and its behavior in Egyptian Female breast cancer patients and its relevance to prognosis.

CK-19 mRNA-positive cells were detected in the peripheral blood of 14 patients (35%). This finding is in agreement with the study by **Stathopoulou, *et al.* [7]** in which CK-19 mRNA-positive cells were detected in 30% of patients using the same technique.

Detection rates of CK-19 mRNA-positive cells in breast cancer have varied in published studies from 21% to 55 % **[7- 9, 16, 23-25]**. This variation could be attributed to different patient population, the different detection techniques used with different sensitivities and specificities, even in different investigators using the same primers may have different results **[7, 8]** depending on the amount of RNA used at the beginning of the reaction or the number of amplification cycles. Alternatively, the false-positive results may be due to the detection of CK-19 pseudo genes a and b **[26]** or even due to sample contamination with epithelial cells of the skin during vein puncture **[27]**. Especially for the nested PCR assay, it is feasible to reduce the sensitivity of detecting these low-level transcripts**. [28]**

In our patients, statistically significant association was found between CK19 mRNA-positivity in the peripheral blood and well established prognostic parameters; namely, tumor size, histologic grade of the tumor, lymph node status, the stage of the disease, and hormonal status. These results were in concordance with other studies that showed statistically significant with tumor size, clinical stage in their cases or association between ck-19 and tumor size **[22, 29]** .In our study the percentages of CK19+ cells in the peripheral blood samples of patients were increased as the illness grew worse. This result was similar with that of Ivy Wong and his group that positive expression level of CK19 correlates strongly with disease stage in colorectal cancer **[30]**.

It has been mentioned that CK19 detection rate increased with tumor size **[31]**. Most of our CK19 positive patients had a tumour size of more than 2 cm. However, other reports found the presence of CK19 positive cells had nothing to do with clinicopathological prognostic factors **[15, 25]**. This could be explained by the fact that solid tumors

usually contain multiple clones; it is possible that only a small subset of cancer cells of the primary tumor have the biologic characteristics to become disseminated tumor cells. Therefore, the likelihood of finding disseminated cancer cells may not necessarily parallel the primary tumor load, nor can it be predicted by the well-known risk factors. Also the lack of correlation might be due to the fact that absence of CK19 transcript in blood samples does not exclude the presence of circulating tumor cells.

Also in this study, detection of CK-19 mRNA in peripheral blood showed direct statistically significant association with lymph node status. This was in contrast to a relative recent report **[29].** This may be due to the different dissemination pathways that breast cancer cells utilize, ie, lymphatic spread of the tumor is independent of hematogenous dissemination. Moreover, it has been revealed that detection of disseminated carcinoma cells in the bone marrow using an antibody directed against a common cytokeratin epitope has an independent prognostic value, which is superior to that of the axillary lymph node status in women with early-stage breast cancer **[32**].

As regards expression of HER2 and in agreement with other studies **[8, 25**], detection of CK-19 mRNA-positive cells showed direct statistically significant association with immunohistochemical expression of HER-2. All our patients with no detectable CK19mRNA were HER-2 negative and vice versa. This supports that early hematogenous dissemination is associated a number of tumor associated characteristics, such as expression of urokinase plasminogen activator receptor, over expression of the erbB2 oncogene, and deficient expression of major histocompatibility complex class I molecules **[21].**

It is noteworthy to mention that at the time of diagnosis, all of our cases were subjected to bone scan as well as imaging studies to detect any metastatic deposits and all of them revealed negative results and showed significant relation with bad prognostic parameters. In this study CK-19 mRNA+ were detected in 14 of 40 (35%) patients, when these positive cases were re-evaluated for possibility of occurrence of metastasis after a period of 12 months, 5 out of 14 patients revealed evidence of metastasis by chest CT and bone scan. A longer period of follow up of the other positive cases might verify if they would metastasize or not. This highlights the paramount importance of application of standard routine techniques for detection of circulating tumor cells in the peripheral blood of newly diagnosed patients in order to identify a group of high risk patients who may need different therapeutic approach.

