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ORIGINAL PAPER

Molecular Markers in Peripheral Blood of Iranian Women with Breast Cancer

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Abstract A biomarker is a quantifiable laboratory measure of a disease specific biologically relevant molecule that can act as an indicator of a current or future disease state. The purpose of this study is to detect the expression of RNA biomarkers using Cytokeratin 19 (CK-19), Mammaglobin (MAM), Carcinoembryonic antigen (CEA), Mucin (MUC), C-Myc, erb-B2, a proliferation marker (Ki-67), Epidermal growth factor receptor (Her2/neu) and Estrogen receptor (ER) in Iranian women who were diagnosed with breast cancer. In this study, 90 samples; 60 cancer patients and 30 healthy controls were considered. 73.4 % patients were in stage I/II and 26.6 % were in stage III/IV. Patients were selected prior to the administration of any adjuvant systemic therapy. Total RNA extraction was obtained from peripheral blood of each patient and healthy control. Reverse transcription polymerase chain reaction (RT-PCR) method was used for detection of mRNA of the selected biomarkers of circulating breast cancer cells in blood. Molecular characterization is assessed as a method for early detection of breast cancer. For this purpose, eleven specific primers were selected and RT-PCR was used. The data of RT-PCR revealed that expression of MUC1, CK19, CEA, MAM, erbB-2, Ki67 and C-Myc biomarkers were significantly different between breast cancer patients and healthy controls. On the other hand, ERα, ERβ and Her2 markers were not significantly different between the two mentioned groups. Biomarkers detection of breast cancer patients could be assessed as a diagnostic factor and its potential for conveying as a prognostic factor require further studies, with a larger number of patients.

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Introduction

Incidence rates of breast cancer are increasing in most countries; for instance, in Asia with 3 % annual increase in incidence as compared to 0.5 % in the rest of the world [1]. Breast cancer is the second in mortality among women in the United States [2, 3]. Breast cancer was also the most common type of cancer in European women, in 2006 [4]. In 2007, Breast cancer was the most common malignancy among women and the second most common cause of cancer-related mortality in the world [1]. In the Eastern Mediterranean region, it remains also as a common and frequent fatal disease, the second cause of cancer death [5].

In Iran, cancer is the third main cause of death [6]. There is no population-based study available for cancer in Tehran (10 % of the Iranian population) [7]. However, based on studies of cancer registry for the period of 1998–2001, breast cancer was one of the major cancers in this population [7]. Moreover, it was the second most common cancer among Iranian women [8]. Early detection of the breast cancer is one of the important national cancer control programs for decreasing the burden of breast cancer in Iran [8].

The global incidence and mortality of breast cancer remains high despite extraordinary progress in understanding the molecular mechanisms underlying carcinogenesis, tumor promotion, and the establishment of molecular targeted therapies.

Early diagnosis is necessary for these high risk cancers at an early stage, which specifies the need for specific and sensitive biomarkers. A biomarker can act as an indicator of a current or future disease state [9].

Cytokeratin 19 (Ck-19) is stably and abundantly expressed in epithelial tumors but not in mesenchymal hemopoietic cells and has been successfully used as a marker for the detection of tumor cells in the bone marrow, lymph nodes and peripheral blood by immunohistochemistry and RT-PCR [10]. Mucins are high molecular weight glycoproteins produced by many epithelial tissues. Overexpression of the mucin proteins, especially MUC1, is associated with many types of cancer [11]. Mucin 1 (MUC1) oncoprotein is aberrantly overexpressed by approximately 90 % of human breast cancers [12]. HER2 is a member of the human epidermal growth factor receptor (EGFR) family [12]. The discovery of the overexpression of ErbB2/HER2/Neu in about 25 % of breast cancers has been focused attention on the ErbB family of receptor tyrosine kinases as a significant contributor to tumor progression. ErbB2 is recognized as an important contributor to some types of breast cancer [13]. Carcinoembryonic antigen (CEA) is a glycoprotein involved in cell adhesion. It has been widely used as a serum tumor marker in breast cancer [14]. Human mammaglobin was reported to be exclusively expressed in mammary epithelium and overexpressed in some breast cancer which makes it a potentially useful RT-PCR target for breast cancer cell detection in hematopoietic products [15].

