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Detection and quantification of mammaglobin in the blood of breast cancer patients: can it be useful as a potential clinical marker? Preliminary results of a GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study

G. Gargano[1#], V. Agnese[1#], V. Calò[1], S. Corsale[1], C. Augello[1], L. Bruno[1], L. La Paglia[1], A. Gullo[2], L. Ottini[3], A. Russo[1], F. Fulfaro[1], G. Rinaldi[1], A. Crosta[1], G. Cicero[1], O. Majorana[1], L. Palmeri[4], C. Cipolla[5], A. Agrusa[6], G. Gulotta[6], V. Morello[2], G. Di Fede[1], V. Adamo[7], G. Colucci[8], R. M. Tomasino[2], M. R. Valerio[1], V. Bazan[1] & Antonio Russo[1]*

[1]Section of Medical Oncology and [5]Section of Surgical Oncology, Department of Surgical and Oncology,Università di Palermo; [2]Cattedra di Oncologia Medica, Deparment od Surgery and Oncology, Università di Palermo; [3]Department of Human Pathology, Università di Palermo; [4]Dipartimento di Medicina Sperimentale e Patologia, Università di Roma 'La Sapienza'; [6]Unit of General Surgery, Università di Palermo; [7]Unit of Medical Oncology, A.O.Universitaria Policlinico 'G.Martino', Università di Messina; [8]Division of Medical Oncology, National Institute of Oncology, Bari, Italy

Background: Mammaglobin is expressed mainly in mammary tissue, overexpressed in breast cancer (BC) and rarely in other tissue. The aim of this study was to assess the sensitivity and specificity of transcript MGB1 detection and to evaluate the role of MGB1 as potential clinical marker for the detection of disseminated cancer cells in the blood of BC patients.

Patients and methods: A consecutive series of 23 BC tissues, 36 peripheral blood BC samples and 35 healthy peripheral blood samples was prospectively recruited to investigate MGB1 expression by means of a quantitative Real Time RT-PCR assay.

Results: MGB1 overexpression in tissue samples of BC patients is significantly associated only with high level of Ki67 ($P < 0.05$). None of the samples from peripheral blood of 35 healthy female individuals were positive for MGB1 transcript. In contrast MGB1 mRNA expression was detected in three of 36 (8%) peripheral blood of BC patients.

Conclusions: Our preliminary results demonstrate that the detection of MGB1 transcript in peripheral blood of BC patients was specific but with low sensitivity. MGB1 overexpression by itself or in combination with Ki67 might be considered an index of BC progression.

Key words: mammaglobin (MGB1), breast cancer, disseminated cancer cells, real time RT-PCR

introduction

Breast carcinoma (BC) is the most common type of neoplasia found in women from highly industrialized countries in the Western world [1]. At the moment of diagnosis, most patients with BC do not present metastases and can therefore be operated on with high hopes of a favorable outcome. Nevertheless, in spite of the strong probability of operative success, 30% of stage I and II patients subsequently regress and die from the disease [2].

Different approaches, both cellular and molecular, have been used for the detection of tumor cells in the blood and it is generally maintained that the technique based on PCR are more sensitive [3].

In spite of the fact that it has been shown that many circulating cells are apoptotic [4], a fraction of these 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. One of the main problems of this type of analysis is the identification of a specific gene target whose expression is associated with the tumor cells and not to an illegitimate transcription of non-epithelial cells [5].

The gene mammaglobin (MGB1) is a member of the uteroglobin family, localized on chromosome 11q12-13; it codifies for a glycoprotein of 23 amino acids whose function is still unknown [6]. This gene is often expressed at basal levels in normal breast epithelium and overexpressed in 23% of

*Correspondence to: Dr A. Russo, Section of Medical Oncology, Department of Oncology, Università di Palermo, Via del Vespro 127, 90127 Palermo, Italy. Tel: +39-091-6552500; Fax: +39-091-6554529; E-mail: lab-oncobiologia@usa.net

#G. Gargano and V. Agnese have equally contributed to this work.

