Mammaglobin: A novel tumor marker for breast cancer

SONIA LABIB EL-SHARKAWY¹, WAFAA EL SAIED ABD EL-AAL¹, MARWA ABD EL MONAEM EL-SHAER¹, NAGLAA FATHY ABBAS¹, MONA FATHY YOUSSEF²

¹National Research Centre, Medical Division, Department of Pathology, Giza, ²Ain Shams University, Department of Clinical Pathology, Cairo-Egypt

ABSTRACT

Breast cancer is a major problem among females all over the world. Despite apparent curative resection, subsequent development of metastatic spread presents a major clinical problem in about 30% of all breast cancer patients. The aim of this study was to investigate the clinical reliability of mammaglobin m-RNA (MAG m-RNA) as a marker of circulating cancer cells in breast cancer patients and to study the relevance of its expression in blood and expression of its protein in breast tissues, with the pathological parameters and its value in evaluating efficiency of treatment. This study was conducted on 48 breast cancer patients and 28 controls (10 healthy controls and 18 patient controls: 6 with fibroadenoma, 4 with uterine carcinoma, 4 with ovarian carcinoma and 4 with colon cancer). For histopathological study, the healthy control group included the normal breast tissue adjacent to fibroadenoma. All breast cancer patients were of the infiltrating ductal carcinoma type and 10 of them had associated areas of intraductal carcinoma. The patient group was classified into 26 patients with localized breast cancer and 22 patients with metastases (9 patients had axillary lymph node metastases and 13 patients had distant metastases). Breast cancer patients were reclassified according to the histologic grade into grade I (8 patients), grade II (26 patients) and grade III (14 patients). All individuals included in this study were subjected to detection of MAG m-RNA in circulating tumor cells in peripheral blood using nested PCR technique. Breast tissue expression of MAG was investigated using immunohistochemistry. Blood and tissue MAG expression were correlated with estrogen receptor and Ki-67 proliferation index. Circulating MAG m-RNA is a highly specific (100%) tumor marker. The detection rate was significantly associated with the histologic grades, ER positivity and low proliferative rate of tumors. The detection rate declines after receiving chemotherapy. Immunohistochemically, the pattern of expression of MAG in breast cancer tissues was characteristically different than that in non-cancer tissues (being diffuse cytoplasmic in the former and scattered in the latter). MAG overexpression in breast tissue was significantly higher in low grade tumors (I and II) than in high grade ones (III). The strong staining intensity was more frequently detected in low grade tumors. Also MAG expression in breast tissue was significantly correlated with ER positivity and low Ki-67 proliferation index of the tumors. MAG is a promising specific tumor marker of breast cancer that could predict the prognosis of breast cancer and its response to hormonal treatment. [Turk J Cancer 2007;37(3):89-97]

KEY WORDS:

Breast carcinoma, immunohistochemistry, PCR, mammaglobin, Estrogen receptor, Ki-67

INTRODUCTION

Breast cancer is a major problem among females all over the world. It is a heterogeneous disease with a varying propensity for spread (1). Despite all efforts done during the past years, the incidence of breast cancer mortality is still rising and represents the leading cause of death in women in their mid-life (2).
At the moment of diagnosis, most patients with breast cancer do not present metastasis and can therefore be operated on with high hopes of a favorable outcome. Nevertheless, despite apparent curative resection, subsequent development of metastatic spread presents a major clinical problem in about 30% of all breast cancer patients (3). For this reason, the detection of circulating carcinoma cells has been suggested to be an important predictor of systemic progression (4).

In recent years, PCR technique has been used as a mean of detecting circulating carcinoma cells. Epithelial markers which have been used include cytokeratins, epidermal growth factors receptor or c-erbB2. However, these markers are not specific for tumor cells (5). Mammaglobin (MAG) is one of the recent markers understudy nowadays (4).

The MAG gene is a member of uteroglobin family, localized on chromosome 11q12-13. It codifies for a glycoprotein of 23 amino acids (6). This gene is often expressed at basal levels in normal breast and its overexpression was assumed to be present in breast cancer (5).

The aim of this study was to investigate the clinical reliability of MAG m-RNA as a marker of circulating cancer cells in breast cancer and to study the relevance of its expression in blood and its protein expression in breast tissue, with the histologic grades, metastases, estrogen receptor status and Ki-67 proliferation index. Moreover, this study aimed to clarify the value of MAG m-RNA in evaluating the efficiency of treatment.

