

Detection of Mammaglobin mRNA in the Blood of Breast Cancer Egyptian Female Patients and Its Relation to Established Prognostic Parameters

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Abstract: Background: Mammaglobin, also known as secretoglobin family 2A member 2 (SCGB2A2), is a member of the superfamily of secretoglobins. Its expression is highly specific for mammary tissue and has been shown to be overexpressed in breast tumor tissue, indicating that mammaglobin might confer a growth advantage to mammaglobin-expressing tumor cells. **Methods:** This study included 46 breast cancer patients, 20 patients diagnosed to have benign breast tumors, and 10 apparently healthy volunteering females as normal controls. Mammaglobin expression was detected in the peripheral blood of both patients and controls using real-time RT-PCR. **Results:** Mammaglobin was detected in 26% of peripheral blood of breast cancer patients studied but not in any of the benign or healthy individuals. It showed statistically significant relations with the positivity of HER2neu expression, the presence of distant metastasis and with CEA (p - value =0.005, 0.013, and 0.001 respectively). On the other hand, it was statistically non- significant for age, grade, stage, ER or PR positivity, size of the tumor, and Lymph node involvement. It showed 26% sensitivity and 100% specificity. **Conclusion:** Our results suggest that mammaglobin is a specific molecular marker for detection of occult mammary carcinoma cells in the peripheral blood of patients with operable breast cancer. It might be of value as a predictor of subsequent metastasis.

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1. Introduction:

Globally, more women are developing breast cancer and more women are dying from it than ever. Between 1980 and 2010, the number of breast cancer cases steadily increased more than two and a half times from 641,000 to 1.6 million annually. This represents an annual increase of 3.1%. The rise in breast cancer cases is happening in every region and in every country, with the number of cases in some countries increasing much faster than the global trend. Four hundred and twenty five thousands women died from breast cancer in 2010. In developing countries, 68,000 of those women were in their reproductive years, aged 15 to 49 [1].

In Egypt, breast cancer is the most common cancer among females accounting for 38.9 % of all cancers affecting females [2].

Breast cancer is considered a systemic disease because early dissemination may occur even in patients with small tumors [3]. Micro-metastases, which are undetectable by the classic images and laboratory studies, can contribute to disease relapse [4]. Therefore, their identification in patients with early breast cancer may have substantial effect on determining prognosis and individualizing treatment for those patients [5]. 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 [6].

In addition to physical examination and mammography, sensitive molecular techniques may be used to detect early-stage breast cancer. Assays for nucleic acid-based markers are valuable tools for the sensitive detection and assessment of disease status in asymptomatic cancer patients [7, 8]. Several techniques have been developed to enrich certain types of cells from blood and to characterize these cells using cytologic and reverse transcriptase-PCR (RT-PCR) assays. Application of these methods may allow for the early detection of cancer when the tumor burden is small and the disease is potentially more curable [9].

In spite of the fact that many circulating cells are apoptotic, a fraction of these cells prove to be still vital and capable of distant metastasis; one of the indexes of cell vitality is the possibility of being transcriptionally active. The presence of circulating tumor cells in the blood might, therefore, be measured by means of the analysis of the expression levels of a specific gene. [10].

Mammaglobin, also known as secretoglobin family 2A member 2 (SCGB2A2), is a member of the

superfamily of secretoglobins, a group of small dimeric secreted and sometimes glycosylated proteins, expressed mainly in mucosa. Secretoglobins seem to be involved in cell signaling, immune response, chemotaxis, and could also serve as transporters for steroid hormones in humans [11]. Mammaglobin expression is highly specific for mammary tissue and has been shown to be overexpressed in breast tumor tissue, indicating that mammaglobin might confer a growth advantage to mammaglobin-expressing tumor cells [12].

Since mammaglobin transcripts are not normally expressed by cells in blood and lymph nodes, it is considered a promising molecular marker for disseminated and circulating breast cancer cells. It also has been suggested to be involved in breast cell proliferation [13].

