Mammaglobin expression in tissue as a predictor of breast carcinoma aggressiveness

Ekspresija mamaglobina u tkivu kao prediktora agresivnosti karcinoma dojke

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Abstract

Background/Aim. Human mammaglobin is considered to be one of the most significant markers of hematogenous dissemination of breast carcinoma. This paper aimed to indicate the important role of peritumoral tissue as an active participant in the tumorigenesis process and the concentration/expression of mammaglobins in the peritumoral tissue as a significant prognostic factor. **Methods.** This research included 64 female patients with primary breast carcinoma during the five-year follow-up period. To determine the concentration of mammaglobin A in samples of carcinoma tissue and peritumoral tissue, Enzyme-linked immunosorbent assay (ELISA) test was used, and for the determination of relative gene expression of mammaglobin A, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used. **Results.** The concentration of mammaglobin A was increased 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 was statistically significantly higher in carcinoma tissue than in peritumoral tissue, regardless of the tumor size, number of affected lymph nodes, number of metastases and tumor type. The concentration of mammaglobin A was higher in peritumoral tissue than in tissue of ductal carcinoma, while in the case of lobular carcinoma the concentration of mammaglobin A was higher in carcinoma tissue than in peritumoral 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. These values may identify patients who are at higher risk of metastatic disease, which would be treated with early radical adjuvant treatment.

Key words:
breast neoplasms; mammaglobin a; neoplasm invasiveness; neoplasm metastasis; prognosis.

Apstrakt

Uvod/Cilj. Humani mamaglobin je jedan od naznačajnijih markera hematogene diseminacije karcinoma dojke. Cilj rada bio je da se ukaže na važnu ulogu peritumorskog tkiva kao aktivnog učesnika u procesu tumorogeneze i koncentracije/ekspresije mamaglobina u peritumorskom tkivu kao značajnog prognostičkog faktora. **Metode.** Ovom studijom su bile obuhvaćene 64 bolesnice sa primarnim karcinomom dojke tokom perioda od pet godina. Za određivanje koncentracije mamaglobina A u tkivu karcinoma i peritumorskom tkivu korišćen je *enzyme-linked immunosorbent assay* (ELISA) test, dok je za određivanje relativne genske ekspresije ovog molekula korišćen *quantitative reverse-transcription-polymerase chain reaction* (qRT-PCR). **Rezultati.** Koncentracija mamaglobina A je rasla 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 bila 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 bila viša u peritumorskom tkivu nego u tkivu duktalnog karcinoma, dok je u slučaju lobularnog karcinoma koncentracija mamaglobina A bila veća u karcinomu, nego u peritumor-

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skom 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 bolesnici koji su pod povećanim rizikom od razvoja metastatske bolesti i koji se mogu tretirati ranim radikalnim adjuvantnim lečenjem.

Ključne reči:
dojka, neoplazme; mamaglobin a; neoplazme, invazivnost; neoplazme, metastaze; prognoza.

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 advances in treating breast carcinoma by applying numerous hormonal, genetic, and molecular markers, such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), Ki67, etc., high rates of mortality and morbidity are obviously related to this disease. Therefore, 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 individualization of therapy, i.e. treatment selection 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 [9] using the polymerase chain reaction (PCR) method. In addition to breast tissue, this uteroglobin protein occurs in two subtypes B1 and B2, detected in ovarian carcinoma [10]. In the literature, other names used for human mammaglobin are also MAM, UGB3, SCGB2A1, MMG, and MGB [11]. Human mammaglobin has been an important predictor for bone metastases in breast cancer [12]. The mRNA expression of mammaglobin may be multiplied in breast cancer versus non-malignant breast tissue [13]. The overexpression of mammaglobin is probably caused by a complex mechanism on the level of transcription [14].

Span et al. [15] demonstrated that mRNA expression of mammaglobin A could be used for individualization of postoperative adjuvant treatment planning. Human mammaglobin (hMAG) was also used to distinguish different breast carcinoma subtypes [16]. It 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. [20] showed a correlation of human mammaglobin with less aggressive forms of the disease. 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 elevated expression in carcinomas and its association with tumor 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 the literature that are related to mammaglobin level of the peripheral blood in BC patients, but there are not so many studies describing mammaglobin level in cancer tissue. Studies concerning mammaglobin level of peritumoral tissue are quite 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 the Laboratory of Cell and Molecular Biology, Biology and Ecology Institute, Faculty of Medical Sciences, 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 the Clinical Centre of Kragujevac. This study was approved by the Ethics Committee of the Clinical Centre of Kragujevac (no. 01-4990) and was carried out in accordance with the Declaration of Helsinki. All patients were given written information about the study details.

