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RESEARCH ARTICLE

EVALUATION THE DIAGNOSTIC AND PROGNOSTIC VALUE OF CYTOKERATIN-19 (*CK19*) GENE EXPRESSION IN IRAQI BREAST CANCER PATIENTS

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ABSTRACT

Breast cancer is the most frequent carcinoma in females and the second most common cause of cancer related mortality in women. Early detection of breast cancer is widely reported to be one of the most effective ways leading to better prognosis and lower death rate. For marker discovery, the analysis of mRNA expression signatures in peripheral human blood has been widely used showing to be a promising technique. Cytokeratin-19 (*CK 19)* is a novel gene that was diagnosed as a highly specific marker for primary breast cancer. The aim of the present study is detecting the expression levels of the *CK-19* gene mRNAs in the peripheral blood of breast cancer patients in comparison with benign and healthy controls as a tool for screening and diagnosis the early stage breast cancers, and estimating the diagnostic and prognostic values of these levels in association with tumor size and lymph node status. The marker was determined in peripheral blood (PB) of 55 patients with Invasive Ductal Carcinoma and samples from 20 healthy donors, and 10 women with newly diagnosed benign breast tumors were served as control group using reverse transcriptase polymerase chain reaction (RT-PCR). *CK-19* was detected in 41(74.54%) peripheral blood of breast cancer patients while 14(25.46%) patients were *CK 19*-negative. *CK-19* also detected in, 1(10%) of the benign tumors and 2(10%) of healthy individuals. It showed statistically significant relations with size of the tumor, and Lymph node involvement. On the other hand, it was statistically non-significant for age of breast cancer patients. The present study results suggest that *CK-19* is a specific molecular marker for detection of breast cancer, discrimination between benign and malignant breast tumors, and it might be of value as a prognostic marker.

INTRODUCTION

Cancer is one of the most important health problems of the current era and also a leading cause of death among populations. Cancer can simply be defined as a class of diseases or disorders that is characterized by uncontrolled division of cells and the ability of these abnormal cells to spread, either by direct growth into adjacent tissues through invasion, or by implantation into distant sites by metastasis (where cancer cells are transported through the bloodstream or lymphatic system (Blachford, 2002). Breast cancer is the most commonly diagnosed malignancy in women around the world, especially in the Western countries. It accounts for almost one fifth of deaths caused by cancer (Winer *et al.*, 2001). Every year, one million new cases are reported worldwide, representing 18% of the total number of cancer in women. In Iraq it has been detected that the number of breast cancer cases are steadily rising since the 1991 war (Jaffer, 1999; Jasim, 2004). Breast cancer is the malignant tumor that forms from the uncontrolled growth of abnormal breast cells. It usually affects tissues involved in milk production (Ductal and lobular tissues) (Madhavan *et al*., 2002). Its originated from the terminal ducto-lobular unit of breast tissue. Breast cancer that has not invaded the basement membrane and thus confined within the terminal ductolobular units is termed carcinoma in-situ. Mainly, there are two types of in-situ cancers; lobular carcinoma in-situ and ductal carcinoma in-situ (Atalay,2004). Beside these common types of invasive breast cancers, there are other rare forms such as medullary, papillary, mucinous, tubular, apocrine and adenoid cystic carcinoma (Winer *et al.*, 2001). As in the case of most of the cancers, staging of breast cancer takes into consideration the size of the tumor (T), the number and location of metastatic lymph nodes (N), and distant organ metastasis (M) (Greene *et al.*, 2002). According to TNM staging system, breast cancer patients are divided into stages I to IV. Stages I and II are called as early stages while stage III designates locally advanced breast cancer. One of the major mechanisms for tumor metastasis is the dissemination of tumor cells from the primary tumor into circulating blood (Botteri *et al.*, 2010). Previous studies have indicated that detection of circulating tumor cells (CTCs) in the peripheral blood can be

