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Evaluation of diagnostic and prognostic value of mucin *(MUC 1)* gene expression in breast cancer patients

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Abstract: The Mucin 1 *(MUC1)* is over-expressed in most human epithelial cancers and has gained remarkable attention as an oncogenic molecule. The aim of the present study is detecting the expression levels of the human Mucin 1 *(MUC1)* 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). Mucin 1 *(MUC1)* was detected in 40 (72.73%) of peripheral blood of breast cancer patients studied, 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 reflected the possibility of detecting of that gene transcript in normal and benign blood samples as well as the breast cancer samples which in turn reflect the value of *MUC1* gene as one of useful tools for discriminating malignant breast tumors from non-malignant ones.

Key words: Breast cancer, RT-PCR, Mucin 1, Diagnostic marker.

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Introduction:

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. 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 (1,2,3).

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) (4). It's originate 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 (5). Beside these common types of invasive breast cancers, there are

other rare forms such as medullary, papillary, mucinous, tubular, apocrine and adenoid cystic carcinoma (6). 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)(7).

Previous studies have indicated that detection of circulating tumor cells (CTCs) in the peripheral blood can be used in staging and prognosis stratification for breast and colon cancer patients (8). *MUC1* gene was mapped on human chromosome 1q21. It has a sequence of 4.4 kb. This gene encodes a membrane-bound protein that is a member of the mucin family. Normally *MUC1* is expressed in the glandular or luminal epithelial cells of the mammary gland, esophagus, stomach, duodenum, pancreas, uterus, prostate, and lungs and to a lesser extent, in hematopoietic cells (10,11, 12). It is absent in the skin epithelium and in mesenchymal cells (13). In healthy tissues, *MUC1* provides protection to the underlying epithelia. Aberrantly glycosylated *MUC1* is over-expressed in most human epithelial cancers and has gained remarkable attention as an oncogenic molecule (14,15). It's also an effective marker for breast cancer CTC and treatment monitor (16). In addition, *MUC1* may regulate the expression of such miRNAs that favors the cancer stem cells (CSCs) to remain in a dedifferentiated 'stem cell like' state. In breast adenocarcinoma and a number of epithelial tumors, *MUC1* is up-regulated with aberrant expression over the entire cell surface (13,17,18,19). *MUC1* is encoded by a gene located on chromosome 1q21, a region frequently altered in breast cancer cells. Therefore, in breast cancer *MUC1* expression is variable and is often over expressed. It has been thought that over-expression of *MUC1* in cancer is caused by increases in gene dosage and level of transcription, and by a loss of post-transcriptional regulation. This characteristic makes the *MUC1* protein valuable as a marker in breast cancer diagnosis and prognosis .

Materials and Methods:

Patients and clinical samples:

The blood samples from 55 patients with different stages of newly diagnosed Invasive Ductal Carcinoma were were provided by certain Iraqi hospitals (including National center for early detection of tumors and Al-Ilweya teaching hospital) after patients underwent cytopathological (Fine needle aspiration FNA) and histopathological examination. Two control groups were used in this study, 10 smples 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 4C^{o} followed by removing the supernatant and adding phosphate buffer saline (PBS) containing 5% Triton X-100 and vortexed to be homogenized then a 0.75 ml of trizol added to each sample in a ratio of 3 TRIzol :1Sample

volume) then the samples were kept at 80Cº. Samples subjected to RNA extraction and molecular study by using Revers 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 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 *MUC1* 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 were prepared for both the target and the endogenous genes. 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 50ºC for 2 minutes, stage 2: 95ºC for 10 min and in a stage 3 in a two-step cycle procedure (denaturation 95C for 15 Sec. and annealing 60ºC for 1 min) repeated for 50 cycles. Melting curve analysis was used to assess the specificity of the amplified products. The expression levels of *MUC1* 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 Cº**
MUC 1-F	5′- GTGCCCCCTAGCAGTACCG -3′	64
MUC 1-R	5′- GACGTGCCCCTACAAGTTGG -3′	64
MUC 1-P	5′- AGCCCCTATGAGAAGGTTTCTGCAGGTAATG -3′	58
ABL-F	5′-TGGAGATAACACTCTAAGCATAACTAAAGGT-3′	57.8
ABL-R	5′-GATGTAGTTGCTTGGGACCCA-3′	54.4
ABL-P	5′-CCATTTTTGGTTTGGGCTTCACACCATT-3′	58.5

Results:

The patients' age ranged between 20 and 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, 40 (72.73%) patients were *MUC 1* -positive which showed statistically high significant differences (*p*= value 0.0024 *p* <0.01) with the percentage of *MUC1*-negative breast cancer patients 15 (27.27%) (Figure 1). According to malignancy status the percentage of patients with high level of *MUC 1* gene expression 22(40%) was significantly higher (*p* value= 0.0026 *p* <0.01) in compare with benign tumor patients 1(10%) and healthy controls 2(10%),(Figure.2). The using of cutoff value (2-fold) of *MUC1* gene expression divided breast cancer samples into high *MUC1* expressing samples 2(5%) and with low *MUC1*expressing 38(95%) (Figure 3). Relation between *muc1* gene expression and clinicopathologic parameters, are listed in Table 4.