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 were provided 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 early diagnosed breast carcinoma. All the patients were examined by the Tumor board meeting of the Clinical Centre of 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. During the surgeries carried out at the General and Thoracic Surgery Clinic, Clinical Centre of Kragujevac, breast carcinoma specimens (n = 64) and peritumoral tissue specimens (n=64) 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 the macroscopic carcinoma margin depending on the size of the excised breast tissue. All specimens were histopathologically examined and verified by the Anatomic Pathology Department of the Clinical Centre of Kragujevac. Specimens were stored at -196 °C until analysis. They were evaluated including the following parameters: histological type of the tumor, the grade of the disease (Nottingham Histological Scores), the condition of the lymph nodes, estrogen, progesterone, and HER2/neu receptors status. The specimens were evaluated according to the American Joint Committee on Cancer (AJCC) protocol [25, 26].

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

Samples were measured and homogenized on ice 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, the 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 the manufacturer's instructions.

Relative expression of messenger ribonucleic acid (mRNA) mammaglobin gene

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

Quantitative mRNA analysis

Quantitative reverse transcription-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 necessary except primers, cDNA specimens, and Rox Low dye, which were added. All qPCR experiments were performed 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 genes. Relative quantification of gene expression was normalized to the β-actin mRNA expression level. The gene-specific qRT-PCR primers were as follows:

Primer	Forward sequence	Reverse sequence
β-actin	5'-AAGCAGGAGTATGACGAGTCCG-3'	5'-GCCTTCATACATCTCAAGTTGG-3'
Mammaglobin-A	5'-CAG CGG CTT CCT TGA TCCTTG-3'	5'-ATA AGA AAG AGA AGG TGT GG-3'

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

Statistical analysis

All data are presented as the mean ± SEM (standard error of the mean). The normality of distribution was tested by the Kolmogorov-Smirnov test. The two-tailed Student's t-test, ANOVA test, or the nonparametric Mann–Whitney rank-sum test were used depending on the normality of distribution. Moreover, the χ^2 test was 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 predicting 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 patients

The levels of mammaglobin in carcinoma tissue and peritumoral tissue were observed, and their prognostic value was analyzed. Correlation between mammaglobin level in carcinoma tissue and peritumoral tissue and certain clinical and pathological characteristics were also the object of the study. Clinical and pathological characteristics of the patients are described in Table 1 .

The average age of patients was 58.95 ± 11.24 years.

The median age of patients was 60.5 years. Most patients (61/64, 96%) were older than 50 years. Fifty five (86%) patients had ductal carcinoma compared to 9 (14%) patients with lobular carcinoma, and this ratio was statistically significant (χ^2 test, $p < 0.01$). A sparing operation was performed in 29 (45.3%) patients who had primary breast cancer of less than 3 cm, compared to 35 (54.7%) patients in whom mutilating 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

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

Table 1

Clinical, pathological, and immunohistochemical and tumor, node, metastasis (TNM) characteristics of breast cancer patients

Characteristics	Number of patients, n (%)
Age of patients (years)	
< 50	3 (4)
> 50	61 (96)
Total number of specimens	128 (100)
peritumoral tissue (PT)	64 (50)
carcinoma tissue (CT)	64 (50)
Histological grade	
low grade (G1 or well-differentiated)	8 (12)
intermediate grade (G2 or moderately differentiated)	36 (56)
high grade (G3 or poorly differentiated)	20 (32)
high grade (G4 or undifferentiated)	0 (0)
Histological type	
invasive ductal carcinoma	55 (86)
invasive lobular carcinoma	9 (14)
Receptor status	
ER+	38 (60)
ER-	26 (40)
PR+	29 (46)
PR-	35 (54)
HER+	31 (48)
HER-	33 (52)
The size of the tumor (cm)	
≤ 2 (T1)	27 (42)
2–5 (T2)	3 (4)
> 5 (T3)	34 (54)
tumor of any size grown into the chest wall (T4)	0 (0)
Regional lymph nodes (N)	
no regional lymph node metastasis (N0)	4 (6)
metastasis to movable ipsilateral axillary lymph node(s) (N1)	31 (48)
metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures (N2)	29 (46)
Distant metastasis (M) developed during a 5-year period	
no distant metastasis (M0)	23 (36)
distant metastasis (M1)	33 (52)
presence of distant metastasis cannot be assessed (Mx)	8 (12)