**MATERIALS AND METHODS**

**Subjects**

**Patient group**

This study was conducted on 48 breast cancer female patients. Their ages ranged from 25 to 60 years. They were referred to the surgery clinics of Ain Shams University Hospital. The diagnosis of breast cancer was based on radiology, mammography, tissue biopsy for histopathological examination, ultrasonography and bone scan for detection of secondaries. The carcinoma cases were of infiltrating ductal carcinoma type and 10 of them showed associated areas of intraductal carcinoma. The patient group was classified into two groups:

1. Localized breast cancer group: This included 26 patients with localized breast cancer, with no evidence of distant metastases.
2. Metastatic group: This group included 22 patients suffering from metastatic breast carcinoma. This group was subdivided into:
   a. Those with axillary lymph node metastases (9 patients).
   b. Those with distant metastases (13 patients).

To study the effect of treatment, 10 patients of the metastatic group who have MAG m-RNA positive cells were selected prospectively after receiving their course of chemotherapy.

Histopathologically, all breast cancer patients were graded according to the system of Bloom and Richardson (7) which was recommended by WHO (8) into:

- Grade I (Well differentiated): It included 8 patients.
- Grade II (Moderately differentiated): It included 26 patients.
- Grade III (Poorly differentiated): It included 14 patients.

**Control group**

This group included 28 subjects: 10 healthy volunteers served as healthy control group and 18 patients served as patient control group (6 patients with fibroadenoma, 4 patients with uterine carcinoma, 4 patients with ovarian carcinoma and 4 patients with colon cancer).

For histopathological study, the healthy control group included the normal breast tissue adjacent to fibroadenomas.

**Sampling**

**Blood samples**

Under complete aseptic conditions, 5 mL of venous blood was collected by venipuncture in sterile EDTA-treated tubes (Bekton Dickenson, OK) from all controls and breast cancer patients before initiation of the first line chemotherapy and/or hormonal treatment. In case of metastatic group, another blood sample was collected from 10 patients after receiving chemotherapy and hormonal treatment.
Tissue biopsies

Breast tissue samples were fixed in 10% formaldehyde, routinely processed to paraffin blocks, then 5 µm thick sections were prepared and stained with hematoxylin and eosin stain for histopathological examination.

Analytical procedures

Nested RT-PCR

Nested RT-PCR for detection of circulating MAG-mRNA was performed as follows:

1) RNA extraction from venous blood sample was immediately performed after sample collection using QIAamp RNA blood kit (QIAGEN Inc., USA). The extracted RNA was diluted prior to its assay. The concentration and purity of RNA extracts were determined by measuring their absorbance at 260 nm (A260) and 280 nm (A280) using a spectrophotometer. Pure RNA has an A260/A280 ratio of 1.6-1.9. An absorbance of 1 µ unit at 260 nm corresponds to 40 µg RNA/mL. The concentration of RNA stock was then determined (concentration of RNA stock = 40 RNA µg/mL x A260 x dilution factor), then the total yield was calculated by multiplying concentration by volume of stock in mL.

The primer and reaction parameters for nested RT-PCR were chosen according to:

- The first primer pair was:
  (5’-CCACCCATGGCAAATTCCATGGCA-3’) and (5’-TCTAGACGGCAGGTCAGGTCCACC-3’).

- The second primer pair used in nested PCR reaction included, the inner upstream primer, which was:
  (5’-AGCACTGCTACGCAGGCTCT-3’) and the downstream primer, which was:
  (5’-ATAAGAAAGAAGTGTTGG-3’).

2) The RNA samples were subjected to reverse transcription and the RNA samples amplification using QIAGEN one-step RT-PCR Enzyme Mix Kit (QIAGEN Inc., USA). Nested PCR was done using Taq PCR Master Mix Kit (QIAGEN Inc., USA).

3) The amplified products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide. A DNA molecular weight marker XIII was also run to identify the site of bands. The band if present was compared to the DNA marker for the site of target DNA that was 202 bp products (Figure 1).

