

## ORIGINAL PAPER

# Analysis of the immunohistochemical expression of mammaglobin A in primary breast carcinoma and lymph node metastasis

M. RAICA<sup>1)</sup>, ANCA MARIA CÎMPEAN<sup>1)</sup>, ADRIANA MECHE<sup>2)</sup>,  
AURORA ALEXA<sup>1)</sup>, C. SUCIU<sup>1)</sup>, ANCA MUREȘAN<sup>3)</sup>

<sup>1)</sup>Department of Histology & Cytology,  
"Victor Babeș" University of Medicine and Pharmacy, Timisoara

<sup>2)</sup>Department of Pathology, County Hospital Arad

<sup>3)</sup>Department of Pathology,  
"Victor Babeș" University of Medicine and Pharmacy, Timisoara

### Abstract

Mammaglobin A is a specific marker of the normal and neoplastic mammary tissue that usually is detected by RT–PCR. Few data are available about the immunohistochemical expression of this marker in mammary carcinoma and about the significance of the positive reaction. Our purpose was to investigate the sensitivity of the mammaglobin expression in breast cancer and to determine its correlations with conventional prognostic parameters. There were investigated 47 patients with breast carcinoma, and slides from paraffin blocks were stained with an antibody against mammaglobin. The immunohistochemical reaction was scored based on the percentage of positive tumor cells in both primary tumors and lymph node metastasis. Positive reaction for mammaglobin was found in the normal mammary tissue adjacent to the tumor in all cases, in 78.72% primary breast carcinoma, and in 58.06% of cases with lymph node metastases. A significant correlation was found between the mammaglobin expression in the primary tumor, grade, and lymph node status, but not with the age of the patient, pathologic subtype of carcinoma and stage of the tumor. The ductal *in situ* carcinoma associated to the invasive tumor did not influence significantly the prognostic value of mammaglobin expression. Our results suggest that mammaglobin is a sensitive marker of breast carcinoma, it defines a subgroup of patients with better prognosis and is a useful method to detect breast cancer metastases.

**Keywords:** breast cancer, diagnosis, immunohistochemistry, lymph node mammaglobin, metastasis, prognosis.

### Introduction

Breast cancer is the most frequent neoplasia in female, and the morbidity and specific mortality continue to increase, in spite of remarkable progresses in the field of early diagnosis and adjuvant therapy. Breast cancer patients with the same pathologic diagnosis and clinical prognostic profile can have different clinical outcomes. This difference is probably because molecularly distinct diseases are grouped into clinical subtypes, based on their morphology [1]. In an attempt to solve this problem, Perou CM *et al.* [2] classified breast cancer based on their gene expression profile and immunohistochemical expression of cytokeratin (5/6 and 8/18), estrogen receptors, and HER2. This classification, which recognizes luminal, basal, and HER2 subtypes, had an immediate impact on therapeutic strategies and it was shown that molecular subtypes respond differently to preoperative chemotherapy [3] and to adjuvant postoperative therapy [4]. These data bring new insights into the natural evolution and biology of breast cancer, but the molecular profile of these tumors is far to be complete. The identification of new proteins in human breast carcinoma continues and results might be important in the detection of novel potential biomarkers and may provide information

on the molecular mechanisms of tumorigenesis [5]. A challenging and promising topic is represented by the discovery of mammaglobin, initially used in the diagnosis and prognosis of breast carcinoma, and soon after that, as a potential target for therapy.

Mammaglobin A is a glycoprotein that belongs to secretoglobulin superfamily and is specifically expressed by normal mammary tissue and overexpressed by breast carcinoma [6, 7]. Although the functional significance of mammaglobin A remains unknown, the restricted expression to the mammary tissue led to the evaluation of this molecule as a possible breast cancer marker [8]. Preliminary results showed that mammaglobin is a useful marker in the diagnosis of primary breast tumors, lymph node metastases, and also in the detection of tumor cells in the peripheral circulation [9–11].

Most of the results were obtained using gene analysis of breast cancer tissue, but this method cannot be applied in all cases, mainly due to the technical procedures. For this reason, immunohistochemistry seems to be easier, more practical and associated to a good sensitivity. The original application of the anti-mammaglobin antibody showed positive reaction in 80% of cases with ductal carcinoma and in all cases of normal mammary tissue but with less than 10% positive

epithelial cells [9]. The antibody against mammaglobin is highly specific for mammary tissue because it does not stain other normal tissues, excepting for sweat glands of the skin.