ER – estrogen receptor; PR – progesteron receptor; HER – human epidermal growth factor receptor.
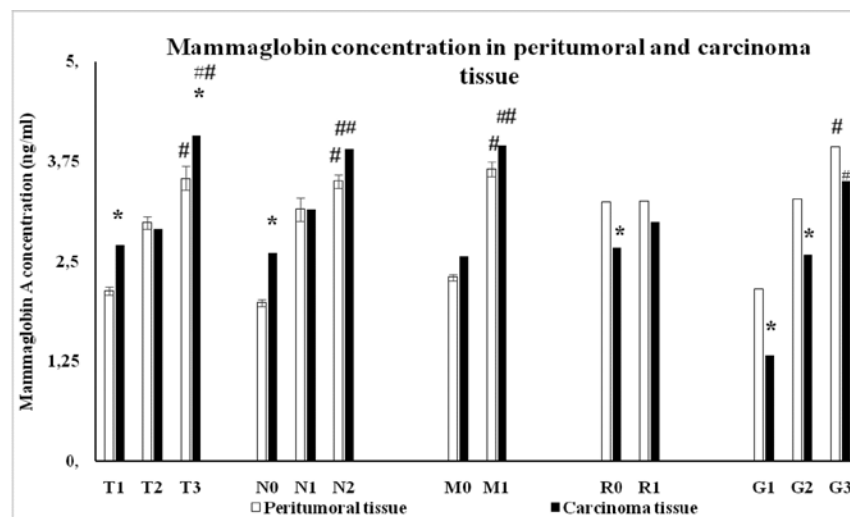
Fig. 1 – Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients related to tumor, node, metastasis (TNM) classification system, histological tumor grade (G1-G3), and status of resection margins (R0 and R1).
T1 – tumor ≤ 2 cm; T2 – tumor 2-5 cm; T3 – tumor > 5 cm; N0 – no regional lymph node metastasis; N1 – metastasis to movable ipsilateral axillary lymph node(s); N2 – metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures; M0 – no distant metastasis; M1 – distant metastasis; R0 – no cancer cells seen microscopically at the primary tumor site; R1 – cancer cells present microscopically at the primary tumor site; G1 – low grade; G2 – intermediate grade; G3 –high grade.
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 a statistically significant difference.
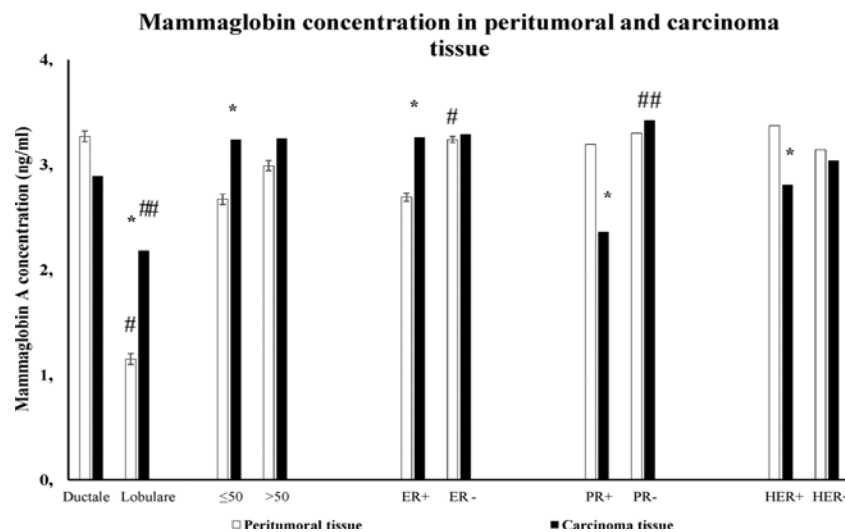
Fig. 2 – Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients related to pathohistological tumor type, patient's age (≤ 50, > 50), hormone receptor [estrogen receptor (ER) and progesterone receptor (PR)], and human epidermal growth factor receptor 2 (HER) status.
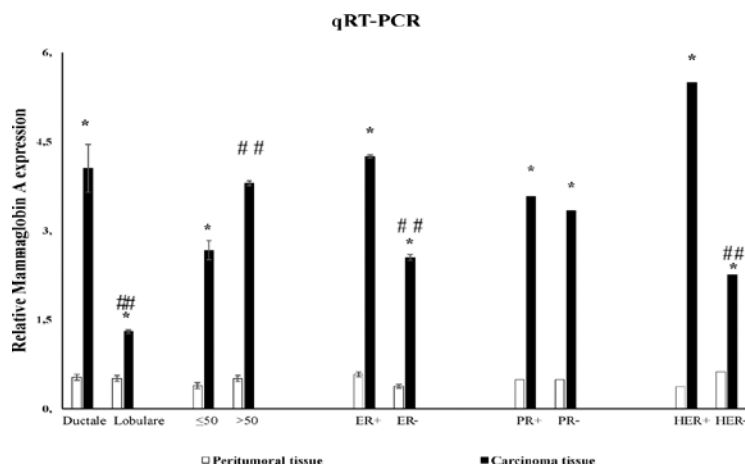The results are given as the mean value ± SE for the examined parameter. *$p < 0.05$ statistically significant difference between carcinoma tissue and peritumoral tissue; # $p < 0.05$ statistically significant difference between peritumoral tissue of different patients; ## $p < 0.05$ statistically significant difference between carcinoma tissue of different patients. The Student's t-test was used, and $p < 0.05$ was regarded as a statistically significant difference.

