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Short title: Mammaglobin and breast carcinoma
Abstract

**Introduction:** Human mammaglobin is considered to be one of the most significant markers of hematogenous dissemination of breast carcinoma. Our goal is to indicate the important role of peritumoral tissue as an active participant in the tumorogenesis process and the concentration / expression of mamaglobins in the peritumoral tissue as a significant prognostic factor.

**Materials and methods:** This research included 64 patients with primary breast carcinoma during five-year follow-up period. To determine the concentration of mamaglobin A in samples of carcinoma tissue and peritumoral tissue, ELISA essay was used, and for the determination of relative gene expression of Mammaglobin A, qRT-PCR was used.

**Results:** The concentration of mamaglobin A increases in both the carcinoma tissue and peritumoral tissue with an increase in tumor size, number of affected lymph nodes, number of metastases, while relative expression of Mammaglobin A is statistically significantly higher in carcinoma tissue than in peritumoral tissue, regardless of tumor size, number of affected lymph nodes, number of metastases and tumor type. The concentration of mamaglobin A is higher in peritumor tissue than in tissue of ductal carcinoma, while in the case of lobular carcinoma the concentration of mamaglobin A is higher in carcinoma tissue than in peritumor tissue.

**Conclusion:** Mammaglobin A concentration in peritumoral tissue higher than 0,6704221 ng/ml, and in carcinoma tissue higher than 0,5784426 ng/ml, as well as Mammaglobin A relative gene expression in carcinoma tissue higher than 1.003 were determined as cut-off values that may identify patients who are at higher risk of metastatic disease, which would be treated with early radical adjuvant treatment.

**Keywords:**

breast carcinoma, mammaglobin, relapse, metastases.
Apstrakt

Uvod: Humani mamaglobin je jedan od naznačajnijih markera hematogene diseminacije karcinoma dojke. Naš cilj je ukazati na važnu ulogu peritumorskog tkiva kao aktivnog učesnika u procesu tumorogeneze i koncentraciju/ekspresiju mamaglobina u peritumorskom tkivu kao značajnog prognostičkog faktora.

Materijal i metode: Ova studija je obuhvatila 64 pacijentkinje sa primarnim karcinomom dojke tokom perioda od pet godina. Za određivanje koncentracije mamaglobina A u tkivu karcinoma i peritumorskom tkivu koristili smo ELISA test, dok je za određivanje relativne genske ekspresije ovog molekula korišćen qRT-PCR.

Rezultati: Koncentracija mamaglobina A raste kako u tkivu karcinoma tako i u peritumorskom tkivu sa porastom veličine tumora, broja zahvaćenih limfnih čvorova, broja metastaza, dok je relativna ekspresija Mamaglobina A statistički značajno viša u karcinomu nego u peritumorskom tkivu, bez obzira na veličinu tumora, broj zahvaćenih limfnih čvorova, broj metastaza i tumorski tip. Koncentracija mamaglobina A je veća u peritumorskom tkivu nego u tkivu duktilnog karcinoma, dok je u slučaju lobularnog karcinoma koncentracija mamaglobina A veća u karcinomu nego u peritumorskom tkivu.

Zaključak: Koncentracije Mamaglobina A u peritumorskom tkivu veće od 0,6704221 ng/ml i u tkivu karcinoma veće od 0,5784426 ng/ml, kao i relativna genska ekspresija Mamaglobina A u tkivu karcinoma veća od 1.003 su cut-off vrednosti na osnovu kojih se mogu identifikovati pacijenti koji su pod povećanim rizikom za razvoj metastatske bolesti i koji se mogu tretirati ranim radikalnim adjuvantnim lečenjem.

Ključne reči: karcinom dojke, mamaglobin, recidiv, metastaze.

Introduction

Breast carcinoma (BC) is the leading cause of cancer death in the USA with over 230,000 estimated new cases in 2014 and 40,000 estimated deaths (1). Despite the achieved advance in the treatment of breast carcinoma by the applications of numerous hormonal, genetic and molecular markers (estrogen receptor (ER), progesteron receptor (PR), HER2, Ki67, etc.), high rates of mortality and morbidity are obvious related to this disease, so
further study in this field is necessary with the aim of finding new markers as predictors of disease aggressiveness (2-4). Breast cancer was classified into invasive ductal carcinoma (over 80% of total BC), invasive lobular carcinoma (10% of total BC) and other BC histological types that are not so common (10% of total BC) according to pathohistological features (5). Improvement of medical achievements led to individualisation of therapy, i.e. selection of treatment tailored to individual patients (6).