Immunohistochemistry is nowadays commonly used to detect micrometastases in axillary lymph nodes, but this assay typically relies on antigens expressed by all epithelial cells. Mamaglobin A was detected by RT-PCR in 66% of the lymph nodes involved by metastases and in 8 to 13% of cases with histologically negative sentinel nodes [10, 12]. These findings suggest that mamaglobin may be useful as a marker of micrometastases. Few and controversial data are available about the diagnostic value of mamaglobin immunohistochemistry in the detection of lymph node metastasis, and virtually, there are no data regarding the correspondence between the expression in the primary and metastases. Because of these reasons, we have been examining the expression of mamaglobin A for its potential utility in the pathologic diagnosis of breast cancer in terms of differentiation, for the possible use in detection of lymph node metastases and the relationships of its expression with commonly used clinical prognostic markers.

## Patients, Material and Methods

### Patient's data

There were investigated 47 patients admitted with breast cancer, aged between 27 and 75 years, without prior chemotherapy or radiotherapy. Primary tumors were surgically removed and lymph node dissection was performed in all cases. Lymph node metastases were found in 31 cases (65.95%). Distribution of cases based on the tumor stage and lymph node metastasis are shown in Table 1.

**Table 1 – Distribution of cases based on tumor stage and lymph node metastases**

Tumor stage/N	N-	N+	Total
T1	6	10	16 (34.04%)
T2	9	14	23 (48.93%)
T3	0	3	3 (6.38%)
T4	1	4	5 (10.63%)

Legend: N, lymph node status; N-, without lymph node metastases; N+, with lymph node metastases.

### Tissue processing

Specimens from the primary tumors and lymph nodes were fixed in buffer formalin and embedded in paraffin. Five-µm thick sections were stained with Hematoxylin–Eosin for the pathological diagnosis and grading of the tumors. The grade was assessed using the Scarff–Bloom–Richardson system.

### Immunohistochemistry

Additional slides from the primary tumors and lymph nodes were stained for mamaglobin. Deparaffinized and hydrated slides were treated with hydrogen peroxide 3% for 5 minutes, and antigen retrieval was performed at microwave in buffer solution pH 9 for 40 minutes. Slides were then incubated for

30 minutes with the antibody against mamaglobin, clone 304–1A5, ready-to-use. The working system was EnVision, and 3,3'-diaminobenzidine was used as chromogen. Nuclei were stained with Lillie's modified Hematoxylin. All reagents used in the present study were from DakoCytomation (Denmark).

### Evaluation

Evaluation was performed with Eclipse 80i Nikon microscope and the reaction for mamaglobin was scored in five microscopic fields (200× magnification) in the tumor area and in three fields of adjacent normal mammary tissue. The same procedure was applied for slides from lymph node metastasis. Results for mamaglobin detection in the primary tumors were compared with those found in corresponding lymph nodes. Finally, we performed a correlation between mamaglobin immunohistochemical expression and stage of the tumor, grade of differentiation, and lymph node status.

### Mamaglobin scoring

The scoring of the mamaglobin reaction was based on the number of positive epithelial cells, as follows: negative (0), weak positive with less than 10% positive cells (+1), moderate positive with 11 to 50% stained cells (+2), and strong with over 50% stained cells (+3). The intensity of the final product of reaction was not taken into account, because all stained cells showed a strong reaction.

### Statistic analysis

Statistical analysis was performed using Stat Plus 2007 program with Spearman's correlation tests. A significant correlation was reported for a value of  $p < 0.05$ . We tried to find if there is any correlation between age, histopathology, tumor stage, grade, association of DCIS with invasive carcinoma, and lymph node status with the expression of mamaglobin.