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

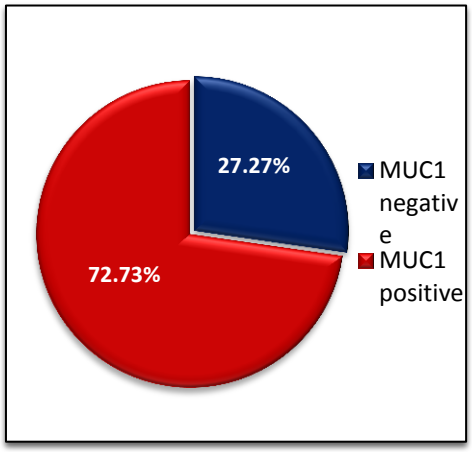

Figure (1): Distribution of breast cancer patients according to *MUC1* gene expression

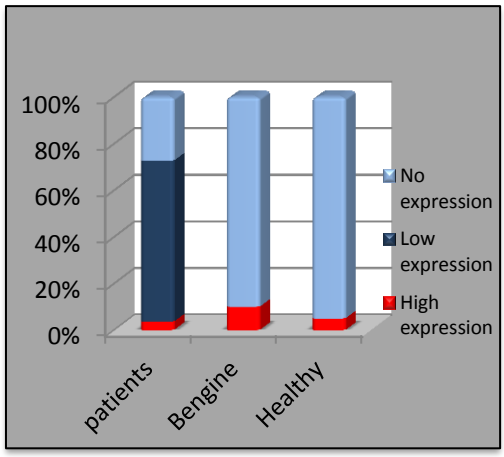

Figure (2): Differences in *MUC1* gene expression among the study groups

In correlation with age groups the present study showed statistically no significant differences in the levels of gene expression with age. In correlation to the lymph node status the results of the present study showed that the highest percentage of *MUC 1* positive patients (84.21%) were few for lymph node status that significantly different from percentage of *MUC 1* positive patients with no or multiple lymph node status (p value =0.0017 $p<0.01$). According to the tumor size the results showed the results showed that there was statistically significant association (p value= 0.0328 $p<0.05$) between the increasing of *MUC1* gene expression and tumor size since the highest percentage of *MUC1* positive patients 3(75%) were with tumor size 4.0-4.9 cm.

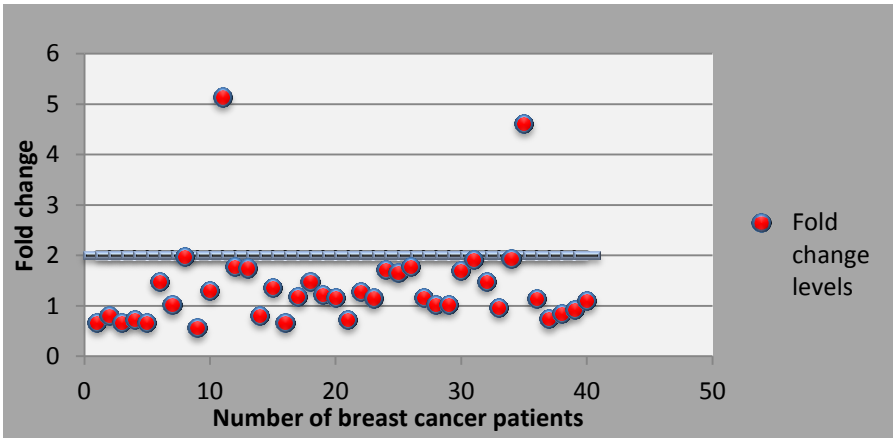

Figure (3): Differences in *MUC1* gene expression levels according to fold change in breast cancer patients (n=40)

Table (4): Effect of clinicopathological features on *MUC1* gene expression in breast cancer patients.