This finding is supported by other studies [21,32] which reported that the detection of occult tumor cells in the bone marrow or peripheral blood of patients with early-stage breast cancer has been shown to be an independent predictive and prognostic factor for early disease recurrence and decreased overall survival either with hormonal receptor positive or negative operable breast cancer. These findings support the role of CTC monitoring as an adjunct to standard clinical and radiographic methods in the evaluation of disease status during follow-up **[24]**.

The detection of CTCs before adjuvant chemotherapy or during tamoxifen administration has been demonstrated to be an independent adverse prognostic factor in women with early-stage breast cancer. The prognostic value of CTC detection is of great significance in subgroups of patients with estrogen receptor-negative and human HER-2/ neu-positive tumors **[24].**

This opens the way to further investigation of important questions such as whether the detection of CTCs should be performed in all patients at the time of primary diagnosis to identify high-risk patients or whether CTCs detection at diagnosis should modify the adjuvant therapeutic strategy or, whether the detection of CTCs during the administration of adjuvant treatment would allow the development of secondary adjuvant therapeutic strategies.

Several groups of researches proposed a number of therapeutic strategies targeting CK19.In cancer cervix chemotherapeutic efficiency is restrained by the overexpression of Ck19 that will minimize the efficacy of chemotherapy in cervical cancers. Nevertheless, overexpression of Ck19 may provide an alternative approach to treat cervical cancers by radiolabelling mAb against Ck19 **[33].** Several similar approaches are undergoing to cancer breast targeting CK19.

Our results strongly suggested that CK19 might be involved with a biologically active subset of breast cancer patients with high tumor burden and bad prognosis, a suggestion that is shared by others who stated that CK19-releasing cells might constitute a biologically active subset of breast cancer cells with high metastatic properties and that $CK19^{+}$ cells in the human breast may have stem cell-like properties **[34**].

Since our findings were obtained using RNA extracted from peripheral blood mononuclear cells; therefore, blood could be a valuable, feasible and inexpensive method for early detection and monitoring CTCs as CK19 mRNA- positive cells. Also, could give a very early indication of possible prognosis of patients and which ones could be at more risk of metastasis than others. This is particularly useful in our category of patients who are

often irregular in their follow up and do not strictly follow treatment schedules.

It is noteworthy to report that none of the 15 patients with benign breast lesions (14fibroadenomas and 1 granulomatous mastitis) were positive for CK-19 while only one of 10 apparently healthy volunteers had positive blood sample. Other investigators have also detected CK-19 transcripts in the PBMCs of healthy breast subjects [35, 3], but none reported if follow up of such women showed development of breast cancer later in life. The high specificity of the method was made possible by avoiding contamination of skin epithelial cells during vein puncture, as well as by carefully designing the primers. Hence, amplification of the known CK-19 pseudo- genes and genomic DNA was avoided **[24].**

We strongly recommend that future studies should examine the use of RT-PCR CK-19 mRNA detection, preferably with a quantitative method, in evaluating CTC prior and after completion of therapy and monitoring minimal residual disease after the administration of novel adjuvant therapies.

Table 1: Patient characteristics

	Patients No (%)
Age(years)	
Median	49
Range	29-80
Menopausal status	
Premenopausal	17 (42.5)
Postmenopausal	23 (57.5)
Tumor size	
<=2cm	16 (40.0)
2-5cm	19 (47.5)
>5cm	5 (12.5)
Pathologic type	
Invasive duct carcinoma (IDC)	30 (75.0)
Invasive lobular carcinoma (ILC)	8 (20.0)
Mixed IDC & ILC	1 (2.5)
Mucinous adenocarcinoma	1 (2.5)
Tumor grade of IDC	
I	1 (3.3)
II	24 (80)
III	5 (16.7)
Axillary lymph nodes	
N0(-ve)	15 (37.5)
N1(1-3)	8 (20.0)
N2(4-9)	13 (32.5)
N3(=>10)	4 (10.0)
Stage	
I	11 (27.5)
II	24 (60.0)
III	5 (12.5)
Hormonal status ER	
Negative	18 (45.0)
Positive	22 (55.0)
PR	
Negative	21 (52.5)
Positive	19 (47.5)
Her2/neu	
Negative	22 (55.0)
Positive	10 (25.0)
equivocal	8 (20)