## Results

### Pathologic report

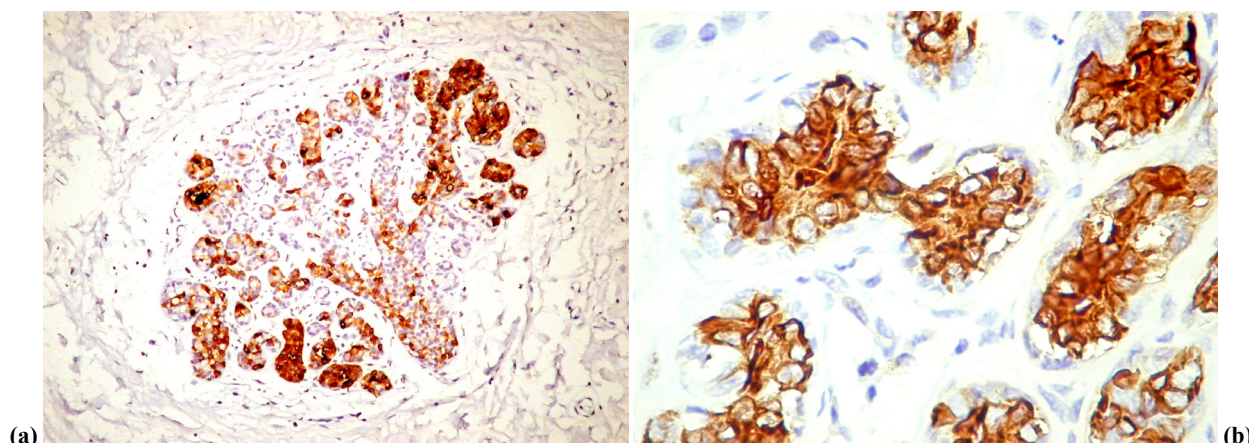
Ductal invasive carcinoma was diagnosed in 41 cases, lobular invasive carcinoma was found in three cases, papillary carcinoma in two cases, and mucinous carcinoma in one case. Four cases were well differentiated (G1), 27 cases were moderately differentiated (G2), and 16 were undifferentiated (G3). Associated ductal *in situ* carcinoma (DCIS) was identified in 25 cases, atypical ductal hyperplasia in four cases and apocrine metaplasia in two cases. Normal mammary tissue adjacent to the tumor was found in 34 patients.

### Mamaglobin expression

The final product of reaction for mamaglobin was intensely stained, with cytoplasmic granular pattern, and restricted to epithelial cells. Normal mammary tissue adjacent to the tumor was positive in all 34 cases, but less than 50% of epithelial cells were stained (Figure 1a). Occasionally, some terminal duct-

lobular units showed homogeneous positive reaction (Figure 1b). Excepting for the mammary tissue, only sweat glands of the skin were found positive. In both cases with apocrine metaplasia almost all

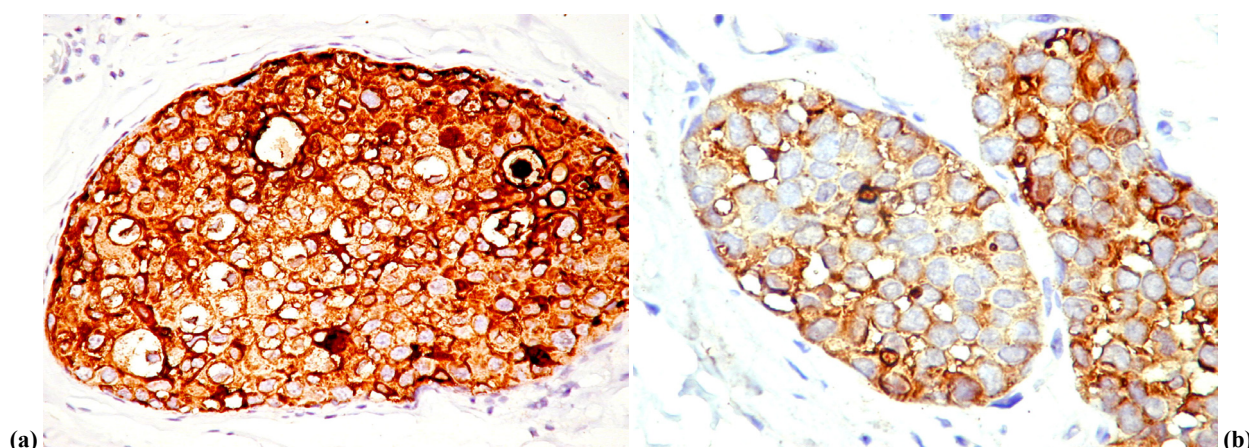
cells were intensely stained, and three from four cases with associated ductal atypical hyperplasia were weak (two cases) and moderately stained (one case).



**Figure 1 – Normal mammary tissue. Terminal duct-lobular unit with heterogeneous positive reaction (a, 40×). Detail on the secretory component (b, 400×).**

DCIS, found in association with the invasive carcinoma in 25 cases, was positive for mammaglobin in 22 cases (88%). Usually, the number of positive cells in DCIS was significantly higher than in the invasive

tumor. Two models of distribution of the final product of reaction were recognized: diffuse, with all tumor cells stained (Figure 2a), and patchy, with heterogeneous feature (Figure 2b).