Endogenous estrogen is thought to play a major role in breast cancer development and estrogen receptors blockers are the most important drugs for cancer treatment. Estrogen mediates its functions through two specific intracellular receptors, ERα and ERβ, which both act as hormone-dependent transcriptional regulators [16].

MYC is a proto-oncogene, its protein product function as a transcription factor, regulating up to 15 % of all human genes. MYC amplification correlates significantly with aggressive tumor phenotypes and poor clinical outcomes [17].

Ki67 protein is a cellular proliferation marker as well as a cancer antigen that is found in growing and dividing cells. However, it is absent in the resting phase of cell growth.

This characteristic makes Ki-67 a good tumor marker. It has traditionally been recognized as a modest prognostic factor, but recent neoadjuvant studies suggest that on-treatment measurement may be a more effective predictor of treatment efficacy [18].

Circulating tumor cell (CTC) detection in peripheral blood of epithelial cancer patients is a recognized indicator for the presence of primary tumors and/or metastasis [19]. RT-PCR technique has been used in the detection of specific tumor cell markers, thus indicating the presence of circulating breast cancer cells in blood. Up-regulated expression level of some of these markers and their relation to breast cancer has been demonstrated in breast cancer cells [20]. The purpose of this study was to detect the expression of mRNA biomarkers; CK-19, hMAM, CEA, MUC-1, C-Myc, erb-B2, Ki67, Her2/neu, and ER simultaneously in Iranian women who were diagnosed with breast cancer prior to the administration of any adjuvant systemic therapy.

In this regard, RT-PCR application was performed to detect disseminated markers in blood of breast cancer patients.

Materials and Methods

Clinical Samples from Patients and Healthy Controls

Clinical evaluation was performed in 60 cancer patients with histological diagnosis of operable breast cancer at different stages. Stages (I–IV) of cancer patients classified according to standard criteria based on data of TNM (Tumor, Nodes and Metastases) and American Joint Committee on cancer staging system (AJCC). Patients had not received any preoperative chemotherapy or hormonotherapy. Written consent form was signed and provided by each patient. Sample acquisition and subsequent use were performed according to the permission from National Ethical Committee from Pasteur Institute of Iran. Twenty milliliters of peripheral blood were obtained from each patient and healthy control, collected in buffered sodium citrate, maintained at 4 °C, within 2 h. Expression of the markers in patients with breast cancer and 30 healthy controls were done prior to the administration of any adjuvant systemic therapy.

RNA Isolation

RNA was isolated from whole blood specimens using the AccuZol™ (BioNEER). Total RNA purification kit (BioNEER) was used according to the manufacturers' instructions for fresh blood samples. According to the manual procedure, 250 μl of blood was used per each round of total RNA isolation. The extracted RNA was assessed by spectrophotometer and concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}). RNA purity was analyzed by the ratio between the absorbance values at 260 and 280 nm. The integrity of total RNA, purified by this kit was checked by agarose gel electrophoresis and ethidium bromide staining. Then, the extracted purified RNA was applied for Reverse Transcription Polymerase Chain Reaction (RT-PCR).

Reverse Transcriptase Polymerase Chain Reactions (RT-PCR)

Total extracted RNA was amplified by AccuPower® RT/PCR PreMix (BioNEER) kit.

It contains all the components necessary for cDNA synthesis and amplification in one tube. The expression of the 11 genes was screened as markers in peripheral blood of collected samples. Table 1 presents the oligonucleotide sequences that were used in this study.