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primary breast tumors. This overexpression does not appear to be correlated with histology, tumor grade, tumor stage or hormone receptor status [7–9]. However, most recent studies have evaluated a series of breast cancer specimens and have reported a correlation between high levels of mammoglobin and expression of estrogen and progesterone receptor, diploid DNA content, low Ki67 labeling index, low nuclear grade and the absence of axillary nodal invasion [10, 11].

The frequency of MGB1 mRNA detection in peripheral blood samples is variable. In fact, while several studies showed that MGB1 mRNA could be detected by RT-PCR in the blood of up to 25% of breast cancer [12, 13], others, using the real time TaqMan RT PCR approach, identified a MGB1 transcription level in only 11% of blood samples [14].

The aim of this study was to assess prospectively mammaglobin expression by means of a quantitative RT-PCR assay in a consecutive series of 22 BC tissues, correlating the resulting expression data with available clinical pathological parameters to clarify the biological role of mammaglobin in BC. Furthermore, the analysis conducted in this study was used to test the sensitivity and specificity of mammaglobin transcript detection by quantitative real time RT-PCR to evaluate the role of MGB1 as a potential clinical marker for the detection of disseminated cancer cells in the blood of BC patients.

patients and methods

specimen collection

BC patients who had undergone mastectomy at the Department of Oncology, University of Palermo, Italy were consecutively recruited for a prospective study. Tumor and corresponding normal tissue were obtained from surgical specimens ($n = 23$). After surgery, the tissue was immediately submerged in RNA*later* (Sigma, St Louis, MO USA) in order to inhibit ribonuclease and stabilize the RNA, which was then stored at −80°C. In order to avoid evaluator variability in the patients, all resection specimens were meticulously examined by two independent pathologists who were not aware of the original diagnosis and of the results of the molecular analyses. All tumors were histologically confirmed to be BC.

About 2.5 ml of peripheral blood were collected from each of 36 women with BC and of 35 healthy women, in Paxgene blood RNA tubes (Qiagen, Hilden, Germany) containing an RNA stabilizing solution. Written informed consent was obtained from all the patients recruited for the study.

RNA extraction and cDNA synthesis

Total RNA from peripheral blood was extracted with the use of the PAXgene blood RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, while total cellular RNA was taken from about 30 mg of tissue, both tumoral and normal, with the Rneasy Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was stored at −80°C until further use.

RNA integrity was verified on the Agilent 2100 bioanalyzer with the use of the RNA 6000 Nano assay protocol (Agilent Technologies, Palo Alto, CA, USA). Only samples with an RNA integrity number (RIN) between 8 and 10 were analyzed.

For cDNA synthesis 5 μg of total RNA were reverse transcribed in a final volume of 50 μl with a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The samples were incubated for 10 min at 25°C and for 2 h at 37°C on the GeneAmp 9700 Applied Biosystem (Applied Biosystems, Foster City, CA).

quantitative determination of MGB1 by real time RT-PCR

Real time RT-PCR was performed with the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) using the TaqMan method, which takes advantage of the 5′–3′ exonuclease activity of the Taq DNA polymerase. In this assay, over the two primers, a fluorescent probe containing a reporter dye at the 5′ end and a quencher dye at the 3′ end was added to the reaction. During PCR, the AmpliTaq Gold enzyme (Applied Biosystems, Foster City, CA) degrades the probe by separating the reporter dye from the quencher dye, which results in increased reporter fluorescence. The accumulation of amplificate is measured by monitoring the actual fluorescence during the exponential PCR phase by means of a charge-coupled device (CCD) camera. Quantification is performed using the threshold cycle (Ct), which is the cycle during which a significant increase in fluorescence is first detected.