The aim of this study is to assess mammaglobin expression by means of a quantitative reverse transcription-PCR assay in the whole blood of breast cancer patients, and comparing them with benign breast patients and normal controls. Also correlating the resulting expression data with available clinical, pathological, and prognostic parameters to clarify the biological role of mammaglobin in Egyptian female breast cancer patients.

2. Patients and Methods

Patients:

This study was conducted at the National Cancer Institute, Cairo University. Forty six breast cancer patients presented to the Surgical Oncology Department, NCI during the period from December 2009 till July 2010 were included in this study. Their age ranged from 30 to 78 years with a mean \pm SD: 52.6 ± 10.7 .

The study also included 20 patients diagnosed to have benign breast tumors by clinical evaluation and fine needle aspiration cytology, malignancy was ruled out by histopathological examination. Also, 10 apparently healthy volunteering females were included as normal controls.

Written informed consent was obtained before enrollment into the study according to ethics committee. Investigations performed included routine hematological and biochemical investigations, CEA and CA15.3 evaluation, and chest x-ray for all patients; imaging diagnoses were utilized for stage IV.

Methods:

Detection of Mammaglobin expression:

RNA Extraction, reverse transcription: [14]

Peripheral blood mononuclear cells (PBMCs) were extracted from 15-20 ml EDTA blood cells (PBMCs), they were obtained by gradient density centrifugation using Ficoll-Hypaque 1077 (Sigma).

Cell pellets were kept at -80°C until RNA extraction was performed using RNA QIA amp RNA Blood Mini Kit Catalog no. 5230 according to the manufacturer's instructions.

PCR amplification:

cDNA-specific hMAM Taqman™ primer and probe sets were developed using Primer Express® software, using the following primers

The forward primer (ATGAAGTTGCTGATGGTCCTCAT) and the probe (FAM-CGGCCCTCTCCCAGCACTGC-TAMRA) are located on exon 1 and the reverse primer (GTCTTAGACACTTGTGGATTGATTGTCT) on exon 2. The nucleotide sequences of the primers and probes were checked for their specificity in the NCBI BLAST® database.

A ready to use primer and probe set pre-designed by Applied Biosystems (Assay-on demand Gene Expression Product number Hs00267190_m1, SCGB2A2) was also used for the detection of hMAM expression. Commercially available primers and probes for GAPDH and β -Actin mRNA were used for normalization (Applied Biosystems). These probes were labelled with a VIC dye and to avoid competition in the multiplex PCR reaction tubes, the concentrations of the primers were limited (Figure 1).

PCR reactions were performed on The Applied Biosystem Step One Plus Real-Time PCR Systems (Applied Biosystems) using the fluorescent Taqman methodology, using the following thermal cycling protocol: 2 min at 50°C , 10 min at 95°C and 50 cycles of 15 s denaturation at 95°C and 60 s annealing at 60°C .

Histopathologic evaluation

All cases have 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) [15]. Tumor staging was evaluated according to American Joint Committee on Cancer (AJCC) [16].

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 ASCO-CAP Guideline Recommendations [17], considering only positive nuclear staining (Figure 2).

HER-2 immunostaining results were estimated according to ASCO-CAP Guideline Recommendations [18] (**Figure 3**).

Statistical Analysis

Data management and analysis were performed using the Statistical Analysis System (SPSS) software. The study of prevalence of the studied marker among the studied groups was done by using Fisher Exact test.

Comparison of groups with respect to numerical variables was done using the Mann Whitney test.

All *p*-values were two sided. *P*-values ≤ 0.05 were considered significant. Sensitivity, specificity and diagnostic accuracy were the validity measures used for testing the studied parameter as diagnostic tools for breast cancer.