Table 1 Marker panel of forward (F) and reverse (R) primers used in this study

	Primers (5′-3′)	References
β-actin		*1
F	5′-CTCTTCCAGCCTTCCTTCCT-3′	
R	5′-AGCACTGTGTTGGCGTACAG-3′	
CK19		25
F	5′-ATGAAAGCTGCCTTGGAAGA-3′	
R	5′-TGATTCTGCCGCTCACTATCAG-3′	
CEA(outer)		*1
F	5′-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3′	
R	5′-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3′	
CEA(inner)		*1
F	5′-GGGCCACTGTCGGCATCATGATTGG-3′	
R	5′-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3′	
MUC-1		*1
F	5′-CGTCGTGGACATTGATGGTACC-3′	
R	5′-GGTACCTCCTCTCACCTCCTCCAA-3′	
Her2		25
F	5′-GGATATCCAGGAGGTGCAGGGTAC-3′	
R	5′-CCTGTGAGGCTTCGAAGCTGCAGCT-3′	
hMAM		25
F	5′-CCATGAAGTTGCTGATGGTC-3′	
R	5′-TCAGAGTTTCATCCGTTTGG-3′	
ERα		*2
F	5′-TGCTTCAGGCTACCATTATGGAGTCTG-3′	
R	5′-GTCAGGGACAAGGCCAGGCTG-3′	
ERβ		*2
F	5′-TTTAAAGAAGCATTCAAGGACATAATG-3′	
R	5′-GAAGTGTGGCTCCCGGAGAGAGAG-3′	
c-myc		*3
F	5′-CAGCTGCTTAGACGCTGGATTT-3′	
R	5′-ACCGAGTCGTAGTCGAGGTCAT-3′	
erbB2		*3
F	5′-CTGGTGACACAGCTTATGCCCT-3′	
R	5′-ATCCCCTTGGCAATCTGCA-3′	
Ki67		*3
F	5′-ATCGTCCCAGGTGGAAGAGTT-3′	
R	5′-ATAGTAACCAGGCGTCTCGTGG-3′	

*1. Berois N, Varangot M, Aizen B, et al. Molecular detection of cancer cells in bone marrow and peripheral blood of patients with operable breast cancer. Comparison of CK19, MUC1 and CEA using RT-PCR. Eur J Cancer. 2000; 36: 717–23.

*2. Jarzabek K, Koda M, Kozlowski L, et al. Distinct mRNA, protein expression patterns and distribution of oestrogen receptors alpha and beta in human primary breast cancer: correlation with proliferation marker Ki-67 and clinicopathological factors. Eur J Cancer. 2005; 41: 2924–34

*3. Mitas M, Mikhitarian K, Walters C, et al. Quantitative real-time RT-PCR detection of breast cancer micrometastasis using a multigene marker panel. Int J Cancer. 2001; 93: 162–71.

The quality of RNA isolates was verified by amplifying of β-actin. The presence of β-actin mRNA (31.2 pg) was used as a control for each molecular marker gene expression. In this experiment, template extracted RNA (1–2 μg) was used with the same amount of β-actin RT-PCR product for obtaining the same sensitivity for each marker. The protocol assures that only mRNA is amplified; the DNA sequences corresponding to the product amplified by the PCR include several introns and the primers spans exons junction. On the other hand, the presence of genomic contamination was assessed in samples by PCR, without reverse transcription.

The sensitivity of RT-PCR has already been reported for detection of disseminated breast cancer cells; for each marker at a density of approximately 1–5 cancer cells per 1–5 ml of blood has been used [21].

The experimental procedure began with mixing the template extracted RNA and the reverse primer in a sterile tube and followed by incubation of the mixture at 70 °C for 5 min. The incubated mixture and the forward primer were transferred to premix tube and then they were filled up with distilled water. The cDNA synthesis was done at, 42 °C for 60 min and at 94 °C for 5 min.

Thereafter, PCR cycles were performed according to PCR condition which was as follows; 94 °C, 60 s, 54 °C 30 s and 72 °C, 60 s. The PCR products hence obtained were visualized by electrophoresis on 2 % agarose gel and under U.V. in gel documentation system.

Statistical Analysis

The analysis of statistical significance of the correlations expression of markers between patients and healthy controls was performed using Pearson's chi-square test. p value of less than 0.05 was considered as significant. Data processing was performed by means of SPSS software.

Results

Patient Analysis

This study is based on 90 samples; 60 cancer patients and 30 healthy controls (non-patients). Average age of the sample population was 44.3 years (Range 22–77), average age of patients was 45.6 years (Range 26–77) and average age of non patients was 40.7 years (Range 22–60). Different stages (I–IV) of solid cancer patients classified according to standard criteria based on data of TNM (Tumor, Nodes and Metastases) and American Joint Committee on cancer staging system (AJCC). In this regard, 54 (90 %) of patients were diagnosed as Invasive ductal carcinoma. Moreover, 19 (31.7 %) of patients were in stage I, 25 (41.7 %) in stage IIA and IIB and 16 (26.6 %) were in stage III and IV. Clinical characteristic of the patients is presented in Table 2.