For the detection of MGB1 and for normalization the following predesigned primer and probe set were used: assay-on-demand Gene Expression Product, number Hs00267190 m_l, SCGB2A2, and assay-on-demand Gene Expression Product, number 4333763F, Hu-PPIA, (Applied Biosystems, Foster City, CA). For the PCR, 100 ng of cDNA in a final volume of 50 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used according to the manufacturer's guidelines.

Each sample was analyzed in triplicate and the mean quantity of each triplicate calculated by the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA).

In our study, the comparative Ct method was used to quantify the relative gene expression with the formula $2^{-\Delta\Delta Ct}$, using cyclophilin A (Hu-PPIA) as the endogenous control and normal tissue as a calibrator.

statistical analysis

Statistical analyses were performed separately for tissues and blood samples of BC patients. Associations between mRNA mammaglobin expression level and clinico-pathological variables were evaluated by the chi-square test with Yates correction, where appropriate.

results

Patients were all female with a mean age for BC tissue patients of 63.3 years (range 35–81) and for BC peripheral blood patients of 58 years (range 38–82). Table 1 shows the clinicopathological features of BC patients.

mammaglobin mRNA expression in breast tissue

RNA extracted from 22 samples of BC tissues was analyzed for MGB! mRNA expression by Real Time RT-PCR. All samples were positive for the MGB1 transcript with regard to normal breast tissue and 11/22 (50%) showed overexpression (above the median for the whole group) (Figure 1).

We used the detection of PPIA mRNA in all reactions to exclude the fact that the absence of MGB1 expression was due to degraded RNA.

The statistical analysis of MGB1 mRNA expression in tissue samples showed that it is associated with higher Ki67 value but not with histological type, tumor grade, TNM stage and 165 steroid hormone receptor status (Table 2).

mammaglobin mRNA expression in peripheral blood samples

None of the samples from the peripheral blood of 35 healthy female patients was positive for MGB1 transcript, while MGB1

Table 1. Clinicopathological features of breast cancer (BC) patients

Clinicopathological features	BC tissue patients *n* (%)	BC peripheral blood patients *n* (%)
Age (years)		
≤50	6 (27)	11 (31)
>50	16 (73)	25 (69)
Mean age in years (range)	63.3 (35–81)	58 (38–82)
Tumor histotype		
Infiltrating ductal	20 (91)	24 (67)
Infiltrating lobular	0	10 (27)
Infiltrating ductal and lobular	2 (9)	1 (3)
Medullar	0	1 (3)
Lymph node metastasis		
Negative	11 (50)	19 (53)
Positive	11 (50)	17 (47)
Histological grade		
Well-differentiated (G1)	2 (9)	1 (3)
Moderately-differentiated (G2)	11 (50)	20 (55)
Poorly-differentiated (G3)	9 (41)	15 (42)
Estrogen receptors		
Negative	8 (36)	13 (36)
Positive	14 (64)	23 (64)
Progesterone receptors		
Negative	8 (36)	13 (36)
Positive	14 (64)	23 (64)
c-ERB		
Negative	11 (50)	19 (53)
Positive	11 (50)	17 (47)
Ki67		
≤20 %	10 (45)	19 (53)
>20 %	12 (55)	17 (47)
Total	22	36

mRNA expression was detected in three of 36 (8%) of peripheral blood BC patients (Table 3). We used the detection of PPIA mRNA in all reactions to exclude the fact that the absence of hMAM expression was due to degraded RNA. Figure 2A shows the amplification plot for MGB1 expression of one of the three positive samples, while Figure 2B shows the MGB1 expression plot of all the three positive samples.

discussion

Even though a great deal of progress has been made in the chemotherapy strategies used in BC patients, especially in advanced tumors, leading to considerable improvement in life style and survival rate, surgical resection remains the most effective treatment for avoiding disease recurrence. Even so, the post-operative clinical course of these patients may be negatively affected by the presence of micrometastases circulating in peripheral blood, not easy to detect with traditional anatomopathological techniques [15].