3.Results

The characteristics of 46 newly diagnosed breast cancer patients enrolled in the study are listed in

[Table 1]. Mammaglobin positivity was detected in the peripheral blood of 12 out of the 46 breast cancer patients studied (26%), but not in any of the benign or healthy individuals.

Mammaglobin expression showed statistically significant relations with the positivity of HER2 expression, and the presence of distant metastasis *p*= (0.005) & (0.013) respectively **[Table 2]**

On the other hand, it was statistically non-significant for age, grade, stage, ER or PR positivity in breast cancer patients, size of the tumor, and lymph node involvement with *p*-value= (0.591), (0.627), (0.405), (0.250), (0.143), (0.163), and (0.252) respectively **[Table 2]**

Comparing mammaglobin positivity according to CEA and CA 15.3 concentrations, it was statistically significant with CEA (Carcino-embryonic antigen) with *p*-value (0.001) **[Table 3]**.

Diagnostic performance of mammaglobin is illustrated in **[Table 4]**

Table (1): Patients characteristics:

Age (years)	Mean \pm SD	52.6 \pm 10.7
		N (%)
Histopathologic type	IDC	43 (93.4)
	ILC	1 (2.2)
	Tubular carcinoma	1(2.2)
	Mixed invasive duct & lobular carcinoma	1 (2.2)
Size	≤ 2 cm	17 (36.9)
	$> 2 -5$ cm	29 (63.1)
*Histologic Grade (IDC&tubular carcinoma) (n=44)	I&II	19 (43.2)
	III	25 (56.8)
Stage	I & II	22(48.0)
	III&IV	24(52.0)
Lymph Node involvement	Positive	36 (78.2)
	Negative	10(21.8)
Distant metastasis	Positive	5(10.9)
	Negative	41(89.1)
ER	Positive	34(74.0)
	Negative	12(26.0)
PR	Positive	32(69.5)
	Negative	14(30.5)
HER- 2/ neu	Positive	9(19.5)
	Negative	37(80.5)
Mammaglobin	Positive	12(26)
	Negative	34(74)

IDC (Invasive duct carcinoma), ILC (invasive lobular carcinoma)

ER (Estrogen receptor), PR (Progesterone receptor)

* Grading is applicable for cases with tubular formation (IDC& tubular carcinoma)

Table (2): Comparison between the clinico-pathologic parameters, hormonal status, and HER-2/neu with mammaglobin mRNA

Clinic-pathologic parameters		Mammaglobin positive	Mammaglobin negative	P-value
		(n=12)	(n=34)	
	Age (Mean±SD)	55.75 ± 9.91	51.58 ± 10.74	0.591
Size of the tumor	≤ 2 cm (n=17)	2	15	0.163
	> 2 -5 cm(n=29)	10	19	
Lymph Node involvement	(N1, 2,3) (n=36)	11	25	0.252
	(N0) (n=10)	1	9	
Distant metastasis	Positive (n=5)	4	1	0.013*
	Negative (n=41)	8	33	
Stage	I & II (n= 22)	4	18	0.405
	III & IV (n=24)	8	16	
ER	Positive (n=34)	7	27	0.250
	Negative (n=12)	5	7	
PR	Positive (n=32)	6	26	0.143
	Negative (n=14)	6	8	
HER2/ neu	Positive (n=9)	6	3	0.005*
	Negative (n=37)	6	31	
Histologic Grade (IDC&tubular carcinoma)	(n=44)	(n=12)	(n=32)	
	I &II (n=19)	4	15	0.627
	III (n=25)	8	17	

*Significant

Table (3): Comparison of mammaglobin positivity according to serum levels of CEA and CA 15.3

	Mammaglobin positive (n=12)	Mammaglobin negative (n=34)	P-value
CEA (ng/ml)	8.5 (2.5 - 30.7)	1 (1 - 2)	0.001 *
CA 15.3 (units/mL)	78 (20 - 195)	18 (12 - 100)	0.068