Table 2 Tumor characteristics of the patients

Characteristics	n=60
Histology	
Invasive ductal carcinoma	54 (90 %)
Invasive lobular carcinoma	3 (5 %)
Invasive medullary carcinoma	3 (5 %)
Tumor grade	
Grade I and II	13 (21.6 %)
Grade III	30 (50 %)
UN	17 (28.3 %)
Stage	
I	19 (31.7 %)
IIA and IIB	25 (41.7 %)
III and IV	16 (26.6 %)
Receptors status (IHC)	
ER^+	16 (26.7 %)
ER^-	14 (23.3 %)
PR^+	16 (26.7 %)
PR^-	14 (23.3 %)
HER2 positive	11 (18.3 %)
HER2 negative	19 (31.7 %)
UN	30 (50 %)

UN unknown; *ER* estrogen receptor; *PR* Progesterone receptor
IHC Immunohistochemistry

Detection of mRNA Specific Marker Gene in Breast Cancer Patients

The data collected from RT-PCR assay is presented in Table 3. Detection of selected markers in peripheral blood of breast cancer patients and healthy control considered as positive and no detection of the corresponding band is regarded as negative. Data analysis was done for each mRNA specific marker. Concerning significant markers, CK19 marker was expressed in 86.7 % patients and in 40 % healthy control (P=0.001) and for MUC1 marker 90 % were positive in patients and 53.3 % in healthy control (P=0.04). In the present study, 71.7 % of the breast cancer cases expressed CEAi, while it was expressed in 6.7 % of healthy control and the difference was highly significant (P=0.001). Regarding CEAo primer, 35 % of cancer cases and 3.3 % of healthy control were positive (P=0.01). Frequency of the Ki67 marker was 58.3 % in patients and MAM marker was expressed in 50 % of patients. Both Ki67 and MAM marker expression was highly significant (P=0.001). C-Myc and erbB-2 were expressed in 38.3 % and 36.7 % of breast cancer cases and difference between patients and healthy control was significant (P=0.019, P=0.024, respectively). Her2 marker was present in only 11.7 % of cases and it was not significantly expressed (P>0.05). ERα was expressed in 55 % and ERβ was overexpressed in 16.7 % of patients while statistically both were not significant between patient and healthy control (P>0.05).

As it is shown in Table 3; for CK-19, hMAM, Ki-67 and CEAi markers *P value* was highly significant (P=0.001). Regarding CEAo, MUC1, C-Myc and erb-B2 markers were also significant with *P value* 0.01, 0.04, 0.019 and 0.024 respectively. However, no significant difference was observed for Her2, ERα and ERβ markers (P>0.05).

The frequency of tumor mRNA marker expression in the blood of the patients and healthy controls is shown in Table 3. MUC1 and CK19 markers expression were the most expressed markers (more than 80 %) in patients. However, the co-expression of MUC1 and CK19 markers occurred in 74.4 % of patients. Simultaneous expression of MUC1, CK19 and CEA markers were also observed in 51.1 % of patients. In healthy control, co-expression of MUC1 and CK19 marker was observed in 6 (20 %), while co-expression of MUC1, CK19 and CEA marker was shown in 1 (3.3 %).

The percentage of positive marker, depending on the range of ages ((22–37), (38–53), (54–69), (70–85)) in

Table 3 Tumor mRNA marker expression in blood from breast cancer patients

mRNA marker	+ No. (%)	– No. (%)
C-Myc		
Patients	23 (38.3)	37 (61.7)
Non-patients	0 (0.0)	30 (100)
Total	23 (25.6)	67 (74.4)
P value	0.019	
Erb-B2		
Patients	22 (36.7)	38 (63.3)
Non-patients	1 (3.3)	29 (96.7)
Total	23 (25.6)	67 (74.4)
P value	0.024	
CK19		
Patients	52 (86.7)	8 (13.3)
Non-patients	12 (40)	18 (60)
Total	64 (71.1)	26 (28.9)
P value	0.001	
hMAM		
Patients	30 (50)	30 (50)
Non-patients	0 (0.0)	30 (100)
Total	30 (33.3)	60 (66.7)
P value	0.001	
Her2		
Patients	7 (11.7)	53 (88.3)
Non-patients	4 (13.3)	26 (86.7)
Total	11 (12.2)	79 (87.8)
P value	0.181	
MUC1		
Patients	54 (90)	6 (10)
Non-patients	16 (53.3)	14 (46.7)
Total	70 (77.8)	20 (22.2)
P value	0.04	
Ki-67		
Patients	35 (58.3)	25 (41.7)
Non-patients	0 (0.0)	30 (100)
Total	35 (38.9)	55 (61.1)
P value	0.001	
ERα		
Patients	33(55)	27 (45)
Non-patients	5 (16.7)	25 (83.3)
Total	38 (42.2)	52 (57.8)
P value	0.056	
ERβ		
Patients	10 (16.7)	50 (83.3)
Non-patients	0 (0.0)	30 (100)
Total	10 (11.1)	80 (88.9)
P value	0.059	
CEA (i)		
Patients	43(71.7)	17 (28.3)
Non-patients	2 (6.7)	28 (93.3)
Total	45 (50)	45 (50)
P value	0.001	
CEA (o)		
Patients	21 (35)	39 (65)
Non-patients	1 (3.3)	29 (96.7)
Total	22 (24.4)	68 (75.6)
P value	0.01	