The main clinicopathological factor to take into consideration in BC treatment is the TNM staging. In particular, a key role is represented by the T and N values, which indicate tumor size and lymph node involvement, thus giving an idea of tumor aggressiveness [16].

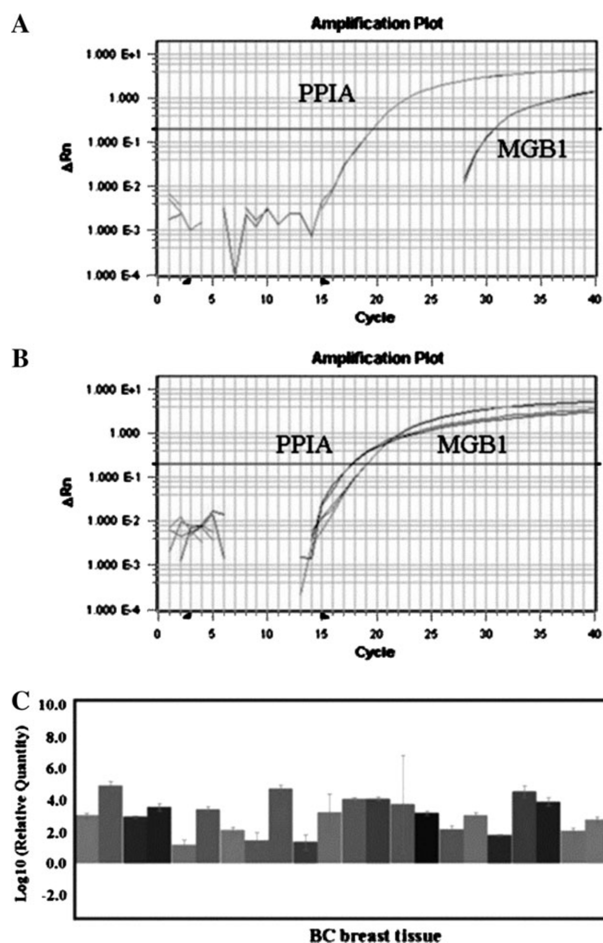

Figure 1. Amplification plot obtained by the ABI PRISM 7900 HT for MGB1 and PPIA mRNA expression in BC tissue samples relatives to the tissues with minor (**A**) and major (**B**) MGB1 expression. (**C**) MGB1 expression plot of 22 BC tissue samples.

Table 2. Association between mammaglobin expression and Ki67 value in 22 breast cancer tissues

Ki67	Low *n* (%)	High *n* (%)	*P* value
≤20%	6 (55)	3 (27)	
>20%	2 (18)	8 (73)	
Unknown	3 (27)	0	
Total	11	11	<0.05

Table 3. Mammaglobin expression in peripheral blood samples of healthy volunteers and breast cancer (BC) patients

	Healthy control *n* (%)	BC peripheral blood sample *n* (%)
	0 (0)	3 (8)
Total	35	36

The relationship between circulating tumor cells and the development of metastatic disease is still not fully understood. In the last few years, a great many biomolecular techniques have been developed and numerous molecular markers have been