Table (2): Relation between clinicopathologic parameters, hormonal status, HER-2/neu and CK-19 mRNA

	Total	CK-19 mRNA(-ve) N (%)	CK-19 mRNA(+ve) N (%)	P value
Age(y)	40	26(65)	14(35)	
Median	49	48	52.2	0.3
Range	29-80	29-80	32-70	
Menopausal status	17	12(70.6)	5(29.4)	0.5
Premenopausal	23	14(60.9)	9(39.1)	
Postmenopausal				
Tumor size				
<2cm	16	16(100)	0(0)	
2-5cm	19	8(42.1)	11(57.9)	**<0.001**
>5cm	5	2(40.0)	3(60.0)	
grade				
I&II	25	18(72)	7(28)	**0.006**
III	5	0	5(100)	
Pathology type				
IDC	30	18(60)	12(40)	
ILC	8	6(75)	2(25)	0.4
Axillary lymph nodes	15	14(93.3)	1(6.7)	**0.005**
N0	25	12(48)	13(52)	
N1,2,3				
Stage	11	11(100)	0	
I	29	15(51.7)	14(48.3)	**0.007**
II,III				
Hormone receptor status				
Both -ve, either -ve	28	14(50)	14(50)	**0.003**
Both +ve	12	12(100)	0	
HER-2/neu				
Negative	22	22(100)	0	
Positive	10	0	10(100)	**<0.001**
equivocal	8	4(50)	4(50)	

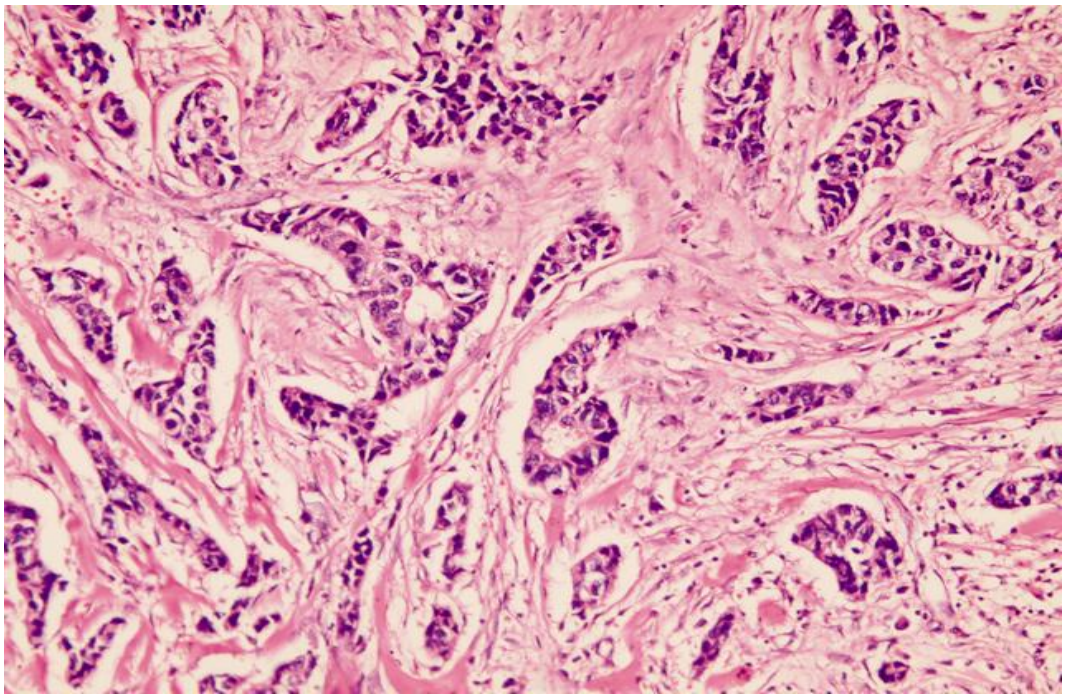

Figure 1: Case of invasive duct carcinoma grade II showing tubular and glandular formation. (H&E x200).