Immunohistochemical study

Streptavidin-biotin technique was used to investigate mammaglobin (MAG), estrogen receptors (ER) and Ki-67 expression. Three slides from each case were deparaffinized, hydrated and incubated in 3% hydrogen peroxide for 30 minutes to block the internal peroxidase activity. Antigen retrieval was done by microwave pretreatment for 10 minutes in 0.01 citrate buffer. For each case, one slide was incubated with anti-mammaglobin monoclonal antibody (Dako Corporation) at a dilution 1:100, the second slide was incubated with mouse monoclonal antibody to ER at a dilution 1:50 (Dako Corporation) and the third one was incubated with MIB1 (Ki-67) at 4 °C overnight. Sections were then washed twice for 5 minutes with PBS and incubated for 10 minutes with biotinylated secondary antibody (Dako Cytomation). The slides were washed twice for 5 minutes in PBS and incubated for 10 minutes in performed avidin-biotin-peroxidase complex (Dako Cytomation). Chromogen development was accomplished by immersion of the sections in 2, 3-Diaminobenzidin tetrahydrochloride (BAB) (Dako Cytomatin) for 5 minutes. The nuclei were counterstained with hematoxylin, dehydrated, cleared and mounted. For negative controls, the primary antibody was omitted and replaced with PBS. Mammaglobin gives cytoplasmic staining while ER and Ki67 appear as nuclear staining (Figures 2, 3).

According to Han et al. (9), the intensity of mammaglobin expression were scored as no staining, weak, moderate and strong staining.

Ki-67 immunostaining was evaluated using Leica Image Processing and Analysis System. In each case, the analysis was done on areas expressing quantitatively the highest number of immunoreactive nuclei (10-20 microscopic fields at x400 magnification were measured for each case). The results were expressed as Ki-67 proliferation index which is defined as the percentage of positively stained nuclei divided by the total number of the counted nuclei. Ki-67 proliferation index was either ≤20 or >20.
According to Eerola et al. (10), estrogen receptor was considered positive when ≥ 10% of cells were stained.

Blood and breast tissue MAG expression were correlated with tissue estrogen receptors and Ki-67 expression.

**Statistical analysis**

Statistical analysis was done using SPSS software package. Results were expressed as number and percentages. The comparison of the frequency of positivity of MAG between the different groups was done by Chi-Square test ($\chi^2$ test) or Fisher exact test where necessary. A p-value of less than 0.05 was considered statistically significant.

The diagnostic performance of MAG mRNA was evaluated. The diagnostic sensitivity, specificity, positive predictive value, negative predictive value and the diagnostic efficiency were calculated.

**RESULTS**

Circulating MAG m-RNA positive cells were not detected in either patient controls nor the healthy controls but were detected in 31 breast cancer patients (64.6%) with highest detection in tumors with low grade, ER positivity and low Ki-67 proliferation index (<20) (Table 1).

Histopathological examination of breast tissues revealed that normal breast tissue and fibroadenoma cases showed lower MAG expression than breast carcinoma cases where scattered positive epithelial cells were seen within the acini with moderate or weak staining intensity. All tissue specimens from uterine, ovarian and colonic carcinoma were negatively stained for MAG.

MAG overexpression in breast tissue was detected in 79% of the carcinoma cases (38 out of 48 cases) where diffuse cytoplasmic staining was seen. All areas of intraductal carcinoma were diffusely stained for MAG (Figure 4). MAG positivity was significantly correlated with tumor grading when comparing high grade tumors (III) with low grade ones (I and II) (Table 1). Also strong staining intensity was significantly correlated with the histologic grade where it was detected in 75% of grade I and only in 17% of grade III. On the contrary, weak staining was not detected in grade I cases, while 50% of grade III were weakly stained (Figure 5). MAG tissue expression was not significantly correlated with the clinical stage.

Table 2 showed the comparative statistics of the frequency of MAG m-RNA expression in peripheral blood and MAG protein expression in breast tissue in breast cancer patients in correlation with pathological grade, ER expression and Ki-67 proliferation index using chi-square test. A significant association of MAG expression was encountered in low grade (Grade I & II), ER positive tumors and tumors with low Ki-67 proliferation index (≤20).

On studying the influence of chemotherapy on the detection of MAG positive cells in peripheral blood of breast cancer patients with metastases, there was a statistically significant decline in the number of patients with MAG positive cells in peripheral blood following chemotherapy and hormonal treatment (Table 3).

Table 4 showed the diagnostic performance of inoculating MAG m-RNA expression in breast cancer patients with a specificity of 100%.

**DISCUSSION**

Even though great deal of progress has been made in the chemotherapy strategies used in breast cancer 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 postoperative clinical course of these patients may be negatively affected by the presence of micrometastases circulating in peripheral blood, not easily to be detected with traditional techniques (3,10,11).

The relationship between circulating tumor cells and the development of metastatic disease is still not fully understood. In the last few years, many biomolecular techniques have been developed and numerous molecular markers have been identified. One of these markers is MAG which is the matter of researches nowadays (3,4,11,12).