One of the specific breast cancer markers is the uteroglobin protein called human mammaglobin. This protein is detected both in normal breast tissue and in breast cancer. Detected blood levels are increased in cancer and have prognostic significance (7, 8). Human mammaglobin was first detected in 1994 by Watson and Fleming using PCR method (9). In addition to breast tissue, this uteroglobin protein occurs in two subtypes B1 and B2 that are detected in ovarian carcinoma (10). In the literature, other names for human mammaglobin are also used: MAM, UGB3, SCGB2A1, MMG, MGB (11). It has been an important predictor for bone metastases in breast cancer (12). mRNA expression of mammaglobin may be multiplied in breast cancer versus non-malignant breast tissue (13). The overexpression of mammaglobin is probably caused by complex mechanism on the level of transcription (14).

Span PN et al. demonstrated that mRNA expression of Mammaglobin A could be used for individualization of postoperative adjuvant treatment planning (15). Human mammaglobin (hMAG) was also used to distinguish different breast carcinoma subtypes (16). Human mammaglobin is positively expressed in 80% of the intraductal carcinoma and 90% of invasive ductal carcinoma (17). The expression of human mammaglobin is in correlation with a high grade of breast cancer (18).

There is no consensus in the literature on the association of human mammaglobin levels and the prognosis of the course of the disease (19). Nunez-Villar et al. showed a correlation of human mammaglobin with less aggressive forms of the disease (20). Many efforts have been made to detect mRNA mammaglobin in lymph nodes, blood and bone marrow in patients with breast carcinoma. The peculiarity of hMAG lies in its almost sole existence in mammary tissue and mammary carcinoma. In addition, the heightened expression in carcinomas and its association with tumour grades renders it an excellent marker for diagnosis and prognosis (21).
BC early detection screening and other detection methods are still being studied. It is reported that expression of hMAG is mostly related to breast carcinoma tissue, and hMAG is defined as one of the first relatively mammary-specific markers (13). There are many studies in literature that are related to mammaglobin level of the peripheral blood in BC patients, while there are not so many studies describing mammaglobin level in cancer tissue; studies concerning mammaglobin level of peritumoral tissue are really rare (22-24).

**Methods**

This study presents a clinical observational cohort study along with an experimental study based on human origin material in vitro. The experimental research was carried out in Laboratory of Cell and Molecular Biology, Biology and Ecology Institute, Faculty of Science, University of Kragujevac. Samples (carcinoma tissue and peritumoral tissue) were taken in corporation with The General and Thoracic Surgery Clinic and Anatomic pathology Department of Clinical Centre in Kragujevac. Researches of this study were carried out in accordance with the Declaration of Helsinki.

*Chemicals and reagents*

Phosphate-buffered saline - PBS was provided by Gibco, the USA; chloroform, ethanol and isopropanol were provided by Serva Company, Germany. Human Mammaglobin-A ELISA kit and monoclonal antibody Anti-Human Antibody by My BioSource, inc. San Diego, CA, the USA.

QuantiTect Reverse Transcription Kit and PCR Kit (Sensiscript Reverse Transcriptase Kit - RT) were provided by Qiagen, Hilden, Germany. The PCR water and TRIzol were provided by Ambion, the USA. Gene expression Kit KapaSYBR® Green PCR Master Mix was provided by KAPA Biosystems, Boston, the USA. PCR primers were provided by Eurofins Genomics, Ebersberg.