**Figure 2 – DCIS, diffuse pattern (a), and heterogeneous (b). Anti-mammaglobin, 400×.**

In invasive carcinoma, the reaction was positive in 37 cases (78.72%) and negative in 10 cases (21.27%). The majority of the cases showed isolated positive cells and less than 10% of the tumor cell population was stained (Figure 3a), and in the other 17 cases, more than 10% of tumor cells were strong positive (Figure 3, b and c). Data on the distribution of cases based on the mammaglobin scoring are shown in Table 2.

**Table 2 – Distribution of cases based on the mammaglobin-scoring**

Score	0	+1	+2	+3
Mammaglobin	10 (21.27%)	20 (42.55%)	8 (17.02%)	9 (19.14%)

No significant correlation was found between the pathologic subtype of carcinoma and mammaglobin expression. Detection of DCIS associated to invasive carcinoma had no statistic significance. Ductal invasive carcinoma with and without associated DCIS was not

correlated with mammaglobin expression ( $p=0.3712$  for invasive ductal carcinoma without DCIS, and  $p=0.5073$  for associated DCIS).

We found mammaglobin-positive reaction in all four well-differentiated tumors, in 23 from 27 G2 cases, and in 12 from 16 undifferentiated breast carcinomas. Significant correlation was found between the tumor grade and mammaglobin expression ( $p=0.0152$ ). No significant correlation was found between mammaglobin expression and stage of the tumor ( $p=0.3597$ ).

We analyzed next the subgroup of 31 patients with lymph node metastases. From them, the primary tumors expressing mammaglobin was found in 23 cases. The corresponding lymph node metastases were found positive in 18 cases (58.06%) (Figure 3d). In only one case, the primary was negative and the metastasis was positive with few tumor cells. From the remaining 16 cases, without lymph node metastases, 14 were positive for mammaglobin and only two negative cases



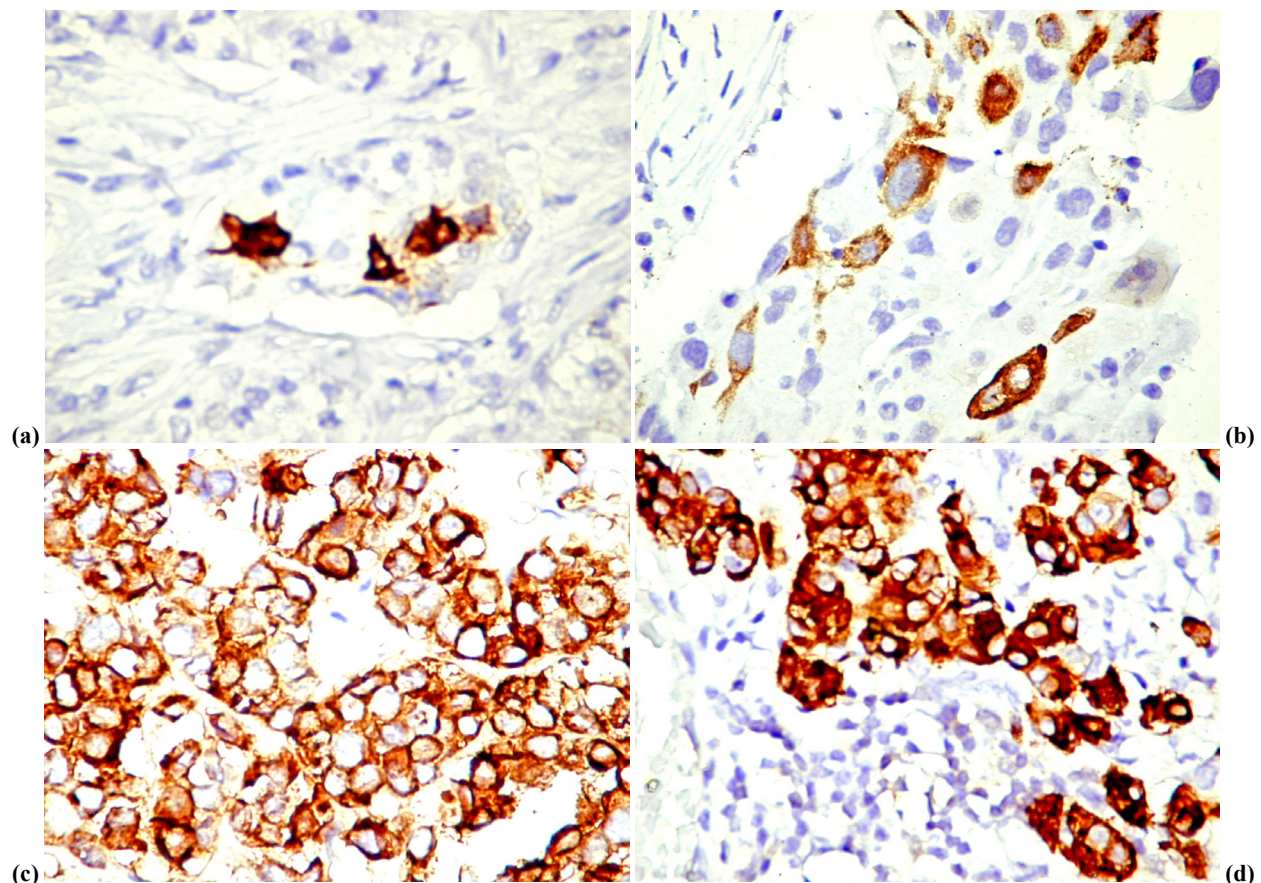
were detected. The relation between the immunohistochemical expression of mammaglobin in the primary and corresponding lymph node metastasis is shown in Table 3.