In the present study, MAG m-RNA positive cells were detected in patients with breast cancer of varying stages and the detection rates were going hand in hand with the progress of the disease. They were detected in the blood of 50% of patients with localized breast cancer and 81.8% of patients with metastasis. In this group, MAG positive cells were detected in 77.8% of patients with axillary lymph node metastasis and 84.6% of pa-
### Table 1
**Descriptive statistics of the frequency of MAG expression in peripheral blood and breast tissue in different patient groups**

<table>
<thead>
<tr>
<th></th>
<th>MAG (+) in PB n (%)</th>
<th>MAG (+) in BT n (%)</th>
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<tbody>
<tr>
<td><strong>Staging</strong></td>
<td></td>
<td></td>
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<tr>
<td>All patients (n=48)</td>
<td>31/48 64.6%</td>
<td>38/48 79%</td>
</tr>
<tr>
<td>Localized (n=26)</td>
<td>13/26 50%</td>
<td>20/26 76%</td>
</tr>
<tr>
<td>Metastatic (n=22)</td>
<td>18/22 81.8%</td>
<td>18/22 81%</td>
</tr>
<tr>
<td>LN (n=9)</td>
<td>8/9 88.9%</td>
<td>7/9 77.8%</td>
</tr>
<tr>
<td>Distant (n=13)</td>
<td>11/13 84.6%</td>
<td>11/13 85%</td>
</tr>
<tr>
<td><strong>Histologic grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I (n=8)</td>
<td>7/8 87.5%</td>
<td>8/8 100%</td>
</tr>
<tr>
<td>Grade II (n=26)</td>
<td>20/26 76.9%</td>
<td>24/26 92%</td>
</tr>
<tr>
<td>Grade III (n=14)</td>
<td>4/14 28.6%</td>
<td>6/14 43%</td>
</tr>
<tr>
<td><strong>ER status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (n=28)</td>
<td>28/28 100%</td>
<td>28/28 100%</td>
</tr>
<tr>
<td>Negative (n=20)</td>
<td>3/20 15%</td>
<td>10/20 50%</td>
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<tr>
<td><strong>Ki-67 proliferation index</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 20 (n=22)</td>
<td>19/22 86.4%</td>
<td>20/22 91%</td>
</tr>
<tr>
<td>&gt; 20 (n=26)</td>
<td>12/26 46.2%</td>
<td>18/26 69%</td>
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PB: Peripheral blood; BT: Breast tissue; LN: Lymph node

### Table 2
**Comparative statistics of the frequency of MAG expression in peripheral blood and breast cancer tissue in breast cancer patients in correlation with pathological grade, ER expression and Ki-67 proliferation index using \( \chi^2 \) test**

<table>
<thead>
<tr>
<th></th>
<th>Peripheral Blood</th>
<th>Breast Cancer Tissue</th>
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<tbody>
<tr>
<td></td>
<td>( \chi^2 )</td>
<td>P</td>
</tr>
<tr>
<td>Grade I vs. II</td>
<td>2.035</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Grade I vs. III</td>
<td>4.361</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Grade II vs. III</td>
<td>4.679</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Grade I + II vs. III</td>
<td>5.617</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ER (+) vs. ER (–)</td>
<td>10.52</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ki-67 &lt;20/&gt;&gt;20</td>
<td>6.109</td>
<td>&lt; 0.001</td>
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P > 0.05: Non-significant difference
P < 0.05: Significant difference
P < 0.01 & 0.001: Highly significant difference
patients with distant metastasis. Our results were in accordance with those of Gargano and his colleagues (4) who detected circulating MAG m-RNA positive cells in 52% of patients with localized breast cancer, 75% of patients with regional lymph node metastasis and 86% of patients with distant metastasis. On the other hand, Watson and his co-workers (12,13) reported that circulating MAG m-RNA positive cells were detected in only 40% of patients with metastatic tumors and could not be detected in any of non-metastatic patients. The difference in the detection rate between the present study and that of Watson et al. (12,13) may be attributed to the difference in the sensitivity of the technique. In the present work and the work performed by Gargano and his colleagues (4) nested PCR technique was applied whereas Watson and his colleagues (12,13) used a single round of PCR which is less sensitive than the former technique.

The frequency reported for MAG expression in breast cancer tissues varies from 20% to 95% (13,14). Such broad range might be due to several factors, such as tumor storage methods (fresh/frozen tissue and paraffin-embedded blocks) and/or the different techniques used for assessing the different expression levels (RT-PCR, immunohistochemical staining or in situ hybridization) (9,14,15).