*Criteria for involving the patients in the study*
In this study we analyzed carcinoma and peritumoral tissue. The study included patients with diagnosed early breast carcinoma. All the patients were examined by the Tumor board meeting of Clinical Centre Kragujevac, and then subjected to the appropriate surgical intervention. After the examination that had been carried out by the Tumor board meeting, tissue samples were taken willingly from patients and approval of the Ethics Committee Clinical Centre of Kragujevac (no. 01-4990). All patients were given written information about the study details. During the surgeries carried out in General and Thoracic Surgery Clinic in Kragujevac, breast carcinoma (n=64) specimens and peritumoral tissue (n=64) specimens were collected. The carcinoma tissue samples appeared to be different in size depending on carcinoma size, and the peritumoral macroscopic unchanged tissue samples were taken to 3 cm from macroscopic carcinoma margin depending on the size of the excised breast tissue. All specimens were pathohistologically examined and verified by Anatomic pathology Department of Clinical Centre in Kragujevac. Specimens were stored at -196 °C until analysis. Specimens were evaluated including these parameters: histological type of the tumor, grade of disease (Nottingham Histological Score), the condition of the lymph nodes, estrogen and progesterone and HER2/neu status that were evaluated according to protocol of American Joint Committee on Cancer - AJCC (25, 26).

The study did not include patients who preoperatively underwent neoadjuvant treatment. The patients with previous history of breast carcinoma, as well as the patients with metastatic deposits were excluded from the study. The study did not affect treatment generally conducted in Clinical Centre of Kragujevac and established on principles of good clinical practice.

**Tissue sample preparation**

The sample was measured and homogenized on ice. Samples were homogenized in 500 μl cold lysis buffer for 0,01g of sample. IKA Homogeniser IKA®-Werke GmbH & Co. KG, Germany and Ultrasonic homogenizers Sonopuls, Bandelin electronic GmbH & Co. KG, Germany were used. Lysis buffer contained 31.25 mM Triss-HCl pH 6.8, 2% SDS, 10% glycerol and dH2O was added up to 100 ml. After centrifugation at 10 000 RPM at 4 °C, 10 min., supernatant was isolated and it presented the cell lysate. In this way the proteins
from carcinoma and peritumoral tissue were isolated. The Lowry method was used to determine protein concentrations (27).

**Determining human mammaglobin-A concentration in carcinoma tissue**

The human mammaglobin-A levels in carcinoma tissue were quantified using Human Mammaglobin-A ELISA kit and monoclonal antibody Anti-Human Antibody (My BioSource, Inc. San Diego, CA, the USA) according to manufacturer’s instructions.

**Relative expression of mRNA mammaglobin gene**

Total RNA from the carcinoma and peritumoral tissue was isolated using the phenol-chloroform method by Chomezinski and Sacchi (28). Concentrations and purity of RNA were measured on biophotometer (Eppendorf BioPhotometer plus). $A_{260/280}$ and $A_{260/230}$ ratios were monitored to assess any possible contamination by protein, organic solvents, salts, carbohydrate etc. The samples were stored at -80 °C until analysis. The RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase (Reverse Transcriptase, RT) (29).

**Quantitative mRNA analysis**

Quantitative polymerase chain reaction (qRT-PCR) cDNA was used for gene expression analysis. Master mix (Universal Kapa Sybr fast qPCR Master Mix 2X) is designed for high-performance real-time PCR containing everything that was necessary except primers, cDNA specimens and Rox Low dye which were added. All qPCR experiments were performed by using the Applied Biosystems, quantitative Real-Time system (Applied Biosystems 7500/7500 Real-Time PCR Software v2.0). Each reaction (a 20 μl reaction mixture) contained 10 μl SYBR Green PCR Master Mix, 1 μl forward and reverse primer (5 pmol/μl) and 2 μl cDNA and 7 μl nuclease-free water. A PCR negative control containing nuclease-free water instead of cDNA and a 2RT control containing 2RT reaction instead of cDNA were included. The thermal cycling conditions included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 seconds, 60 °C
for 30 seconds, and 72 °C for 30 seconds. To analyze the qPCR results, we used the relative quantification method, which is based on the expression levels of a target gene versus reference genes (housekeeping gene).

There are 2 replicates in each combination of gene. Relative quantification of gene expression was normalized to the β-actin mRNA expression level. The gene-specific qRT-PCR primers were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-AAGCAGGAGTATGACGAGTCCG-3'</td>
<td>5'-GCCTTCATACTCTCAAGTTGG-3'</td>
</tr>
<tr>
<td>Mammaglobin-A</td>
<td>5'-CAG CGG CTT CCT TGA TCCTTG-3'</td>
<td>5'-ATA AGA AAG AGA AGG TGT GG-3'</td>
</tr>
</tbody>
</table>

To calculate the expression of a target gene in relation to a reference gene, we used $2^{-\Delta \text{CT}}$ method (30).