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used in staging and prognosis stratification for breast and colon cancer patients (Wülfing *et al*., 2006, AlFaisal *et al*., 2009, Allen *et al*., 2010;). Disseminated tumor cells (DTC) in bone marrow and circulating tumor cells (CTC) in peripheral blood (Stathopoulou *et al.,* 2002; Xenidis *et al*., 2006) of patients with breast cancer have been shown to be independent adverse prognostic factors for disease recurrence and disease-related death. The detection of mRNA transcripts for specific epithelial markers by using reverse transcriptase polymerase chain reaction (RT-PCR) and, more recently, the quantitative real-time RT-PCR (QPCR) seems to have higher diagnostic sensitivity (Ring *et al*., 2005). The major advantage of RNA-based approaches is related to the rapid degradation of RNA released from cells in the blood by RNAses; therefore, the origin of detectable blood RNA transcripts is considered to be viable cells. Cytokeratin-19 (*CK-19*), cytoskeletal component present in normal and cancerous epithelial cells, has been extensively used for the detection of breast cancer cells in mesenchymal tissues and seems to be the most sensitive and reliable tumor marker in both patients with operable and metastatic breast cancer (Brown *et al*., 2006; Stathopoulou *et al*., 2003) Several studies have shown the prognostic significance of *CK-19* mRNA-positive CTC in patients with operable breast cancer (Xenidis *et al*., 2009; Xenidis *et al*., 2003). The aim of the present study was to evaluate the diagnostic and prognostic values of the *CK-19* gene by comparing the levels of *CK-19* gene expression of breast cancer patients, benign breast tumors and healthy controls in relationship with certain clinical characteristics (tumor size and lymph node status).

MATERIAL AND METHODS

Patients and clinical samples

Blood samples from 55 patients with different stages of newly diagnosed Invasive Ductal Carcinoma were provided by certain Iraqi hospitals (including National center for early detection of tumors and Al-Elweya teaching hospital) after patients underwent cytopathological (Fine needle aspiration FNA) and histopathological examination. Two control groups were used in this study, 10 samples of patients with benign breast tumors and 20 samples from healthy donors. The required information about the patients and the histopathologic properties of the tumors were recorded from the patients' files. The samples preservation with TRIzol was done at the Genetic lab of National center for early detection of tumors in Baghdad medical city. Out of 2ml of peripheral blood that drawn into EDTA tubes, 0.5 ml was preserved as whole blood after treating with trizol (sample which was centrifuged at 1,000 xg for 5 min. at 4Cº followed by removing the supernatant and adding phosphate buffer saline (PBS) containing 5% Triton X-100 and vortexed to be homogenized, a 0.75 ml of trizol was added to each sample in a ratio of 3 TRIzol :1 Sample volume and the samples were kept at -80Cº. Samples were subjected to RNA extraction and molecular study by using Reverse Transcription and Real Time PCR at Molecular Oncology Unit in Guy´s hospital – Kings college/London.

RNA extraction, reverse transcription and real-time RT-PCR assay

The total RNA of breast cancer, benign tumors and healthy control samples was extracted using the TRIzol® LS Reagent (Life Technologies - Ambion CO.) following the protocol provided by the manufacturer. Total RNA was reversely transcribed using High-Capacity cDNA Reverse Transcription Kit. The procedure was carried out in a reaction volume of 20 µl following the protocol provided by the manufacturer (Applied Biosystem) cDNA was stored at -80 °C until use. Expression of *CK19* gene was analyzed using specific primers and probes (Table 1). Serial dilutions of primers and probes were used for preparing of standard curve. standard curve was prepared for both the target and the endogenous genes(Figure 3.1,3.2). The data generated from serial dilution of standard curve were excellent means which determined the overall performance of QPCR assay. In this assay, the housekeeping gene ABL was used as an internal control to normalize variations in integrity and the total amount of cDNA. Quantitative real-time PCR assays were performed in duplicate using TaqMan master mix (Applied Biosystem/ USA) in 20 µl reaction volume containing10 µl of master mix (TaqMan master mix),1 µl of primer mixes, 5µl of RNase free water and 4µl of cDNA template on the 7900 HT Fast Real-time PCR system (Applied Biosystem/ USA). Real-Time PCR protocol was as follows; stage 1: 50 ºC for 2 minutes, stage 2: 95 ºC for 10 min and in a stage 3 in a two-step cycle procedure (denaturation 95 ºC for 15 Sec. and annealing 60 ºC for 1 min) repeated for 50 cycles. Melting curve analysis was used to assess the specificity of the amplified products. The expression levels of *CK 19* gene from the cDNA were measured by quantitative real-time PCR using the relative quantification method ($2^{-\Delta\Delta Ct}$ method). The fold-change in gene expression was normalized to a housekeeping gene ABL and relative to a calibrator sample.