Analysis of mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients

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

Relative expression of mammaglobin A was 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).

Fig. 3 – Relative mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients related to pathohistological tumor type, patient's age ($\leq$ 50, > 50), hormone, receptor receptor [estrogen receptor (ER) and progesterone receptor (PR)] and human epidermal growth factor receptor 2 (HER) status. The results are given as the mean value ± SE for the examined parameter; *$p < 0.05$ 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.
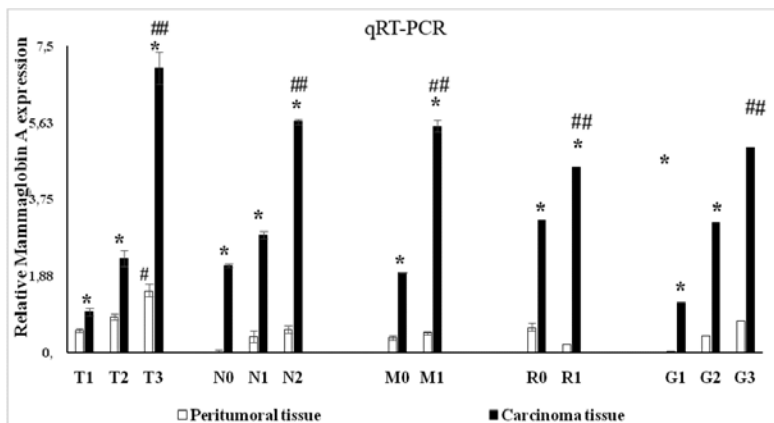

Fig. 4 – Mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients related to tumor, node metastasis (TNM) system classification, positive resection margins (R0 and R1), and histological tumor grade (G1-G3).
T1 – tumor $\leq$ 2 cm; T2 – tumor 2-5 cm; T3 – tumor > 5 cm; N0 – no regional lymph node metastasis; N1 – metastasis to movable ipsilateral axillary lymph node(s); N2 – metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures; M0 – no distant metastasis; M1 – distant metastasis; R0 – no cancer cells seen microscopically at the primary tumor site; R1 – cancer cells present microscopically at the primary tumor site; G1 – low grade; G2 – intermediate grade; G3 –high grade.
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, $p = 0.001$).
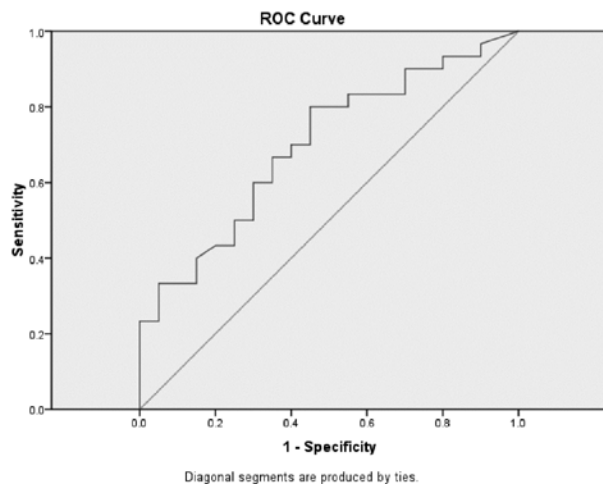
Prognostic significance of mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients

As shown in Figure 5, mammaglobin concentration in peritumoral tissue had a propensity for distant metastasis (binary logistic regression, $p = 0.024$). Mammaglobin borderline value in carcinoma tissue was 0.67 ng/mL for sensitivity 0.58 and specificity 0.59.
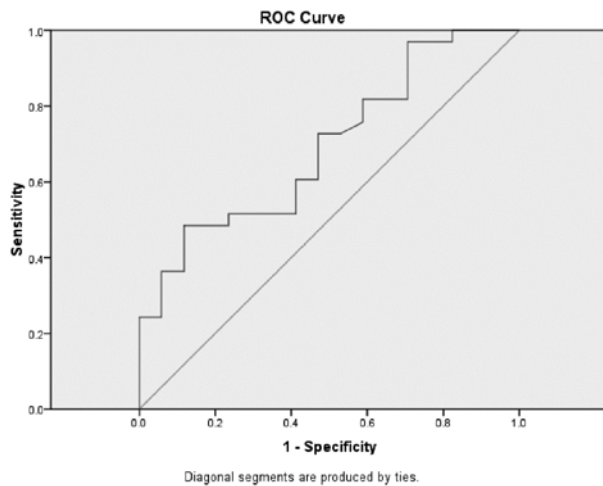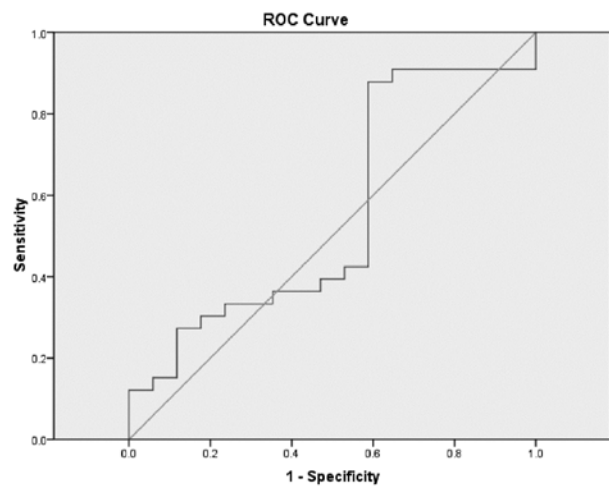