**Statistical analysis**

All data are presented as the mean±SEM (standard error of the mean). The normality of distribution was tested by Kolmogorov-Smirnov test. The two-tailed Student’s t-test, ANOVA test or nonparametric Mann–Whitney rank-sum test were used depending on the normality of distribution. Also, Chi-square test used for categorical variables. A binary logistic regression model was used to evaluate prediction between two variables. A receiver operating characteristic (ROC) curve analysis was employed to assess the diagnostic capabilities of the variables for prediction of distant metastasis. The results were considered significantly different when $p<0.05$. The data were analyzed using SPSS version 20, statistical package.

**Results**

*Clinical and pathological characteristics of breast cancer (BC) patients*
The levels of mammaglobin in carcinoma tissue and peritumoral tissue were observed and their prognostic value was analysed. Correlation between mammaglobin level in carcinoma tissue and peritumoral tissue and certain clinical pathological characteristics was also the object of the study. Clinical and pathological characteristics of the patients are described in Table 1 and 2.

Average age of patients was 58.95 ± 11.24 years old. Median age of patients was 60.5 years, 55 (86%) of patients had ductal carcinoma compared to 9 (14%) of patients with lobular carcinoma, and this ratio was statistically significant (X², p<0.01). A sparing operation was performed in 29 (45.3%) patients who had primary breast cancer of less than 3cm, compared to 35 (54.7%) patients in whom mutilatory surgery was performed. Adjuvant chemotherapy had 46 (72%) patients. Postoperative radiotherapy was used in 43 (67%) patients.

*Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients (Figure 1 and 2).*

The concentration of mammaglobin A grows both in carcinoma tissue and peritumoral tissue with an increase in tumor size, the number of affected lymph nodes, the number of metastases and tumor grade (Figure 1).

The concentration of mammaglobin A is higher in peritumoral tissue than in carcinoma tissue in ductal carcinoma, while in the case of lobular carcinoma the concentration of mammaglobin A is higher in carcinoma than in peritumoral tissue (Figure 2).

*Analysis of Mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients (Figure 3 and 4)*

Relative expression of *Mamaglobin A* is statistically significantly higher in carcinoma than in peritumoral tissue, regardless of the histological type of tumor, age of the patient, hormone or HER status (Figure 3).

Relative expression of Mamaglobin A is statistically significantly higher in carcinoma than in peritumoral tissue, regardless of tumor size, number of affected lymph nodes, number of metastases and tumor grade (Figure 4).

*Prognostic significance of Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients*
As shown in Figure 5, mammaglobin concentration in peritumoral tissue has a propensity for distant metastasis (binary logistic regression, p=0.024). Mammaglobin borderline value in carcinoma tissue is 0.67ng/ml for sensitivity 0.58 and specificity 0.59.

Mammaglobin concentration in carcinoma tissue has a propensity for distant metastasis (binary logistic regression, p=0.025). Mammaglobin borderline value in carcinoma tissue is 0.578ng/ml for sensitivity 0.67 and specificity 0.65 (Figure 6).

**Prognostic significance of Mammaglobin A gene expression in peritumoral and carcinoma tissue in breast carcinoma patients**

As indicated in Figure 7, mammaglobine gene expression in peritumoral tissue has no significant influence on occurrence of distant metastasis (binary logistic regression, p=0.307).

Mammaglobin gene expression in carcinoma tissue identified by PCR method has a propensity for distant metastasis (binary logistic regression, p=0.043) (Figure 8). Mammaglobin gene expression borderline value in carcinoma tissue is 1.003 for sensitivity 0.73 and specificity 0.76.