Statistical Analysis

The Statistical Analysis System- SAS (2010) was used to effect of difference factors in study parameters or percentage. The chi-square test at the comparative between percentage & least significant difference –LSD test to the comparative between means in this study.

Table 1.Primers and Probes sequences

Primers and Probes used with RT-qPCR		
Primer	Sequence	Melting temperature
CK19-F	5′- TGCGGGACAAGATTCTTGGT -3′	60.0 Cº
CK19-R	5′- TCTCAAACTTGGTTCGGAAGTCA -3′	48.4 Cº
CK19-P	5′- ACCATTGAGAACTCCAGGATTG -3′	55.5 Cº
ABL-F	5′- TGGAGATAACACTCTAAGCATAACTAAAGG T-3′	49.9 Cº
ABL-R	5′-GATGTAGTTGCTTGGGACCCA-3′	47.3 Cº
ABL-P	5′-CCATTTTTGGTTTGGGCTTCACACCATT-3′	52.5 Cº

Ethical use of data

Informed consent was obtained from all the study participants and the guidelines set by the ethics committee of our institute and hospitals were applied.

RESULTS

The patients' age range was 24-70 years and the median is 49 years with high frequency of patients in the range of 40-59 years. According to the family history, 50(90.91%) of patients were have negative family history which statistically high significance differences (X^2 =13.473 **, p<0.01) in comparison with patients that have positive family history. According to the lymph node status, the percentage of patients with multiple lymph nodes was higher than those with few or no lymph nodes which showed statistically high significant differences (p value 0.0017**p<0.001), (Table.2). In regard to the tumor size the highest percentage of patients showed the tumor size 2.0-2.9 cm. which showed statistically high significant differences (p value 0.0014**p<0.001), (Table.3). Out of 55 patients, 41 (74.54%) patients were *CK19*-positive while 14(25.46%) patients were *CK19*-negative. According to malignancy status the percentage of patients with high level of *CK19* gene expression 30(54.45%) was significantly higher in compare with benign tumor patients and healthy controls in which the percentages of samples that showed high level of *CK19* gene expression were 1(10%) and 2(10%) respectively, (Figure.1). In correlation with age groups the present study showed statistically no significant differences in the levels of gene expression with age, (Figure 2).

Table 2. Distribution of patients according to lymph node status

Lymph node status	Patients	
	No.	%
No	9	16.36
Few	19	34.54
Multiple	27	49.1
Total	55	100
Chi-square value		11.092 **
P-value		0.0017

Table 3. Distribution of patients group according to tumor size

Tumor size (cm)	Patients	
	No.	%
1.0-1.9	14	25.45
2.0-2.9	19	34.55
3.0-3.9	18	32.73
4.0-4.9	4	7.27
Total	55	100
Chi-square value		11.267 **
P-value		0.0014

In correlation to the lymph node status the results of the present study showed that the highest percentage of *CK19* positive patients 24(88.89%) were multiple for lymph node status that significantly highly different from patients with no or few lymph node status (p value 0.00026 **p<0.0001), (Figure 3). According to the tumor size the results showed that there was increasing in the *CK19* gene expression with increasing of tumor size since the highest percentage of *CK-19* positive patients 4(100%) were with tumor size 4.0-4.9 cm. which showed statistically high significant differences (p value 0.0026 **p<0.001), (Figure 4).