*Significant: Median and interquartile range in parenthesis

CEA: Carcino-embryonic antigen; CA 15.3: Carbohydrate antigen 15.3

Table (4): Measurement of diagnostic performance of mammaglobin

	Mammaglobin
Sen %	26
Sen (95% CI)	(15 -41)
Spe %	100
Spe (95% CI)	(76 -100)
PPV	100
PPV (95% CI)	(73 -100)
NPV	32
NPV (95% CI)	(21- 46)
Diagnostic accuracy %	45

Sen: Sensitivity; Spe: Specificity; PPV: Positive predictive value, NPV: Negative predictive value; DA: Diagnostic accuracy, 95% CI: 95% confidence interval.

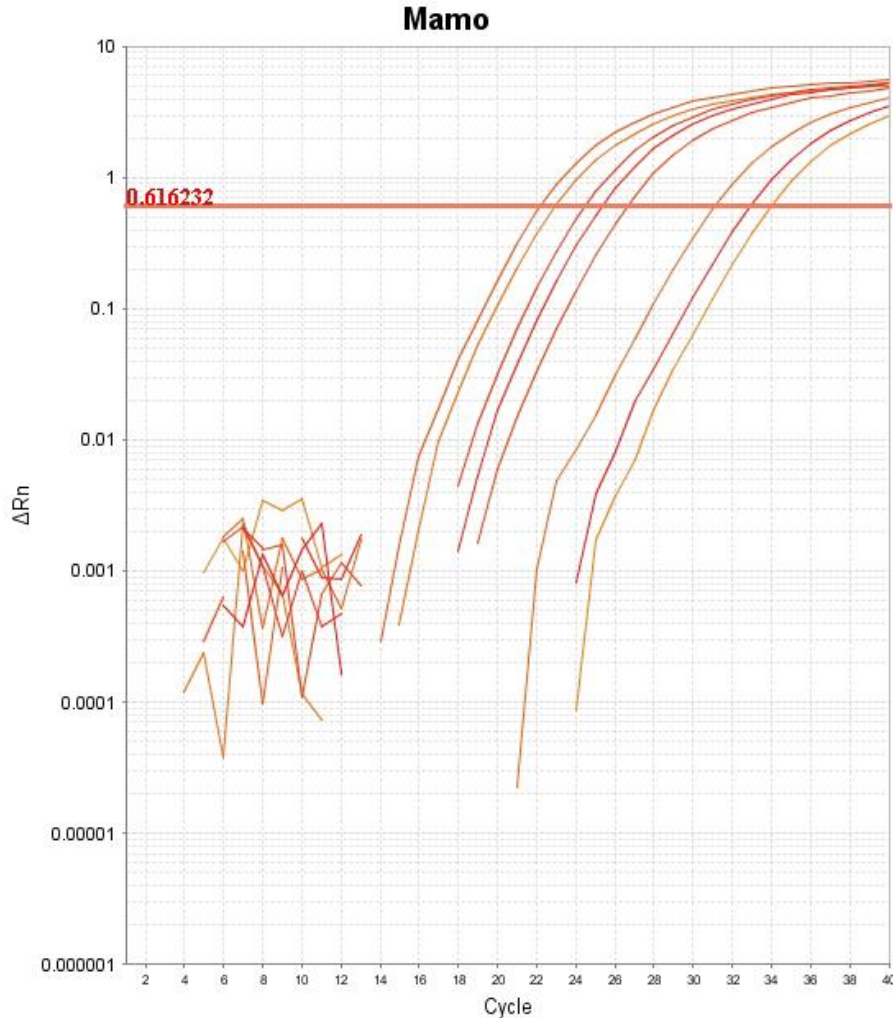


Figure 1: Real time PCR of mamaglobin gene in cancer breast

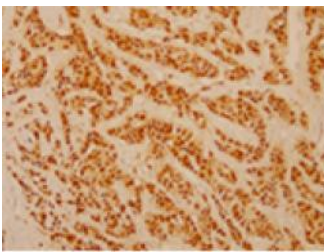


Figure 2: Invasive duct carcinoma grade 2, strongly expressing ER, x200

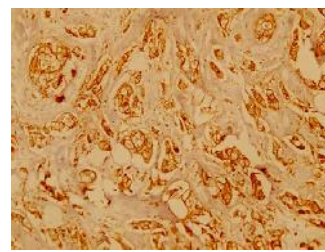


Figure 3: Invasive duct carcinoma grade 2 with strong complete membranous immunoreactivity to HER2, x100