In this experiment, 60 breast cancer patients and 30 healthy controls were considered

Non-patients considered as healthy control

sample population, was shown that 38 to 53 is the most populated age with the most positive markers. It is shown that CK19, MUC1 and CEA(i) markers are relatively the most positive markers in each age group.

However, the co-expression of MUC1 and CK19 markers was observed among different age group. They occurred in 82.4 %, 62 %, 52.4 % and 100 % of each age group, respectively. Simultaneous expression of MUC1, CK19 and CEA markers were observed in 76.5 %, 38 %, 57.1 % and 50 % of these age groups. In comparison with healthy control age group, expression of MUC1, CK19 and CEA markers were prevalent.

The distribution of positive markers in different stages of patients is shown in Table 4. Table 5, shows the percentage of positive markers in patients with Invasive ductal carcinoma.

As it is shown in Table 4, the same pattern of marker expression is observed in different stages. In stage IIA and IIB, the most positive markers are shown. Frequency of the most prevalent markers (MUC1, CK19) are 10(62.5 %) in stage I, 17(73.9 %) in stage II and 5(83.3 %) in stage III and IV. Co-expression of MUC1, CK19 and CEA marker is shown in 7(43.8 %) patients for stage I, 10(43.5 %) for stage II and in 4(66.6 %) for stage III and IV patients (data not shown).

Invasive ductal carcinoma is the most frequent type of tumor in our study. MUC1 and CK19 expression in this group was observed in 40(74 %) while MUC1, CK19 and CEA co-expression was shown in 30(55.6 %) patients.

Discussion

In this study, the detected transcripts of molecular markers could drive from different origins; including the tumor tissues [22, 23]. There is also a possibility of correlation between the detection of these markers and the presence of CTCs in the peripheral blood. There has been an enormous

Table 4 Percentage of positive markers distribution in different stages of patients

Stage of patients (n=60)	Expression of markers										
	C-Myc	erb-B2	CK19	hMAM	Her2	MUC1	Ki-67	ERα	ERβ	CEA(i)	CEA(o)
Stage I n=19 (31.7 %)	30.4	31.9	36.5	30	42.9	35.2	28.6	15.2	20	32.5	28.5
Stage IIA/IIB n=25 (41.7 %)	56.5	54.5	48.1	60	57.1	46.3	57.1	63.6	60	53.5	62
Stage III/IV n=16 (26.6 %)	13.1	13.6	15.4	10	–	18.5	14.3	21.2	20	14	9.5

effort to develop special and sensitive biomarkers for precise and accurate screening, diagnosis, prognosis and monitoring of high risk cancer to assist with therapeutic decisions [24]. In the future, a combination approach will be simultaneously measure multiple markers. Probably, it would be the most successful in detecting breast cancer in early phases. Ideally, such biomarkers could be able to detect breast cancer in asymptomatic patients, even in the setting of normal mammogram and physical examination results [16]. The detection of circulating mRNA by RT-PCR allows the amplification of genes that are specially expressed or the expression of which is significantly upregulated in tumor cells. Detection of tumor specific mRNA could be indicative of viable tumor cells actively shedding nucleic acids.