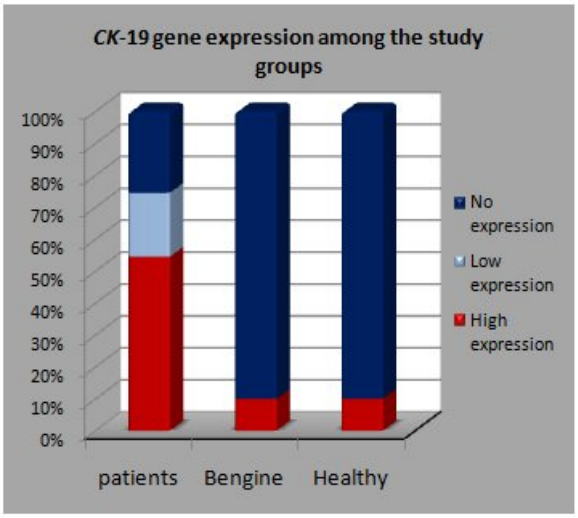

Figure 1. Differences in *CK-19* gene expression among the study groups

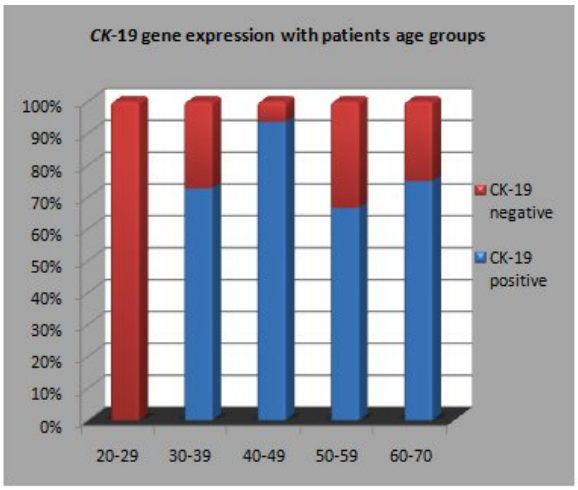

Figure 2. Differences in *CK-19* gene expression with patients age groups

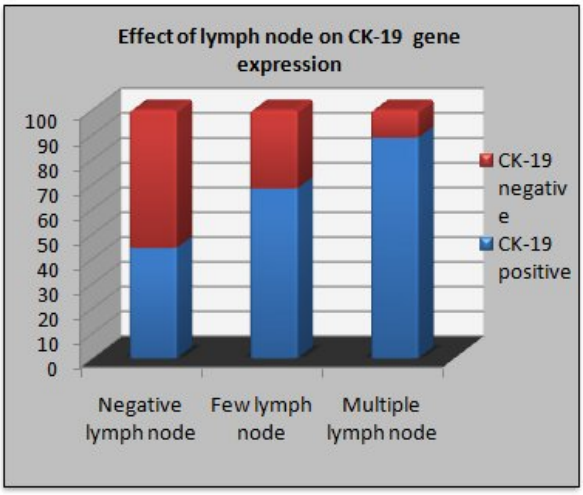

Figure 3. Correlation of *CK-19* gene expression with lymph node status

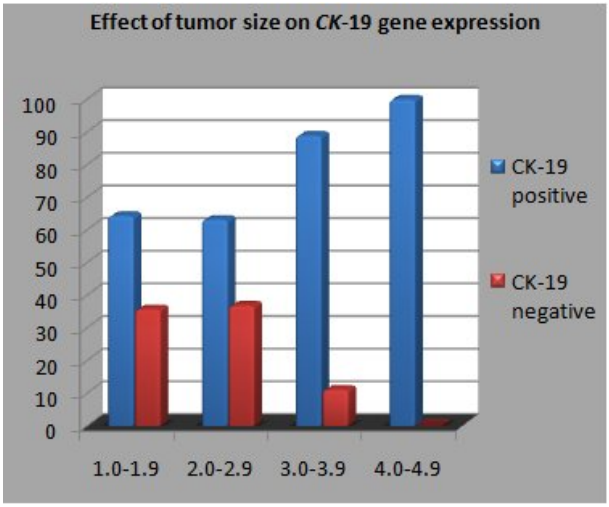

Figure 4. Correlation of *CK-19* gene expression with tumor size

DISCUSSION

The relationship between circulating tumor cells and the development of metastatic disease is not fully understood, but the ability to detect very small numbers of breast carcinoma cells in circulation could have both prognostic and therapeutic implications, as has already been shown for some hematologic malignancies (van Dongen *et al*., 1999). *CK-19* has been reported before to be the superior marker for breast cancer especially in metastatic disease in sentinel lymph nodes (Schoenfeld *et al*., 1999; Visser *et al*., 2008). Based on previous data, it has also been demonstrated as a highly sensitive marker for the detection of micrometastases in axillary lymph nodes and breast cancer cells in peripheral blood by using real time RT-PCR (Stathopoulou *et al*., 2003; Aerts *et al*., 2001). Interestingly, correlation of *CK-19* positive cells in the peripheral blood with breast cancer patients stage I or II was demonstrated as a marker of poor clinical outcome (Stathopoulou *et al*., 2002).