4. Discussion:

The detection of tumor cells in the peripheral blood or in the bone marrow of patients with breast cancer may be a marker for impending disease relapse [19, 20]. At least for some hematological malignancies, treatment of patients in such a state of minimal residual disease can lead to a better clinical outcome. Thus, the availability of sensitive and

specific markers for minimal residual disease may allow the development of treatment strategies for patients with breast cancer and low tumor burden [21, 22].

The identification of markers to distinguish between normal cells, tumorigenic cells and different stages of cancer is of critical importance for cancer diagnosis and prognosis. Detection of cancer-

associated cellular markers is difficult due to the very low numbers of the circulating cancer cells in the blood. Humoral cancer markers, however, are characterized by their appearance in body fluids in amounts exceeding the normal physiologic concentrations. These markers can be released on tumor disintegration or may be secreted by tumors [23].

The frequency reported for mammaglobin expression in breast cancer varies from 10% to 80%; such a broad range might be due to several factors, such as tumor storage methods (fresh/frozen tissue and paraffin-embedded blocks), the different techniques used for assessing the different expression levels (RT-PCR, quantitative real time PCR, immunohistochemical staining, in situ hybridization), tumoral heterogeneity and the specific features of the patient cohorts included in the study [24]. Also the amount of mammaglobin mRNA molecules in peripheral blood samples of breast cancer patients might depend not only on the number of tumor cells but also on the extent of mammaglobin mRNA expression per single cell. Therefore, the individual detection limit for mammaglobin mRNA may vary from patient to patient [25].

In this work, mammaglobin (MG) transcript was detected in 12 out of 46 breast cancer cases (26%), but not in the benign or healthy individuals indicating its high specificity as a marker gene for cells derived from mammary glands. Our results are comparable to studies by *Zach et al.* [25] and *Silva et al.* [26] who reported mammaglobin positivity to be 25% and 24.4% respectively by standard nested RT-PCR.

Lower levels of mammaglobin expression were detected by many researchers: *Gruenewald et al.* [27] and *Michail et al.* [28], both detected (8%) expression of mRNA encoding mammaglobin in 11/133 and 14/175 blood samples from patients with invasive breast cancer respectively. Consistently, studies by *Kaidi et al.* [29], *Roncella et al.* [30] and *Benoy et al.* [31], *Gargano et al.* [32], *Mikhitarian et al.* [33], and *Paola et al.*, [34], reported mammaglobin positivity to be (13.5%, 12%, 14%, 8%, 13.9%, and 13.5%) respectively.

The lower mammaglobin positivity rate of these previous studies may be due to many factors; conducting the study on patients in early stages only, using a small amount of blood sample,

or using different primer sets for mammaglobin.

Other studies have showed mammaglobin - based CTC detection ranging from 41% to 62% [35, 36, 37, and 38]. This may be attributed to the late disease stages of the cases included in these studies.

Data showing the correlation of the mammaglobin transcript level with clinical and pathological prognostic markers is not very clear.

While in some cases,, the mammaglobin over expression did not appear to correlate with histological type, tumor grade, TNM stage or hormone receptor status [30], in others the over expression has been associated with a less aggressive tumor phenotype characterized by a low Ki67 labeling index, low nuclear grade, estrogen and progesterone receptor expression[39].

In this study, on estimating the relation of mammaglobin positivity to tumor size, grade, nodal status and stage in breast cancer patients, no statistically significant results were obtained.