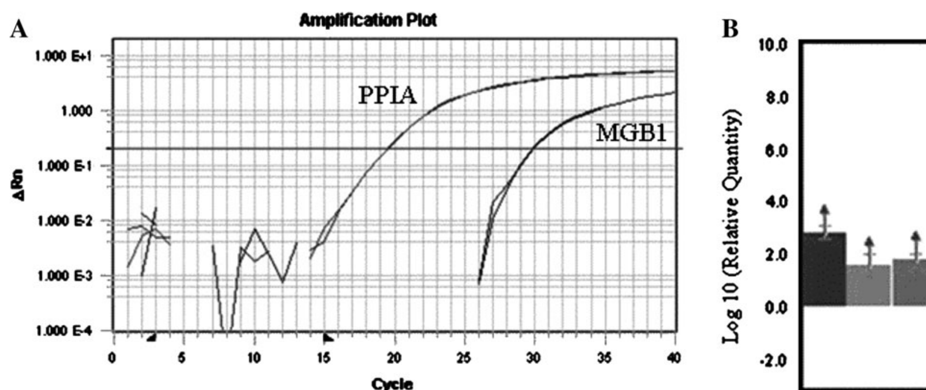

Figure 2. (**A**) Amplification plot obtained by the ABI PRISM 7900 HT for MGB1 and PPIA mRNA expression in one of the three BC peripheral blood sample. (**B**) MGB1 expression plot of three positive BC peripheral blood samples.

identified, making it possible for a clearer classification of a subgroup of BC patients who might show a more favorable prognosis and therefore benefit from more specific drug therapy [17].

The aim of this study was first to analyze the expression level of the mammaglobin (MGB1) gene in BC tissue samples of BC patients and in the blood samples of BC patients at any stage of the disease, in order to identify a possible marker of cancer aggressiveness and to consider it as a possible index of BC progression.

The frequency reported for MGB1 expression in BCs varies from 20% 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 [7, 9]. For example, Watson et al. reported overexpression of mammaglobin in only 23% of the breast cancer tissues analyzed. This probably depends on the low sensitivity of the method utilized, which was the Northern blot hybridization [8]. In our study, the analysis of tissue samples has shown that, compared with normal breast tissue, all the BC tumors also had MGB expression and 50% (11/22) of the samples showed overexpression. Of these, five of 11 (45%) were node-positive and six of 11 (54%) were node-negative tissues. The follow-up information of the patients showing MGB1 overexpression are related to a very short time, and do not allow for statistical analyses.

Compared with reports in literature, our study shows a lower rate of MGB1 transcript alteration in the blood samples. Suchy et al. reported the detection of MGB1 expression in only 11/98 (11%) blood samples of BC patients using the real time TaqMan RT-PCR approach [14]. In our study no MGB1 expression was ever detected in the 35 peripheral blood samples obtained from healthy volunteers. This result reinforces the notion that MGB1 is a specific marker in the identification of BC cells in peripheral blood. Furthermore, MGB1 expression was detected in only three of 36 (8%) blood samples obtained from BC patients at any stage. However, the absence of MGB1 transcript in blood samples does not exclude the presence of circulating tumor cells; this low rate of MGB1 expression might possibly have been compromised by the small amount of blood tested in each reaction, or by the number of cells present in the peripheral blood or by the MGB1 expression level for single cells [18].

Recent data also show that the correlation of the MGB1 transcript level with clinical and pathological prognostic markers is not very clear. While in some cases, in fact, the MGB1 overexpression did not appear to correlate with histological type, tumor grade, TNM stage or hormone receptor status [8], in others the overexpression has been associated with a less aggressive tumor phenotype characterized by a low Ki67 labeling index, low nuclear grade, estrogen and progesterone receptor expression [10]. In our study the MGB1 overexpression in the tissue samples of BC patients seemed to be related only to the high level of the proliferative index Ki67. The role of mammaglobin in cancer progression is still unclear [6]. The finding that MGB1 expression is associated with mammary gland proliferation and terminal differentiation [19] might explain our results, if it is considered as strictly correlated with a process of differentiation brought about by malignant transformation.

In conclusion, our preliminary results of the analysis of MGB1 expression level in BC tissue and blood samples obtained from BC patients show that MGB1 expression is mammary-specific and may define a unique phenotype to a subset of BC. Moreover, MGB1 overexpression, by itself or in combination with Ki67, might be considered a variable index of BC progression in BC tissues. Additional studies of a long-term clinical follow-up and larger samples are needed to clarify the biomolecular, diagnostic and prognostic role of mammaglobin in BC progression.

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