Fig. 5 – Receiver operating characteristic (ROC) curve: mammaglobin A concentration in peritumoral tissue in breast carcinoma patients.

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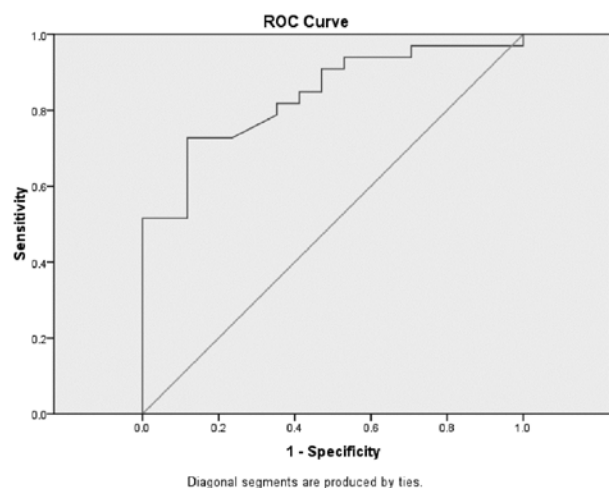

Fig. 6 – Receiver operating characteristic (ROC) curve: mammaglobin A concentration in carcinoma tissue in breast carcinoma patients.

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

Fig. 7 – Receiver operating characteristic (ROC) curve: mammaglobin A gene expression in peritumoral tissue in breast carcinoma patients.

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

Fig. 8 – Receiver operating characteristic (ROC) curve: mammaglobin-A gene expression in carcinoma tissue in breast carcinoma patients

Discussion

Examination of high mobility group A (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 mammaglobin level in patients' serum but not so many studies related to the mammaglobin 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 patients, is the study of Zafracas et al. [11]. They found that mammaglobin was abundantly expressed in both malignant and normal breast tissues. Our goal was to analyze the 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 high 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 research, dealing with the analysis of the potential correlation between mammaglobin expression in carcinoma tissue and peritumoral tissue and certain clinical and pathological characteristics 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 the studied data, serum concentrations of mammaglobin were 0.07–9.6 ng/mL compared to 0–0.07 ng/mL of the control group [35]. Our study showed that there was no statistically significant difference in mammaglobin concentrations 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 was higher than the range of healthy persons, 0–0.07 ng/mL, pointing out the prognostic value of mammaglobin concentrations in tissues.

Data in the studies related to the serum concentration of mammaglobin have been contradictory. Zehentner et al. [36] claimed that ELISA test data showed that mammaglobin level was not dependant on the disease stage. The ROC curve showed the values of 1.71 ng/mL of cut-off; the test was considered to be positive when values of mammaglobin were higher than the given ones [36]. In our study, the ROC curve showed that mammaglobin concentration value in peritumoral tissue can be used as a prognostic factor of distant metastasis [area under curve (AUC) = 0.693, $p = 0.027$]. Moreover, the ROC curve showed that mammaglobin concentration value in carcinoma tissue could be used as a prognostic factor of distant metastasis (AUC = 0.698, $p = 0.019$).

However, Bernstein et al. [37] claimed that patients at stages I–III had mammaglobin values of 0.9–1.4 ng/mL, and at stage IV the value of 2.3 ng/mL. There was a strong positive correlation between mammaglobin values and carcinoma size; patients with a tumor of large diameter had higher serum concentrations of mammaglobin. 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 T1 stage up to those of 3.8 ng/mL at T3 stage. Values in peritumoral tissue were 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 through the available databases; therefore, it was impossible to compare the results. To our knowledge, this is the first study of this kind that dealt with determining mammaglobin tissue concentration.

Our results showed that there is a gradual increase of mammaglobin protein expression in carcinoma tissue and peritumoral tissue, with a higher possibility of lymphatic metastasis. For peritumoral tissue, the values were in the range of 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 et al. [38] cited statistically higher mammaglobin concentration in patients with positive lymph nodes. Tafreshi et al. [39] demonstrated that the level of mammaglobin was significantly higher in affected lymph nodes compared with healthy lymph nodes and showed that breast cancer targeted agent based on mammaglobin could be used for the non-invasive, *in vivo* detection of cancer altered axillary lymph nodes.