The present study examined the levels of *CK-19* mRNA in peripheral blood of breast cancer patients using qRT-PCR technique and estimation its diagnostic and prognostic values. The percentage of breast cancer patients that showed *CK19*-positive was higher (74.54%) than those of benign tumors and healthy controls (10% for both groups). There are many other studies which detected that the *CK19*-positive was high in breast cancer patient with relatively slight expression in benign tumors or healthy controls including Stathopoulou *et al*. (2003) who detected *CK19*-mRNA in 37.7% of breast cancer patients and only 2.2% of the healthy control subjects, Stathopoulou *et al*. (2002) reported that *CK19* mRNA was detected in the peripheral blood of 3.7% of healthy blood donors, and in 63%,73% and 52%,30% of marrow and blood samples of patients with early or metastatic breast cancer respectively, Aerts *et al*. (2001) who reported that significantly elevated *CKI9* transcript levels in 10% of the volunteers, and in 30% of stage I-III and 70% of the stage IV breast cancer patients. Other studies showed that neither benign breast tumors nor healthy volunteers had *CK19* mRNA-positive blood sample including Said *et al*. (2012), Wang *et al*. (2009). The identification of distribution according to the age groups of the present study showed that no significant correlation between *CKI9* gene expression levels and patients age groups which similar to that reported by other studies including Said *et al*. (2012); Kummalue *et al*. (2012); Saloustros *et al*. (2011); Ignatiadis *et al*. (2007), all these studies detected that *CK19* mRNA transcription did not vary significantly with the age of breast cancer patients.

The lymph node status results of the present study showed that there was statistically significant association between the percentage of *CK19* mRNA-positive samples and the lymph node involvement, these results comparable to those of Said *et al*. (2012); and Saloustros *et al*. (2011). On the other hand, the present study results were different from results reported by other studies that showed no significant effect of lymph node involvement on *CK19* expression including Kummalue *et al*. (2012); Ignatiadis *et al*. (2007); and Stathopoulou *et al*. (2002) who showed that no significant association between the detection of *CK19* mRNA-positive cells and number of involved axillary lymph nodes. According to the tumor size the results showed that there was significant association between the increasing of *CK19* gene expression and tumor size since the highest percentage of *CK19* positive patients were with tumor size 4.0-4.9 cm. These results comparable to those of Said *et al*. (2012); and Wang *et al*. (2009) who both showed that statistically significant association between the presence of *CK19* gene expression and the patients' tumor size. The results of present study contradicting to those reported in other studies which showed statistically non- significant between *CK19* mRNA expression and tumor size including Kummalue *et al*.,(2012); Ignatiadis *et al*.,(2008) and Stathopoulou *et al*.,(2002). There are several studies that have focused on the *CK19* expression in breast cancer, but the clinical significance of *CK19* expression is still unclear. The present study was designed to investigate the diagnostic and prognostic value of *CK19* gene expression in breast cancer patients and since peripheral blood of *CK19* mRNA reflecting the activity of *CK19* gene and levels of gene expression, the present study used real-time RT-PCR as a powerful and rapid tool for determination of mRNA which indicated the sensitivity of RT-PCR for detection even low *CK19* mRNA levels in breast cancer samples as well as in benign tumor and healthy control. On the other hand, from the results of the present study it can be concluded that the analysis of *CK19* mRNA levels in breast cancer blood samples obtained from Iraqi breast cancer patients showed that *CK19* gene expression can be a useful tool for discrimination between malignant and benign ovarian tumors, which in turn reflecting the diagnostic value of *CK19*. It's also can be considered as potential prognostic marker since its overexpression associated with multiple lymph node status and the greatest tumor size which reflect its role in metastasis and prognostic breast cancer.

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