CK-19 has been shown as a candidate for a general marker of epithelial cancers [25]. It is an acidic protein of 40 kDa that is part of the cytoskeleton of epithelial cells. It is highly expressed by all epithelial cells and represents a useful indicator of epithelial differentiation [10]. CK-19 has been successfully used as a marker for detection of certain tumor cells in blood [26]. In addition, it has been studied as potential marker for minimal residual disease in blood [27]. CEA mRNA is another marker which can be detected in almost all epithelial cells including breast cancer [28]. Human mammaglobin (hMAM) is another breast-specific and breast cancer-associated marker in breast epithelial cells and it is over-expressed in breast cancer. hMAM known for its mammary tissue specificity and it has been discussed as a promising diagnostic marker in breast cancer [29]. In our study, the importance of these markers was also shown (p=0.001 and p=0.01) in the blood of breast cancer patients. MUC1 is a heterodimeric membrane mucin which presents in simple epithelia and in many carcinomas. It is present in about 90 % of breast cancers; its expression was also significant (p<0.05) in this study. It has been reported that Erbs can bind to the cytoplasmic tail of MUC1 [13]. This relation was also observed in this study since ErbB-2 expression marker was significant (p=0.024) [30]. The detected RNA transcripts in this study could derive from the tumor tissue as a promising diagnostic tool for non invasive and cost effective cancer detection. On the other hand, RT-PCR using tumor cells circulating in the blood offer increased sensitivity and would be useful for patient treatment and management.

Markers of proliferation, such as Ki67, Human epidermal growth factor receptor (HER) family of receptor tyrosine kinase, forms part of a complex signal cascade modulating cell proliferation, survival, adhesion, migration and differentiation [31]. HER-2 positive sera were already reported in the serum of 27 Iranian breast cancer patients with metastasis [19]. The expression of this marker was not significant in our study (p=0.181), while Ki67 biomarker expression was highly significant (p=0.001). Ki67 has been appeared as a time-varying biomarker of breast cancer [32].

Early detection of breast cancer is a critical determinant in the outcome of therapies [33, 34]. It is clear that patients with early detection of cancer have better rate of recovery and survival than patients with more advanced cancer [35]. It has already been shown that approximately 15–20 % of breast cancers have amplification of the HER2/neu gene or over expression of its protein product. An inverse relationship between the level of HER2 expression and the expression of cytokeratin has been shown [35]. In addition, evaluation of changes in markers during the process of the disease and prognostic value of the markers needs more survey. In this study, it was explained that the type of primer used for marker detection in the blood can be important. HER2 is recognized as an important marker of breast cancer; however it might not be detected in the blood of Iranian women. Nonetheless, erb-B2 marker (another fraction of tyrosine kinase) is detected among breast cancer subjects. In

Table 5 Percentage of each positive marker in Invasive ductal carcinoma and non ductal carcinoma

C-Myc	erb-B2	CK19	hMAM	Her2	MUC1	Ki-67	ERα	ERβ	CEA(i)	CEA(o)
Invasive ductal carcinoma n=54 (90 %)										
24	29.6	74	35.2	3.7	79.6	46.3	40.7	9.2	55.6	29.6
Non ductal carcinoma n=6 (10 %)										
50	50	66.7	66.7	–	50	66.7	33.3	–	66.7	33.3

addition, in the present study ER receptors were not significantly expressed in the blood of breast cancer patients.

In summary, expression of MUC1, CK19, CEA, MAM, erb-B2, Ki67 and C-Myc biomarkers between breast cancer patients and healthy controls were detected ($P<0.05$). In this study, CK19, MUC1 and CEA were the most frequent markers in prevalent age group (38–53). Moreover, the most frequent expression of these markers was also observed in stage II. C-myc, hMAM, ki-67, ERβ markers are not expressed in healthy subjects while expression of these markers were 38.3 %, 50 %, 58.3 % and 16.7 % in patients, respectively. In this regard, these markers are considered as more specific markers. Moreover, multiple marker assays may significantly improve the sensitivity of detecting heterogeneous tumor cells compared with single marker assays. Detection of molecular markers can represent CTC in blood as a simple diagnostic test of breast cancer patients.

However, subtle patterns of multiple genes could help identify new prognostic markers in the clinical setting.

Most probably, the expression of the specific markers increases with the progress of the breast cancer. The molecular diagnostic and thereby staging methodologies via specific biomarkers could be used to monitor patients in future and to detect recurrent disease prior to clinical manifestations. Breast cancer is the most common cancer among Iranian women (24 per 100 000) [6]. Therefore, this kind of research is highly recommended because it could be helpful for detecting and consequently decreasing the burden of breast cancer.

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