Consistently, many studies couldn't detect any significant association between mammaglobin expression and the grade, the stage and lymph nodes involvement in breast cancer cases [26, 30, 36, 40, and 41]. On studying 40 Egyptian female patients with primary breast cancer, *El Attar et al.*, [41] reported no significant difference between level of plasma mammaglobin mRNA expression and tumor's size. On their research on detection of circulating tumour cells in breast cancer patients using human mammaglobin RT-PCR on their study done on 190 patients with invasive and 12 patients with in situ breast cancer, *Ferro et al.* [42] reported that multivariate logistic regression analysis indicated its association with lymph node involvement ($P=0.009$) and tumor size ($P=0.207$).

There are reports of statistically significant association between mammaglobin based circulating tumor cells detection and tumor size [34,36], clinical stage [24,25], grade[29,33], nodal status [27,34] and distant metastases [25,27] supporting the concept of mammaglobin gene being a poor prognostic indicator.

There is a lack of a solid and agreed upon correlation between mammaglobin expression and the hormone receptors' status and HER-2 receptors in many previous studies [43, 44, 45, 46, and 47]. Other than that, mammaglobin remains an issue for study because of its closeness to breast biology, and also because uteroglobin, one of the members of the secretoglobin family, plays a role in the regulation of progesterone. These steroid hormones have been implicated in the development of breast cancer [48, 49]. Hence, the question of the role of the secretoglobin mammaglobin in breast cancer development remains unanswered and requires more research.

In this study, when studying the prevalence of mammaglobin according to ER, PR, Her2 neu positivity, presence or absence of distant metastasis, the comparison was statistically significant for Her2 neu, and distant metastasis (P value =0.005 and 0.013 respectively).

Similar to our results, **Gruenwald et al. [27]** found that mammaglobin mRNA expression in blood correlated with clinical parameters such as nodal status, and metastasis. They mentioned that mammaglobin transcripts detectable in blood by RT-PCR represent a specific molecular marker for hematogenous spread of breast cancer in his study done on blood samples from 12 patients with ductal carcinoma in situ, 133 patients with invasive breast cancer, 20 patients with hematological malignancies, and 31 healthy volunteers. Also, no associations between mammaglobin positivity in peripheral blood and pathologic and/or molecular (multi-marker RT-PCR) status of axillary lymph nodes were detected by **Kaidi et al. [29]**.

In this study, 20.6% of ER positive tumors and 41.6 % of ER negative tumors were also mammaglobin positive, however this relation did not reach statistical significance.

Mikhitarian et al. [33] postulated that there was a trend of the overexpression of the mammaglobin gene towards ER-negative tumors (21.7% ER negative versus 11.0% ER positive). This trend might denote a higher propensity of ER negative tumor to be mammaglobin positive and to disseminate in circulation as indicated by the detection of mammaglobin in peripheral blood. This might be potentially associated with a higher incidence of metastasis. Similarly, **Paola et al., [34]** found that expression of mammaglobin was significantly correlated with negative ER status but not with PR or HER2/ neu expression in their study done on peripheral blood samples from 190 patients with invasive and 12 patients with in situ breast cancer, before therapy tested for mammaglobin expression by a nested (RT-PCR) assay.

At present, there are no established circulating tumor markers available for clinical use in the determination of cancer susceptibility, screening and diagnosis. Tumor markers currently available lack sensitivity for early cancer detection and specificity for malignancy; therefore, there is a continuing quest to identify a more sensitive and accurate circulating marker specific or applicable for a range of human cancers. The identification of such a marker would be of great clinical value [23].