**Discussion**

Examination of hMGA mRNA levels of patients‘ peripheral blood results in 38, 2% sensitivity, 100% specificity, 100% positive prognostic value (PPV) and 61, 8% negative prognostic value (NPV) (31). There have been many studies describing mammaglobine level in patients‘ serum but not so many studies related to the mammaglobine level in carcinoma tissue; studies examining mammaglobin level in peritumoral tissue are very rare. The peritumoral tissue is a relatively new research topic, and recent studies have presented its important role in breast cancer formation and development (32). One of the studies that investigated mammaglobin levels in peritumoral, as well as tumoral tissue in breast cancer patient is the study of Zafracas et al. They found that mammaglobin was abundantly expressed in both malignant and normal breast tissues (11). Our goal was analysis of gene and protein expression of mammaglobin in carcinoma tissue and peritumoral tissue. We also managed to determine specific values of these parameters in carcinoma tissue and peritumoral tissue that appeared to be of highly prognostic value.
The goal of modern oncology is personalized therapy, which presents the optimal method for a patient (33). This study contributes to personalized therapy researches, dealing with analysis of potential correlation between mammaglobin expression in carcinoma tissue and peritumoral tissue and certain clinical pathological characteristics that are specific for each patient. We also managed to define specific values of mammaglobin levels (cut-off values) in carcinoma tissue and peritumoral tissue, having statistically proved prognostic values related to some of the most important prognostic parameters (e.g. distant and lymph nodes metastasis) for the outcome (34).

According to studied data, serum concentrations of mammaglobine were 0,07 - 9,6 ng/ml compared to 0 - 0,07 ng/ml of the control group (35). Our study shows that there was no statistically significant difference in mammaglobin concentration in carcinoma and peritumoral tissue. ELISA test was used to determine this difference. We got the values 2, 4 ng/ml - 3, 8 ng/ml, which is more than the range of healthy persons 0 - 0, 07 ng/ml, pointing out the prognostic value of mammaglobin concentrations in tissues.

Data in studies related to serum concentration of mammaglobin have been contradictory. Zehentner et al. claim that ELISA test data showed that mammaglobin level was not dependant on disease stage. ROC curve showed the values of 1,71 ng/ml of cut-off; the test considered to be positive when values of mammaglobine were higher than the given ones (36). In our study ROC curve showed that mammaglobin concentration value in peritumoral tissue can be used as a prognostic factor of distant metastasis (AUC= 0.693, p=0.027). Also, ROC curve showed that mammaglobin concentration value in carcinoma tissue can be used as a prognostic factor of distant metastasis (AUC= 0.698, p=0.019).

However, Bernstein JL rt al. claim that patients at stages I – III had mammaglobin values of 0,9 - 1,4 ng/ml, and at stage IV the value 2,3 ng/ml. There was a strong positive correlation between mammaglobine values and carcinoma size; patients with a tumor of large diameter had higher serum concentrations of mammaglobin (37). Our results match these data. There was an increased level of mammaglobin in carcinoma tissue and peritumoral tissue in patients with a larger breast tumor. As for the serum concentrations, results of our study showed concentrations of 2,6 ng/ml at stage T1 up to those of 3,8 ng/ml at stage T3. Values in peritumoral tissue were in significantly lower than those in carcinoma tissue- 2,4 ng/ml at T1 up to 3,6 ng/ml at T3. We did not find similar studies while searching the available data bases so it was impossible to compare the results. To our
knowledge, this is the first study of this kind that dealt with determining of mammaglobin tissue concentration.

Our results showed that there is a gradual increase of mammaglobin protein expression in carcinoma tissue and peritumoral tissue with higher possibility of lymphatic metastasis. For peritumoral tissue the values were at range 2,3 ng/ml - 3,7 ng/ml, and for carcinoma tissue 2,6 ng/ml- 3,8 ng/ml concerning N0 and N2 disease stages, respectively. These differences are statistically significant giving tissue mammaglobin concentrations a prognostic role. These results correspond with the ones we found in other studies. Liu Y. et al. cite statistically higher mammaglobin concentration in patients with positive lymph nodes (38). Tafreshi et al. demonstrated that the the level of mammaglobin is significantly higher in affected lymph nodes comparing with healthy lymph nodes and showed that breast cancer targeted agent, based on mammaglobin can be used for the non-invasive, in vivo detection of cancer altered axillary lymph nodes (39).

Mean value of serum concentration of mammaglobin in patients with metastatic breast carcinoma was 9,38 ng/ml (7,9 ng/ml in the control group). Sensitivity was 68% and specificity 88,8%. Slight differences may appear because of different antibodies that were used in various studies (36). Our results showed that mean value of mammaglobine determined by protein expression in patients with metastatic disease was 2,4 ng/ml - 3,75 ng/ml in peritumoral, and 2,55 ng/ml - 3,8 ng/ml in carcinoma tissue. Determining protein expression with ELISA test shows prognostic value related to distant metastasis in BC patients. Further, we defined specific cut-off values of mammaglobin concentration which indicate distant metastasis occurrence risks. This value was 0,6704221 ng/ml in peritumoral tissue, and 0,5784426 ng/ml in carcinoma tissue. We did not find similar studies while searching the available data bases so it was imposible to compare the results.