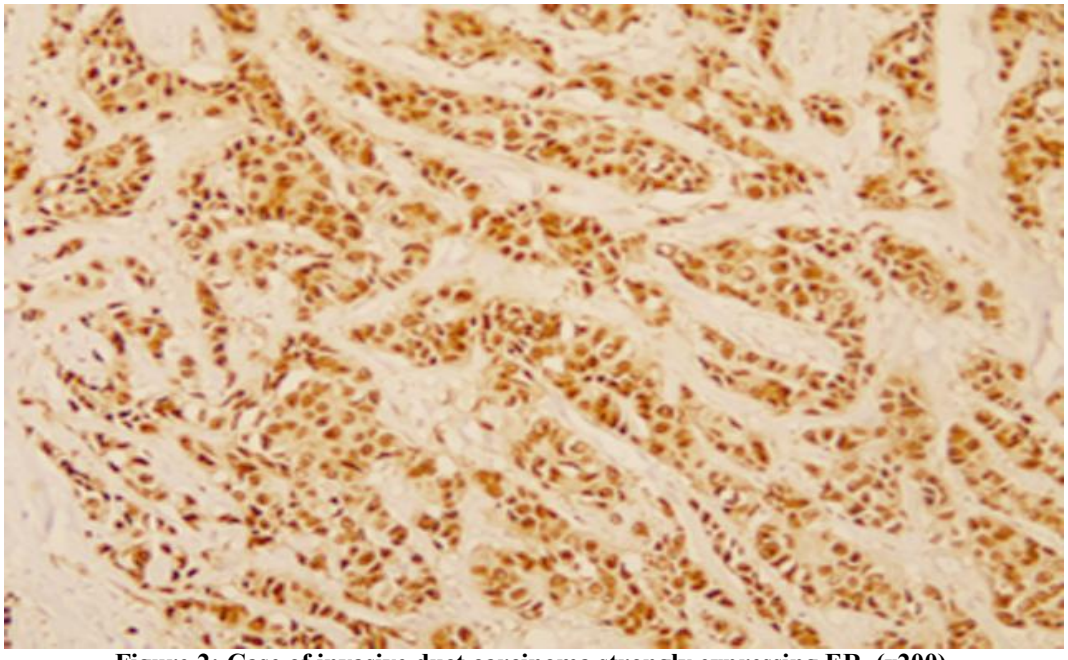

Figure 2: Case of invasive duct carcinoma strongly expressing ER. (x200).