In our study, comparison of mammaglobin positivity according to serum levels of CEA and CA15.3 revealed significant results only with CEA (p value 0.001). It is known that CEA is a marker used to detect metastasis in different types of cancer, and mammaglobin expression in this study showed significant results when compared to distant metastasis. This proves that mammaglobin could be a reliable marker for detecting metastasis in breast cancer patients. The use of these 2 markers in

combination may be useful in detecting the presence of distant metastasis in breast cancer patients. As regards CA15.3, higher levels were obtained in mammaglobin positive compared to mammaglobin negative patients, although these results didn't reach statistical significance. Further studies on a larger scale are needed to confirm these findings.

Contrary to these results, no correlation was observed with plasma mammaglobulin mRNA levels and both CEA and CA15.3 in a study done by **El Attar et al. [41]** on 40 Egyptian females with primary breast cancer using nested (RT-PCR). On the other hand, **Gruenwald et al. [27]** found that hMAM mRNA expression in blood correlated with CA 15-3 serum levels.

In our study, mammaglobin recorded high specificity (100%) and low sensitivity (26%) which is comparable with the results obtained by **Li et al. [3]** which also showed 100% specificity for mammaglobin. This high specificity makes mammaglobin a potential reliable and non invasive marker to differentiate between breast cancer and benign breast diseases.

There is growing evidence that cancer cells invade peripheral blood in a very early stage of the disease [13]. Only about one per 1000000 - 10000000 will get to and settle in distant organs and a small percentage of those will develop metastasis. Therefore the detection of breast cancer cells in peripheral blood is not equivalent to distant metastasis. Yet, it indicates the possibility of developing metastasis in distant sites of the body. This may be of prognostic significance and will allow monitoring of disease progression. It may also help in the selection of a group of patients who are highly likely to develop systemic disease [10], statement that is supported by **Slade et al. [5]**, who reported that more than half of this subset of patients will later have metastatic disease even after radical surgery. Thus, follow up for this subset of patients is necessary to clarify whether the positive results obtained are related to the existence of occult metastasis [4].

Eight out of 12 (67%) breast cancer cases showing positive mammaglobin expression had no distant metastasis at the time of this study. Strict follow up of these patients for developing metastasis is recommended, as early interference may save or at least improves the quality of their lives.

When evaluating the potential usefulness of mammaglobin expression as a marker of disseminated breast cancer, one should be aware that the number of mammaglobin transcripts in the peripheral blood depends not only on the number of cancer cells in circulation but also on mammaglobin expression level in neoplastic cells. Its prognostic

value especially in clinically localized disease must be evaluated after long-term clinical follow-up of the patients [12].

Several groups of researchers proposed a number of therapeutic strategies targeting mammaglobin molecule. Some of the strategies are based upon the hypothesis that mammaglobin is associated with the surface of breast cancer cells. **Zuo et al. [50]** study showed that some of the mammaglobin proteins are directly associated with the surface of breast cancer cells; therefore, it may be utilized as a useful molecular marker for breast cancer targeted drug delivery. **Tiriveedhi et al. [51]** could identify HLA-A24-restricted CD8 (+) cytotoxic T-cell epitopes derived from mammaglobin, which offers a novel therapeutic target as a breast cancer vaccine.

In conclusion, our preliminary results of the analysis of mammaglobin expression level in breast cancer blood samples obtained from Egyptian breast cancer patients showed that mammaglobin expression is mammary specific and may define a unique phenotype to a subset of breast cancer. It also showed significant correlations with a number of prognostic factors of breast cancer which makes it a potential prognostic marker. Its high specificity may qualify it to be a reliable non invasive marker to differentiate between benign and malignant breast diseases, but its low sensitivity makes it unsuitable for early diagnosis of breast cancer. Its high expression among the metastatic breast cancer patients may qualify it to be a predictor marker for developing metastasis among breast cancer patients. Combination of both CEA and mammaglobin may be used as a panel for detecting distant metastasis among Egyptian breast cancer patients.

Additional studies of long-term clinical follow-up and larger sample sizes are needed to clarify the biomolecular, diagnostic and prognostic role of mammaglobin in breast cancer progression.

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