As for the tumor grade, our study showed that increased mammaglobin protein expression in carcinoma tissue and peritumoral tissue affected the tumor grade. Higher concentration of mammaglobin can affect tumor metastatis in distant organs; threerfore, determining protein expression of mammaglobin can have a prognostic value. Similar results can be found in a few studies that examined mammaglobin expression in carcinoma tissue using the method of immunohistochemistry; we determined both protein and gene expression in carcinoma and peritumoral tissue. Rehman F. et al. also noticed increased
mammaglobin concentration in carcinoma tissue while changing the tumor grade and size (40).

Our results showed that protein expression of mammaglobin in carcinoma tissue and peritumoral tissue was higher in patients with ductal tumors than in those with lobular tumors. The results of some other studies were different. Watson and Nunez-Villar found no significant difference in mammaglobin expression considering histological types of breast cancer (7, 20). On the other hand, there are studies like us that confirmed increased protein expression in ductal tumors (40). This confronts with the study by Bhargava et al.; this study showed that infiltrated lobular carcinoma had the highest mammaglobin expression (41).

We did not present correlation between hormonal status (ER and PR) and HER2 and protein expression. There are different data in the studies related to this. O’Brien et al. cite that the presence of mammaglobin in patients with ER+ and PR+ a good prognostic indicator (42). Guan et al. show that the presence of mammaglobin protein and gene expression correlates with ER positivity (43).

We did not show age dependence in mammaglobin expression, since the results were like those of other studies (38). We defined specific values of mammaglobin concentration in carcinoma tissue and peritumoral tissue, and we showed that there were patients who were potentially at risk of disease development. They were suggested an adjuvant cancer treatment. Protein expression value was 0.6704221 ng/ml in peritumoral tissue, and 0.5784426 ng/ml in carcinoma tissue (ELISA test).

We also dealt with mammaglobin gene expression in carcinoma tissue and peritumoral tissue and the results were quantitatively different as described in other studies (42). Nevertheless, Chen G et al. showed that only 21% of results of gene examination correlated with protein expression (adenocarcinoma lung) (44).

Possible reasons are well known phenomena of post transcriptional and post translational regulation and modification; in some cases, it is not certain that protein would be functional and detectable (45, 46).

As for the gene expression in carcinoma tissue and peritumoral tissue, our studies shows gradual increase of mammaglobin gene expression depending on the tumor size, though it is less shown in peritumoral tissue. Mammaglobin gene expression values of concentration
in carcinoma tissue were 1.5 at T1 stage up to 7.4 at T3 stage, being lower in peritumoral tissue: 0.9 at T1 stage up to 1.7 at T3 stage.

Mammaglobin gene expression is in correlation with lymph nodes status. Results showed significantly lower values in peritumoral tissue: 0.1 at N0 up to 0.4 at N2 stage; in carcinoma tissue these values were 1.9 at N0 up to 5.6 at N2 stage. These data are like the ones from the other studies (36). Marchetti A et al. consider mammaglobin one of the most sensitive and most specific markers for lymph nodes micrometastasis detection (47).

Gene expression of mammaglobin was slightly increased in carcinoma tissue of patients with metastasis. In peritumoral tissue it had values of 0.3 at M0 up to 0.5 at M1, and in carcinoma tissue 2.0 at M0 up to 5.5 at M1. 12% of cases M status was not defined so these patients were excluded from the study.

ROC curve shows that the value of mammaglobin gene expression in peritumoral tissue cannot be used as a prognostic factor of distant metastasis (AUC= 0.553, p=0.546).

ROC curve shows that the value of mammaglobin gene expression in carcinoma tissue can be used as a prognostic factor of distant metastasis (AUC=0.838, p<0.01).