**Table 3 – Expression of mammaglobin in the primary tumors and corresponding lymph node metastases (n=31)**

Tumor	Mammaglobin positive	Mammaglobin negative
Primary	23 (74.19%)	8 (25.80%)
Lymph node metastasis	18 (58.06%)	13 (41.93%)

The lymph node status correlated with mammaglobin expression ( $p=0.0215$ ). The mammaglobin reaction was negative in all 16 cases without lymph node metastasis.

From all 37 cases positive for mammaglobin, 28 aged over 40-year-old and the distribution of cases between subgroup scored with +1 and subgroup scored with +2 and +3 was similar. No significant correlation was found between age and expression of mammaglobin in primary breast tumors included in our study ( $p=0.09$ ).



**Figure 3 – Primary breast carcinoma with few positive cells (a), more than 10% positive cells (b), and almost all positive tumor cells (c). Lymph node metastases, all tumor cells are positive (d). Anti-mammaglobin, 400×.**

## Discussion

In the current study, we describe the immunohistochemical expression pattern of mammaglobin A in the primary breast carcinoma and lymph node metastases. Watson MA and Fleming TP [6] identified a gene expressed only in the breast tissue, using a modified display technique. This gene was called mammaglobin and encodes a 93-amino acid protein, known today as mammaglobin A. Mammaglobin A gene is localized on chromosome 11q13 together with other human secretoglobins, where they form a dense cluster [13]. Until now, no gene amplification or rearrangement was detected in breast carcinoma, and therefore, the over-expression found in many cases seems to be the consequence of a change in transcriptional regulation, as suggested by Watson MA *et al.* [14].

The original scoring system for mammaglobin immunohistochemical expression [9], based on the

cut-off point of 50% positive tumor cells and the intensity of reaction differentiates five tumor subclasses. Our results showed that the positive reaction is always strong with diffuse, cytoplasmic pattern, even in cases with few positive cells, and therefore, the intensity is less useful for the scoring. On the other hand, we found a clear correlation between mammaglobin expression and grade of the tumor, and this is why we used another scoring system with only four subclasses and based strictly on the number of positive tumor cells.

Mammaglobin A was detected by RT-PCR and immunohistochemistry in 55.4 to 81% of the cases with breast carcinoma [9, 15] and in only 43% of the cases of nonmalignant breast by RT-PCR [16]. We found positive reaction for mammaglobin in all cases in the normal mammary tissue adjacent to the tumor (n=34). This finding might be explained either by low serum levels undetectable by RT-PCR, or by the secretory

activation of apparently normal mammary tissue. The first hypothesis is indirectly supported by the study of O'Brien NA *et al.* [16], who detected mammaglobin expression by RT-PCR in 59% of cases with fibroadenoma and in less than half of the cases with nonmalignant breast. There is a general agreement in the literature regarding the high number of mammaglobin-expressing breast cancer. Mammaglobin is a more sensitive marker than gross cyst-disease fluid protein-15 (GCDFFP-15) for breast carcinoma, but lacks the specificity of GCDFFP-15. On the other hand, only very few cases are negative for both markers, and therefore, the combination is extremely helpful for the diagnosis [17].