Variable Age groups\year	Number of cases	*MUC1-positive* No. (%)	*MUC1-egative* No. (%)
20-29	2	2(100)	0(0)
30-39	11	7(63.64)	4(36.36)
40-49	15	9(60)	6(40)
50-50	15	13(86.67)	2(13.33)
60-70	12	9(75)	3(25)
NS (No Significance)			
Lymph node status			
Negative	9	4(44.44)	5(55.56)
Few	19	16(84.21)	3(15.79)
Multiple	27	20(74.07)	7(25.93)
p value 0.0017 ** <0. 001			
Tumor size/cm			
1.0-1.9	14	10(71.42)	4(28.58)
2-2.9	19	14(73.68)	5(26.32)
3-3.9	18	12(66.67)	5(33.33)
4-4.9	4	3(75)	1(25)
p value 0.0328 * <0. 05			

Discussion:

The results of the present study showed that the percentage of *MUC 1*-positive breast cancer patients 40 (72.73%) was significantly higher when compared with benign tumor patients and healthy controls, which indicates high specificity of as a marker gene for cells derived from mammary glands. The present study results have some similarity to that reported by other studies including Zaretsky *et al* and Baker *et al*.,(20,21) who found that *MUC1* over expressed in (69%) of breast cancer patients but none of healthy volunteers, Mitas *et al.*, (22) who found that *MUC1* gene expression diagnosed in (81.5%) of breast cancer patients. Pereira *et al.*, (23). who found that *MUC1* detected in 50 of the 67 cases of invasive carcinoma, but expression was also detected in benign epithelium.

On the other hand, the present study results were different from results that reported by De Cremoux *et al*, (24) who found that *MUC1* transcripts were detected in 2 (24%) and in 27 (45%) patients of two breast cancer groups who studied, but also found that 3 (11%) of patients with benign breast disease were positive for *MUC1* transcripts, and Mikhitarian *et al*,(25) who showed that *MUC1* positivity was 0% in peripheral blood and bone marrow samples of breast cancer patients. The identification of distribution according to the age groups of the present study showed no significant correlation between *MUC1* gene expression levels and patients age groups. These results were similar to that of Pereira *et al*,(23) who found no significant correlations between *MUC1* expression and age of breast cancer patients. The lymph node status, results of the present study showed that the percentage of *MUC1* positive patients with few for lymph node status (84.21%) were higher than the patients with multiple lymph node (74.07%) or no lymph node status (44.44%), which showed statistically high significantly differences. The present study results were different from that reported by other studies that showed no significant correlations between *MUC1* expression and lymph node status, including Pereira *et al*,(23) and Mikhitarian *et al*,(25). On the other hand, studies including Mitas *et al*,(26) who showed a significant association between mucin 1 expression and increasing of breast cancer node status and metastasis, and Jang *et al*,(27) who found that *MUC1* expression was associated with a higher frequency of lymph node metastasis. According to the tumor size the results showed that there was statistically significant association between the increasing of *MUC1* gene expression and tumor size since the highest percentage of *MUC1* positive patients 3(75%) were with tumor size 4.0-4.9 cm. The present study results were different from most of other studies including Pereira *et al*,(23) and Mikhitarian *et al*,(25) who both found that no significant correlations between mucin expression and tumor size. The *MUC1* gene is expressed in breast tumors, with a high, but variable, level of transcripts, but mainly up-regulated (28). The exact role of this overexpression and the regulation of *MUC1* expression is not completely understood. Its role in tumor progression is evoked because it has been demonstrated that entire cell membrane expression of *MUC1* reduces cell-cell and cell-extracellular matrix interaction (29). *MUC1* is mainly expressed in breast and ovarian tissue, and much lower in other epithelial tissues where other *MUC1* genes are mainly expressed. The identification of genes overexpressed in breast cancer(including *MUC1*), combined with advances in molecular biology, provides the opportunity to establish more sensitive, specific, and costeffective ways of identifying metastatic disease (30). Thus, the development of a molecular diagnostic assay capable of detecting breast cancer-associated gene expression in the peripheral blood has the potential to vastly improve breast cancer staging and treatment (31). In the present study, the levels of *MUC1* gene expression in breast cancer patients as well as in benign tumors and healthy controls were examined. In conclusion the results reflected the possibility of detecting of that gene transcript in

normal and benign blood samples as well as the breast cancer samples but with wide differences in the sample percentages and level of gene expression which in turn reflect the value of *MUC1* gene as a useful tool for discriminating malignant breast tumors from non-malignant ones. The results may also indicate that the *MUC1* gene has no prognostic value as mentioned for other two previous genes (*MGB1* and *CK19*) since the highest percentage of *MUC1* gene expression was detected in patients with few rather than multiple lymph node. Nevertheless, it can be said that this study results provide evidences that *MUC1* as well as other study genes can be applied as a part of genes panel for breast cancer detection.

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