The study by Span et al. showed that increased gene expression was independently associated with longer non-relapse period. This was particularly evident in patients taking Tamoxifen, indicating relation to hormonal status of tumor. Therefore, mammaglobin gene expression is considered to be a good prognostic marker (15). There are some authors, nevertheless, stating that there was not a statistically significant correlation between gene expression level and hormonal status (48, 49).

Our results match previously described data. A Korean group found a statistically significant correlation between mammaglobin level of peripheral blood and ER, PR status of patient and hormonal status. No connection existed related to HER2 status (50).

Our study indicates that gene expression is significantly higher in carcinoma tissue than in peritumoral tissue, and statistically more significant in ductal than in lobular breast cancer. These data correspond to those of other researches (51).

It can be concluded that mammaglobin gene expression in carcinoma tissue (not the one in peritumoral tissue) can be used as a prognostic marker of of hematogenous dissemination of breast carcinoma. We determined specific values of gene expression for carcinoma tissue. Thus, it is possible to identify high-risk patients of metastatic disease; these patients are suggested adjuvant cancer treatment. Boundary value of mammaglobin
gene expression is considered to be 1.003 for sensitivity of 0.73 and specificity of 0.76 in carcinoma tissue.

In summing up, the basic results of this study are:

- Protein expression of mammaglobin in peritumoral and carcinoma tissue can be used as a prognostic marker for hematogenous dissemination of breast carcinoma.
- Specific values of mammaglobin concentration in peritumoral and carcinoma tissue were defined above which it can be assumed that metastatic dissemination of disease would occur.
- In peritumoral tissue mammaglobin concentration determined with ELISA test was 0.6704221 ng/ml, and in carcinoma tissue this value was 0.5784426 ng/ml.
- Mammaglobin gene expression can be a prognostic marker for hematogenous dissemination of breast carcinoma concerning carcinoma tissue.
- Determined value of mammaglobin gene expression in carcinoma tissue was 1.003.
- Analysis of mammaglobin in peritumoral and carcinoma tissue makes it possible to define high-risk patients of disease development and we suggest adjuvant cancer treatment in order to prevent disease development.

Declaration of interest

The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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postmenopausal patients, and has independent prognostic value for relapse-free survival
time. Journal of clinical oncology : official journal of the American Society of Clinical
between molecular subtype of invasive breast carcinoma and expression of gross cystic
Center for Biotechnology Information (US); 2004.
Detection of mammaglobin mRNA in peripheral blood is associated with high grade breast
immunohistochemical expression of mammaglobin A in primary breast carcinoma and
lymph node metastasis. Romanian journal of morphology and embryology = Revue
Elevated mammaglobin (h-MAM) expression in breast cancer is associated with clinical