The 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 the mean value of mammaglobin determined by protein expression in patients with metastatic disease was 2.4–3.75 ng/mL in peritumoral and 2.55–3.8 ng/mL in carcinoma tissue. Determining protein expression with ELISA test shows prognostic value related to distant metastasis in BC patients. Furthermore, 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 through the available databases; therefore, it was impossible to compare the results.

As for the tumor grade, our study showed that increased mammaglobin protein expression in carcinoma tissue and peritumoral tissue affects the tumor grade. A higher concentration of mammaglobin can affect tumor metastasis in distant organs. Therefore, determining the 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 et al. [40] also noticed increased mammaglobin concentrations in carcinoma tissue while changing the tumor grade and size.

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 Fleming [7] and Nunez-Villar et al. [20] found no significant difference in mammaglobin expression considering histological types of breast cancer. On the other hand, there are studies like ours that have confirmed increased protein expression in ductal tumors [40]. This confronts with the study by Bhargava et al. [41]. Their study showed that infiltrated lobular carcinoma had the highest mammaglobin expression.

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

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 et al. [44] showed that only 21% of results of gene examination correlated with protein expression (adenocarcinoma lung).

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 study showed a gradual increase of mammaglobin gene expression depending on the tumor size, though it was 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 was 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 the N2 stage. These data are like the ones from the other studies [36]. Marchetti et al. [47] consider mammaglobin one of the most sensitive and most specific markers for lymph nodes micrometastasis detection.

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. In 12% of cases, M status was not defined. Therefore, these patients were excluded from the study.

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

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

The study by Span et al. [15] showed that increased gene expression was independently associated with a longer non-relapse period. This was particularly evident in patients taking tamoxifen, indicating a relation to the hormonal status of the tumor. Therefore, mammaglobin gene expression is considered to be a good prognostic marker. Nevertheless, some authors stated that there was no statistically significant correlation between gene expression level and hormonal status [48, 49].

Our results match previously described data. A Korean group [50] found a statistically significant correlation between mammaglobin level of peripheral blood and ER, PR status of patients and their hormonal status. There was no connection related to HER2 status.

Our study indicated that gene expression was 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 studies [51].

It can be concluded that mammaglobin gene expression in carcinoma tissue (not the one in peritumoral tissue) could be used as a prognostic marker 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 the sensitivity of 0.73 and specificity of 0.76 in carcinoma tissue.

Conclusion

To sum up, the basic results of this study are the following: protein expression of mammaglobin in peritumoral and carcinoma tissue can be used as a prognostic marker for dissemination of breast carcinoma; specific values of mammaglobin concentration in peritumoral and carcinoma tissue have been defined above, from which can be assumed that metastatic dissemination of disease could 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; the determined value of mammaglobin gene expression in carcinoma tissue was 1.003; the 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.