Almost all studies showed that mammaglobin expression in breast tumors was observed at a high frequency, as we also demonstrated in the present finding. In our study, mammaglobin was expressed in 78.72% of cases and this suggests that it is a useful diagnostic marker but lacks absolute sensitivity. On the other hand, some authors found no correlation between its expression and stage, grade, or histology of the tumor [9, 18]. In only one study, it was shown that mammaglobin expression detected by RT-PCR is restricted to cases with G3 grading [19]. Our results support these findings only in terms of the tumor stage and histology ( $p=0.3597$ , and  $p=0.3712$  respectively), but not concerning the grade, for which we found a significant correlation in our patients ( $p=0.0152$ ). Our results confirm previous studies that demonstrated that high mammaglobin expression reflects a better differentiation, a low proliferation rate and high hormone dependence [18, 20, 21]. It was demonstrated that mammaglobin protein exists in two main forms in breast tissue, and only the high molecular weight form was found more frequently in receptor-positive cancers [22]. The high molecular weight expression of mammaglobin correlated positively with hormone receptors and negatively with tumor grade and seems to be associated with better prognosis. All together, these results demonstrate that the assessment of mammaglobin expression could be useful to stratify patients for adjuvant therapy.

Although expressed in many cases with breast carcinoma, mammaglobin is not entirely specific. Some studies have shown that mammaglobin is also expressed by other normal tissues, like the endometrium, ovary or sweat glands, and in tumors of the sweat gland, lung and ovary [16, 23–25]. All tumors of the thyroid, pancreas, gastrointestinal tract, head and neck and uterine cervix were negative [26]. Excepting for the tumors of the sweat glands and lungs, the number of positive cases and the level of expression was very low as compared with breast cancer. In tumors of the lung, only some cases with primary adenocarcinoma were found positive and all metastatic breast tumors expressed mammaglobin [27]. This suggests that mammaglobin expression is useful in the differential diagnosis. On the other hand, the expression of mammaglobin in normal tissues restricts its use as immunotherapeutic target, as proposed by some authors [28–30].

Min CJ *et al.* [31] evaluated seven potential breast

cancer tumor markers and from these only mammaglobin was detected by RT-PCR in carcinoma cell lines but not in normal lymph nodes. In lymph nodes of patients with breast carcinoma, the concordance between mammaglobin detection by RT-PCR and histopathological findings range between 79 and 93.4% [32–34]. These results suggest the inclusion of mammaglobin as a component of panels evaluating metastatic tumors of unknown primary [35]. In our patients, no expression of mammaglobin was found in lymph node without metastases and positive reaction was found in 18 from 23 cases with positive primary tumors. In only one case, the primary tumor was negative and the lymph node metastasis showed positive reaction. These data, together with our results, demonstrated that mammaglobin expression is very useful to detect metastases, but the method is less sensitive than initially believed. Similar studies were performed on bone marrow aspirates from patients with residual breast cancer and only 12% were mammaglobin-positive [36], and on circulating tumor cells with a sensitivity of 63.3% [37]. These results demonstrate that molecular characterization of epithelial cells using mammaglobin offers potential for early detection of invasive and metastatic breast carcinoma.

Based on previous studies and our results, we may conclude that mammaglobin is a useful and specific marker of breast carcinoma. Detection of mammaglobin A by immunohistochemistry is helpful in the diagnosis of lymph node metastases and the finding of mammaglobin-positive cells in a distant metastasis with unknown primary would suggest a breast origin.

## Conclusions

In summary, the immunohistochemical expression of mammaglobin was found in 78.72% of the cases with primary breast carcinoma, and correlated with lymph node status and grade, but not with the stage of the tumor. Mammaglobin was expressed in lymph node metastases in 58.06% of the cases and results strongly correlated with the positive reaction in the primary tumor. Our findings suggest the mammaglobin expression defines a subgroup of patients with better prognosis and is a useful method to detect breast cancer metastases.

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**Corresponding author**

Marius Raica, Professor, MD, PhD, Department of Histology & Cytology, “Victor Babeş” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timișoara, Romania; Phone +40722–438 170, Fax +40256–490 626, e-mail: raica@umft.ro

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