Table 1.
Clinical, pathological and TNM characteristics of breast cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients-N, (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of specimens</td>
<td>128 (100%)</td>
</tr>
<tr>
<td>• Peritumoral tissue (PT)</td>
<td>64 (50%)</td>
</tr>
<tr>
<td>• Carcinoma tissue (CT)</td>
<td>64 (50%)</td>
</tr>
<tr>
<td><strong>Histological grade</strong></td>
<td></td>
</tr>
<tr>
<td>• Low grade (G1 or well differentiated)</td>
<td>8 (12%)</td>
</tr>
<tr>
<td>• Intermediate grade (G2 or moderately differentiated)</td>
<td>36 (56%)</td>
</tr>
<tr>
<td>• High grade (G3 or poorly differentiated)</td>
<td>20 (32%)</td>
</tr>
<tr>
<td>• High grade (G4 or undifferentiated)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>The size of the tumor</strong></td>
<td></td>
</tr>
<tr>
<td>• Tumor $\leq$ 2 cm (T1)</td>
<td>27 (42%)</td>
</tr>
<tr>
<td>• Tumor 2-5 cm (T2)</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>• Tumor $&gt; 5$ cm (T3)</td>
<td>34 (54%)</td>
</tr>
<tr>
<td>• Tumor of any size grown into the chest wall (T4)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Regional lymph nodes (N)</strong></td>
<td></td>
</tr>
<tr>
<td>• No regional lymph node metastasis (N0)</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>• Metastasis to movable ipsilateral axillary lymph node(s) (N1)</td>
<td>31 (48%)</td>
</tr>
<tr>
<td>• Metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures (N2)</td>
<td>29 (46%)</td>
</tr>
<tr>
<td><strong>Distant metastasis (M)-developed during 5-year period</strong></td>
<td></td>
</tr>
<tr>
<td>• No distant metastasis (M0)</td>
<td>23 (36%)</td>
</tr>
<tr>
<td>• Distant metastasis (M1)</td>
<td>33 (52%)</td>
</tr>
<tr>
<td>• Presence of distant metastasis cannot be assessed (Mx)</td>
<td>8 (12%)</td>
</tr>
</tbody>
</table>
Table 2. Clinical, pathological and immunohistochemical characteristics of breast cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients-N, (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total number of specimens</strong></td>
<td></td>
</tr>
<tr>
<td>• Peritumoral tissue (PT)</td>
<td>64 (50%)</td>
</tr>
<tr>
<td>• Carcinoma tissue (CT)</td>
<td>64 (50%)</td>
</tr>
<tr>
<td><strong>Histological type of cancer</strong></td>
<td></td>
</tr>
<tr>
<td>• <em>Invasive Ductal Carcinoma</em></td>
<td>55 (86%)</td>
</tr>
<tr>
<td>• <em>Invasive Lobular Carcinoma</em></td>
<td>9 (14%)</td>
</tr>
<tr>
<td><strong>Receptor status</strong></td>
<td></td>
</tr>
<tr>
<td>• ER+</td>
<td>38 (60%)</td>
</tr>
<tr>
<td>• ER-</td>
<td>26 (40%)</td>
</tr>
<tr>
<td>• PR+</td>
<td>29 (46%)</td>
</tr>
<tr>
<td>• PR-</td>
<td>35 (54%)</td>
</tr>
<tr>
<td>• HER+</td>
<td>31 (48%)</td>
</tr>
<tr>
<td>• HER-</td>
<td>33 (52%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>• &lt; 50</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>• &gt; 50</td>
<td>61 (96%)</td>
</tr>
</tbody>
</table>
Figure 1. Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients related to TNM system classification and histologic tumor grade. R0 and R1 - status of resection margins. The results are given as the mean value ± SE for the examined parameter number of samples (N); *p<0.05 statistically significant difference between carcinoma tissue and peritumoral tissue; #p<0.05 statistically significant difference between peritumoral tissue of different patients; ##p<0.05 statistically significant difference between carcinoma tissue of different patients. Student’s T Test and ANOVA were used, and p<0.05 was regarded as statistically significant difference.
Figure 2. Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients related to pathohistological tumor type, patient’s age, hormone and HER 2 status. The results are given as the mean value ± SE for the examined parameter number of samples (N); *p<0.05 statistically significant difference between carcinoma tissue and peritumoral tissue; #p<0.05 statistically significant difference between peritumoral tissue of different patients; ##p<0.05 statistically significant difference between carcinoma tissue of different patients. Student’s T Test was used, and p<0.05 was regarded as statistically significant difference.
Figure 3. Relative Mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients related to pathohistological tumor type, patient’s age, hormone and HER 2 status. The results are given as the mean value ± SE for the examined parameter number of samples (N); *p<0.05 statistically significant difference between carcinoma tissue and peritumoral tissue; #p<0.05 statistically significant difference between peritumoral tissue of different patients; ##p<0.05 statistically significant difference between carcinoma tissue of different patients.
Mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients related to TNM system classification, positive margins and histologic tumor grade. R0 and R1 - status of resection margins. The results are given as the mean value ± SE for the examined parameter number of samples (N); *p<0.05 statistically significant difference between carcinoma tissue and peritumoral tissue; #p<0.05 statistically significant difference between peritumoral tissue of different patients; ##p<0.05 statistically significant difference between carcinoma tissue of different patients. Mammaglobin gene expression is significantly higher in carcinoma tissue (Mann-Whitney Test, U= 754, p= 0.001).
Figure 5. ROC curve- Mammaglobin A concentration in peritumoral tissue in breast carcinoma patients.
Figure 6. ROC curve- Mammaglobin A concentration in carcinoma tissue in breast carcinoma patients.
Figure 7. ROC curve- Mammaglobin A gene expression in peritumoral tissue in breast carcinoma patients
Figure 8. ROC curve- Mammaglobin-A gene expression in carcinoma tissue in breast carcinoma patients

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