R E F E R E N C E S

1. *Siegel RL, Miller KD, Jemal A*. Cancer statistics, 2015. CA Cancer J Clin 2015; 65(1): 5–29.
2. *Zambrano J, Yeh ES*. Autophagy and Apoptotic Crosstalk: Mechanism of Therapeutic Resistance in HER2-Positive Breast Cancer. Breast Cancer (Auckl) 2016; 10: 13–23.
3. *Inoue K, Fry EA*. Novel Molecular Markers for Breast Cancer. Biomark Cancer 2016; 8: 25–42.
4. *Lorincz AM, Sukumar S*. Molecular links between obesity and breast cancer. Endocr Relat Cancer 2006; 13(2): 279–92.
5. *Toss A, Cristofanilli M*. Molecular characterization and targeted therapeutic approaches in breast cancer. Breast Cancer Res 2015; 17: 60.
6. *Koren S, Bentires-Alj M*. Breast Tumor Heterogeneity: Source of Fitness, Hurdle for Therapy. Mol Cell 2015; 60(4): 537–46.
7. *Watson MA, Fleming TP*. Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer. Cancer Res 1996; 56(4): 860–5.
8. *Lacroix M*. Significance, detection and markers of disseminated breast cancer cells. Endocr Relat Cancer 2006; 13(4): 1033–67.
9. *Watson MA, Fleming TP*. Isolation of differentially expressed sequence tags from human breast cancer. Cancer Res 1994; 54(17): 4598–602.
10. *Bellone S, Tassi R, Betti M, English D, Cocco E, Gasparrini S*, et al. Mammaglobin B (SCGB2A1) is a novel tumour antigen highly differentially expressed in all major histological types of ovarian cancer: implications for ovarian cancer immunotherapy. Br J Cancer 2013; 109(2): 462–71.
11. *Zafrakas M, Petschke B, Donner A, Fritzsche F, Kristiansen G, Knuchel R*, et al. Expression analysis of mammaglobin A (SCGB2A2) and lipophilin B (SCGB1D2) in more than 300 human tumors and matching normal tissues reveals their co-expression in gynecologic malignancies. BMC Cancer 2006; 6: 88.
12. *Li G, Zhang J, Jin K, He K, Wang H, Lu H*, et al. Human mammaglobin: a superior marker for reverse-transcriptase PCR in detecting circulating tumor cells in breast cancer patients. Biomark Med 2011; 5(2): 249–60.
13. *O'Brien N, Maguire TM, O'Donovan N, Lynch N, Hill AD, McDermott E*, et al. Mammaglobin a: a promising marker for breast cancer. Clin Chem 2002; 48(8): 1362–4.
14. *Watson MA, Darrow C, Zimonjic DB, Popescu NC, Fleming TP*. Structure and transcriptional regulation of the human mammaglobin gene, a breast cancer associated member of the uteroglobin gene family localized to chromosome 11q13. Oncogene 1998; 16(6): 817–24.
15. *Span PN, Waanders E, Manders P, Heuvel JJ, Foekens JA, Watson MA*, et al. Mammaglobin is associated with low-grade, steroid receptor-positive breast tumors from postmenopausal patients, and has independent prognostic value for relapse-free survival time. J Clin Oncol 2004; 22(4): 691–8.
16. *Lewis GH, Subhawong AP, Nassar H, Vang R, Illei PB, Park BH*, et al. Relationship between molecular subtype of invasive breast carcinoma and expression of gross cystic disease fluid protein 15 and mammaglobin. Am J Clin Pathol 2011; 135(4): 587–91.
17. Molecular Imaging and Contrast Agent Database (MICAD). Bethesda (MD): National Center for Biotechnology Information (US); 2004.
18. *Mikhitarian K, Martin RH, Ruppel MB, Gillanders WE, Hoda R, Schutte del H*, et al. Detection of mammaglobin mRNA in peripheral blood is associated with high grade breast cancer: interim results of a prospective cohort study. BMC Cancer 2008; 8: 55.
19. *Raica M, Cimpean AM, Meche A, Alexa A, Suciu C, Muresan A*. Analysis of the immunohistochemical expression of mammaglobin A in primary breast carcinoma and lymph node metastasis. Rom J Morphol Embryol 2009; 50(3): 341–7.
20. *Nunez-Villar MJ, Martinez-Arribas F, Pollan M, Lucas AR, Sanchez J, Tejerina A*, et al. Elevated mammaglobin (h-MAM) expression in breast cancer is associated with clinical and biological features defining a less aggressive tumour phenotype. Breast Cancer Res 2003; 5(3): R65–70.
21. *O'Brien N, O'Donovan N, Foley D, Hill AD, McDermott E, O'Higgins N*, et al. Use of a panel of novel genes for differentiating breast cancer from non-breast tissues. Tumour Biol 2007; 28(6): 312–7.
22. *Bozhenko VK, Kharchenko NV, Vaskevich EF, Kudinova EA, Oorzhak AV, Rozhkova NI*, et al. Mammaglobin in peripheral blood and tumor in breast cancer patients. Biomed Khim 2016; 62(4): 453–7. (Russian)
23. *Gargano G, Agnese V, Calo V, Corsale S, Augello C, Bruno L*, et al. 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. Ann Oncol 2006; 17 Suppl 7: vii41–5.
24. *Cerveira N, Torres L, Rocha P, Bizarro S, Pereira D, Abreu J*, et al. Highly sensitive detection of the MGB1 transcript (mammaglobin) in the peripheral blood of breast cancer patients. Int J Cancer 2004; 108(4): 592–5.
25. *Elston CW EI, Goulding H*. Role of pathology in the prognosis and management of breast cancer. In: *Elston CW, Ellis IO*, editors. The Breast. London: Churchill Livingstone; 1998. p. 385–433.
26. *Edge SB, Compton CC*. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 2010; 17(6): 1471–4.
27. *Lowry OH, Rosebrough NJ, Farr AL, Randall RJ*. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193(1): 265–75.
28. *Chomczynski P, Sacchi N*. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162(1): 156–9.

29. *Bustin SA*. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000; 25(2): 169–93.

30. *Schmittgen TD, Livak KJ*. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008; 3(6): 1101–8.

31. *Aristizabal-Pachon AF, de Carvalho TI, Carrara HH, de Andrade JM, Takahashi CS*. Detection of human mammaglobin A mRNA in peripheral blood of breast cancer patients before treatment and association with metastasis. J Egypt Natl Canc Inst 2015; 27(4): 217–22.

32. *Lapeire L, Hendrix A, Lambein K, Van Bockstal M, Braems G, Van Den Broecke R*, et al. Cancer-associated adipose tissue promotes breast cancer progression by paracrine oncostatin M and Jak/STAT3 signaling. Cancer Res 2014; 74(23): 6806–19.