In our study, the analysis of tissue samples has shown that compared with normal breast tissue, 79% of the breast cancer tumors had diffuse cytoplasmic MAG expression. On the contrary, results of Gargano et al. (4) showed that 90% of breast cancers showed diffuse cytoplasmic expression of MAG. This controversy may be attributed to...

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<th>Table 3</th>
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<tr>
<td>Influence of chemotherapy on the detection of MAG positive cells in peripheral blood of breast cancer patients with metastases</td>
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<tr>
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<tr>
<td>Before chemotherapy</td>
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<td>After chemotherapy</td>
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\(P < 0.001\): highly significant differences

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<th>Table 4</th>
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<tr>
<td>Diagnostic performance of inoculating MAG m-RNA expression in breast cancer patients</td>
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<tr>
<td>Validity test</td>
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</tr>
<tr>
<td>Sensitivity</td>
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<tr>
<td>Specificity</td>
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<td>(+) predictive value</td>
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<tr>
<td>(-) predictive value</td>
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<tr>
<td>Diagnostic efficiency</td>
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Fig 1. Photomicrograph of 2% agarose gel showing:
- The DNA molecular weight marker showing the band of 202 bp (lane 1);
- The positive results (lane 2,3,4,5,6,8&11);
- The negative results (lane 7,9,10&11)
the real time PCR method used in our study which is more sensitive than the immunohistochemical technique.

The existence of MAG m-RNA positive cells in the blood of 13 out of 26 patients with no apparent metastases in the present work is of the most importance as Shira and his co-workers (15), 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).

The presence of circulating MAG m-RNA positive cells in the blood during the clinical course of various treatment regimens may provide information about the disease status. The disappearance of MAG positive cells from the blood of patients who showed a favorable response to treatment may indicate that the metastatic disease is consolidated and less active (15, 16). On the other hand, the persistence of MAG positive cells may suggest the incomplete eradication of tumor cells even if the patient is clinically free. This may explain the results of our study, which showed a significant decrease in the number of metastatic patients with MAG m-RNA positive cells in peripheral blood after receiving chemotherapy. Our results are in agreement with those of Gilbey and his colleagues (3).

The relevance of the tumor grade to the expression of MAG was a matter of research by Gilbey et al. (3), Garagna et al. (4) and Bernstein et al. (12). Results of the lat-

Fig 2 (A,B). (A): Infiltrating ductal carcinoma of the breast showing positive staining for mammaglobin; (B) Negative staining for mammaglobin in uterine carcinoma (Immunoperoxidase, x200)

Fig 3 (A,B). Immunohistochemical staining of mammaglobin in: (A): Grade II breast carcinoma with diffuse strong staining; (B): Grade III breast carcinoma with diffuse weak staining (Immunoperoxidase, x300)
Mammaglobin in Breast Cancer

Researchers revealed a significant association between the expression of MAG mRNA and the histologic grade of tumor. These results were confirmed by ours. Thus, it could be speculated that MAG gene expression is highly restricted to low grade tumors.

Estrogen is an important growth factor for breast tumor playing an important role in regulating the proliferation and differentiation of normal and malignant mammary epithelial cells (16-18). The assessment of estrogen receptor status of breast cancer is now routinely performed on paraffin sections by means of immunohistochemistry and the results predict the tumor response to endocrine treatment (18,19).

The present study showed that all positive ER tumors express MAG whether in peripheral blood or in breast tissues. This finding was consistent with that of Gargano et al. (4) and Span et al. (20) who suggested that overexpression of MAG is associated with good prognosis.

Proliferative activity is an important criterion for assessment of prognosis and therapy of malignant tumors (20-22). High expression of proliferating antibodies, such as Ki-67, has been reported to identify the aggressive attitude of the tumors and to provide a guide to their proliferation rate (22-24).

The present study showed that the detection of MAG mRNA in peripheral blood or its protein expression in breast tissue is more in tumors with low proliferative activity (Ki-67 ≤20), a finding previously demonstrated by Bernstein et al. (12). The present study revealed an overall sensitivity 64.6%. No MAG mRNA positive cells were detected in the blood of the healthy control group or in patients with carcinomas other than breast cancer. This finding means that circulating MAG mRNA is a specific marker for breast cancer. The 100% specificity of the marker is supported by Bernstein et al. (12).

In conclusion, MAG is a mammary specific tumor marker, its detection in peripheral blood and/or its overexpression in breast tissues is associated with a better differentiation, a higher hormone dependence and a lower proliferation, all of which together define a better prognosis. Moreover, the circulating MAG mRNA may be used as a tumor marker to monitor the efficiency of therapy.

References