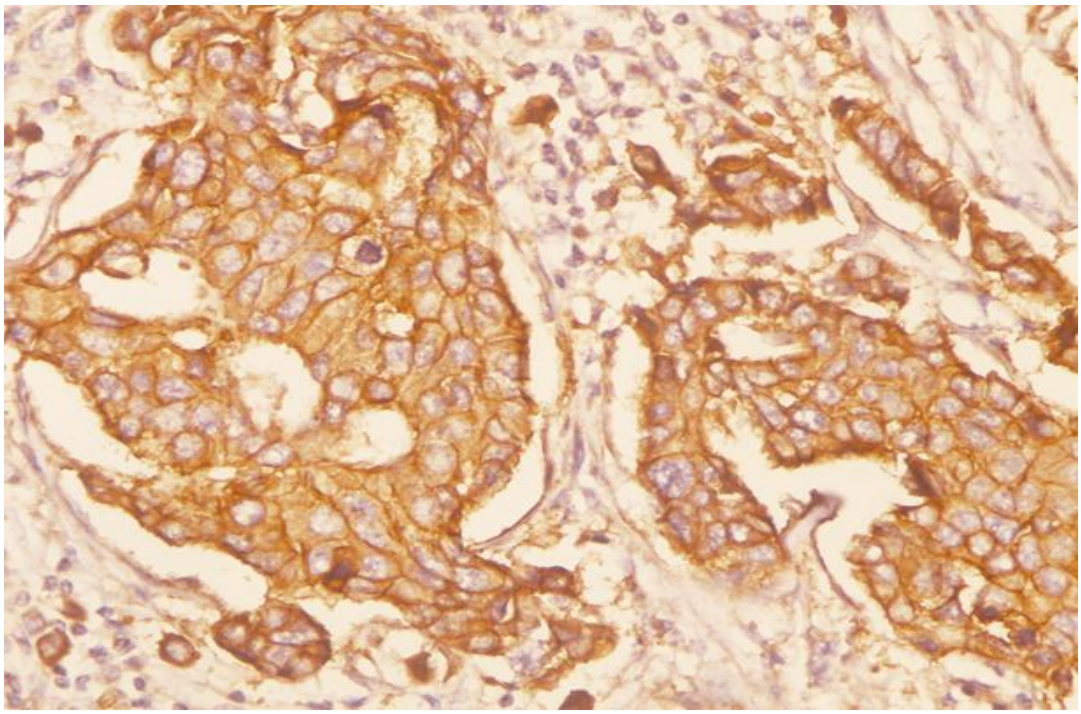

Figure 3: Strong positive membranous expression for HER 2 (score 3) in case of high grade invasive duct carcinoma. (x 400)

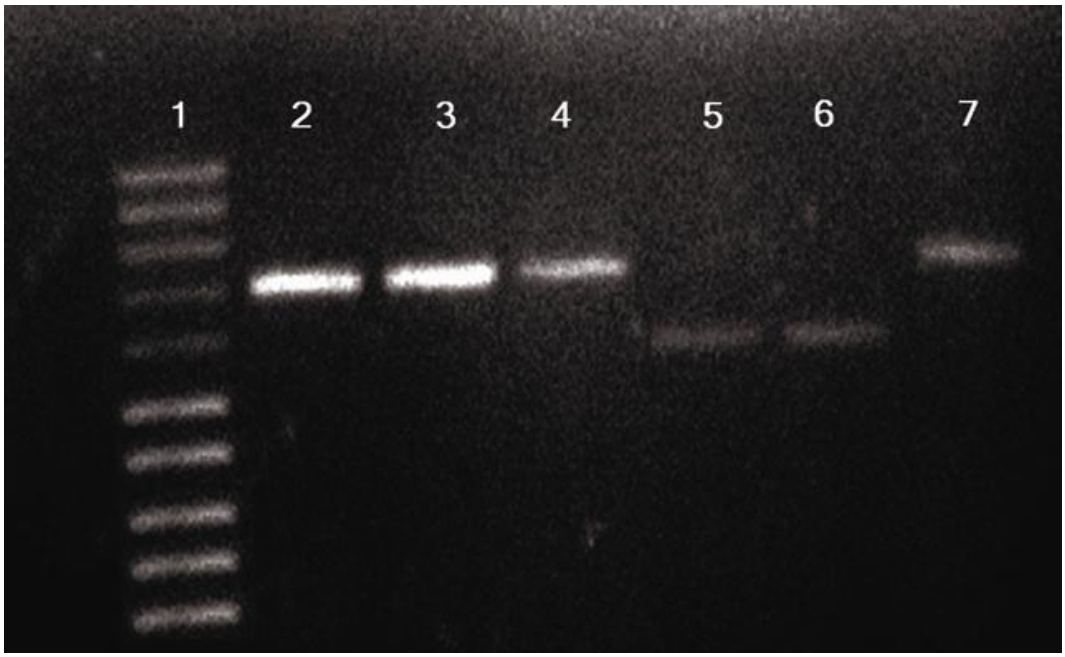

Figure 4: Post PCR products CK19 in breast cancer patients.

Lane 1 DNA molecular weight 100 base pairs plus ladder (100 lanes)
Lane 2 MCF7 positive control (720 base pairs)
Lanes 3- 4 -7 CK19 positive (720 base pairs)
Lanes 5-6 B -actin positive (540 base pairs)

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