33. *Trifiletti DM, Sturz VN, Showalter TN, Lobo JM*. Towards decision-making using individualized risk estimates for personalized medicine: A systematic review of genomic classifiers of solid tumors. PloS One 2017; 12(5): e0176388.

34. *Tseng LM, Hsu NC, Chen SC, Lu YS, Lin CH, Chang DY,* et al. Distant metastasis in triple-negative breast cancer. Neoplasma 2013; 60(3): 290–4.

35. *Fanger GR, Houghton RL, Retter MW, Hendrickson RC, Babcook J, Dillon DC*, et al. Detection of mammaglobin in the sera of patients with breast cancer. Tumour Biol 2002; 23(4): 212–21.

36. *Zehentner BK, Dillon DC, Jiang Y, Xu J, Bennington A, Molesh DA*, et al. Application of a multigene reverse transcription-PCR assay for detection of mammaglobin and complementary transcribed genes in breast cancer lymph nodes. Clin Chem 2002; 48(8): 1225–31.

37. *Bernstein JL, Godbold JH, Raptis G, Watson MA, Levinson B, Aaronson SA*, et al. Identification of mammaglobin as a novel serum marker for breast cancer. Clin Cancer Res 2005; 11(18): 6528–35.

38. *Liu Y, Ma L, Liu X, Wang L*. Expression of human mammaglobin as a marker of bone marrow micrometastasis in breast cancer. Exp Ther Med 2012; 3(3): 550–4.

39. *Tafreshi NK, Enkemann SA, Bui MM, Lloyd MC, Abrahams D, Huynh AS,* et al. A mammaglobin-A targeting agent for noninvasive detection of breast cancer metastasis in lymph nodes. Cancer Res 2011; 71(3): 1050–9.

40. *Rehman F, Nagi AH, Hussain M*. Immunohistochemical expression and correlation of mammaglobin with the grading system of breast carcinoma. Indian J Pathol Microbiol 2010; 53(4): 619–23.

41. *Bhargava R, Beriwal S, Dabbs DJ*. Mammaglobin vs GCDFP-15: an immunohistologic validation survey for sensitivity and specificity. Am J Clin Pathol 2007; 127(1): 103–13.

42. *O'Brien NA, O'Donovan N, Ryan B, Hill AD, McDermott E, O'Higgins N*, et al. Mammaglobin a in breast cancer: existence of multiple molecular forms. Int J Cancer 2005; 114(4): 623–7.

43. *Guan XF, Hamedani MK, Adeyinka A, Walker C, Kemp A, Murphy LC*, et al. Relationship between mammaglobin expression and estrogen receptor status in breast tumors. Endocrine. 2003; 21(3): 245–50.

44. *Chen G, Gharib TG, Huang CC, Taylor JM, Misek DE, Kardia SL*, et al. Discordant protein and mRNA expression in lung adenocarcinomas. Mol Cell Proteomics 2002; 1(4): 304–13.

45. *Wei C, Luo Q, Sun X, Li D, Song H, Li X*, et al. MicroRNA-497 induces cell apoptosis by negatively regulating Bcl-2 protein expression at the posttranscriptional level in human breast cancer. Int J Clin Exp Pathol 2015; 8(7): 7729–39.

46. *Vasudevan D, Hickok JR, Bovee RC, Pham V, Mantell LL, Bahroos N*, et al. Nitric Oxide Regulates Gene Expression in Cancers by Controlling Histone Posttranslational Modifications. Cancer Res 2015; 75(24): 5299–308.

47. *Marchetti A, Buttitta F, Bertacca G, Zavaglia K, Bevilacqua G, Angelucci D*, et al. mRNA markers of breast cancer nodal metastases: comparison between mammaglobin and carcinoembryonic antigen in 248 patients. J Pathol 2001; 195(2): 186–90.

48. *Marques AR, Teixeira E, Diamond J, Correia H, Santos S, Neto L*, et al. Detection of human mammaglobin mRNA in serial peripheral blood samples from patients with non-metastatic breast cancer is not predictive of disease recurrence. Breast Cancer Res Treat 2009; 114(2): 223–32.

49. *Fortunato L, Mascaro A, Baldi A, Farina M, Cortese G, Ventrone MA*, et al. Positive bone marrow biopsy is associated with a decreased disease-free survival in patients with operable breast cancer. Ann Surg Oncol 2009; 16(11): 3010–9.

50. *Lee GW, Kim JY, Koh EH, Kang D, Choi DS, Maeng KY,* et al. Plasma human mammaglobin mRNA associated with poor outcome in patients with breast cancer. Genet Mol Res 2012; 11(4): 4034-42.

51. *Al Joudi FS*. Human mammaglobin in breast cancer: a brief review of its clinical utility. Indian J Med Res 2014; 139(5): 675–85.