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ORIGINAL ARTICLE

Estimation of maspin's subcellular localization in invasive ductal breast cancer via light microscopy and computerized image analysis: a comparative study

M. Panou[1,4*], N. Kavantzas MD.[1*], T. Sergentanis[2], S. Sakellariou[1], G. Agrogiannis[1], P. Chatzipantelis[3], E. Anastasopoulou[3], V. Soranoglou[4], F. Zagouri[5], P. Korkolopoulou[1], E. Patsouris[1], I. Bramis MD.[5], G. C. Zografos[5]

[1]1st Department of Pathology, School of Medicine, National and Kapodistrian University of Athens, Athens; [2]Department of Hygiene, Epidemiology and Medical Statistics, School of Medicine, National and Kapodistrian University of Athens, Athens; [3]Department of Pathology, "Hippokrateion" General Hospital, Athens; [4]2nd Department of Orthopaedics, "G. Gennimatas" Athens General Hospital, Athens; [5]1st Department of Propaedeutic Surgery, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

**Authors equally contributed*

Summary

Purpose: *Contradictory results have been reported concerning the role of maspin and its cellular distribution in breast cancer. The purpose of this study was to examine the subcellular localization (nuclear–cytoplasmic) of maspin in breast cancer and to compare the evaluation of maspin immunostaining via light microscopy (LM) to the estimation via computerized image analysis (CIA) system. We also examined correlations between maspin expression and several clinicopathological parameters.*

Methods: *The sample consisted of 48 primary invasive ductal carcinomas (IDC) of the breast. Maspin immunostaining was quantified and graded via LM by two pathologists, separately in the nuclear and cytoplasmic compartments. Total maspin expression was also estimated via CIA system. Univariate non-parametric statistics and stepwise multivariate ordinal logistic regression were performed.*

Results: *Both maspin components (nuclear and cytoplasmic) were closely associated with each other (p<0.001). Total maspin score was positively and closely associated with nuclear maspin (p<0.001) and cytoplasmic maspin (p<0.001). Total maspin , nuclear maspin and cytoplasmic maspin did not correlate significantly with either age, grade, T, N and M status, stage, micro vessel density (MVD) (CD34), ki-67, p53, estrogen receptor (ER) and HER-2 status, or with any of the 4 groups of the molecular classification. The only factor that showed a borderline inverse correlation with nuclear maspin (p=0.059) was progesterone receptors (PR) positivity.*

Conclusion: *The cytoplasmic and nuclear fractions of maspin seem to be closely interwoven. Evidently, both mutually intertwined counterparts were independently reflected upon the total maspin levels measured by CIA. Future studies should ideally encompass all three approaches (nuclear, cytoplasmic, total) adopted herein.*

Key words: *breast cancer, image analysis, immunohistochemistry, maspin, progesterone receptor, subcellular localization*

Introduction

Clade B serpin family of proteins regulates a variety of cellular functions including cell adhesion and motility. One key member of the family is maspin (Mammary Serine Protease Inhibitor) or SERPINB5 [1]. Maspin plays a role in the development of the mammary gland and is expressed in myoepithelial cells and normal secretory epithelial cells [2].

To date, an impressive list of biological functions has been attributed to both intercellular and

Correspondence to: Marios Panou, MD. 1st Department of Pathology, School of Medicine, National and Kapodistrian University of Athens. Moschopoulou 3 street, 18547 N. Faliro, Athens, Greece. Tel: +30 6972600283, Fax: +30 2107462157, E-mail: marios.panou@yahoo.gr
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extracellular maspin, which includes promoting cell adhesion and apoptosis and inhibiting cell motility, invasion and angiogenesis [3], suggesting that maspin is a tumor suppressor gene [4]. Maspin expression may predict a better prognosis for several types of carcinomas including breast, prostate, colon and oral squamous cell carcinoma [5]. However, in other malignancies, such as pancreatic, lung, thyroid, ovarian and endometrial cancers [6-9], maspin expression was paradoxically increased in malignant cells compared to their normal cells of origin. Concerning the role of maspin in breast cancer and its prognostic impact, contradictory results have been reported. Some earlier studies on maspin demonstrated its tumor-suppressive properties [2,10,11] mediated by several mechanisms including anti-angiogenesis [12], anti-invasion and anti-metastatic functions [2,13,14] and induction of p53-mediated apoptosis [15]. Conversely, Umekita et al. [16,17] reported that expression of maspin in breast cancer is associated with significantly shorter relapse-free survival and that the expression of maspin is up-regulated during the progression of ductal breast carcinoma. Other researchers have also associated maspin expression with higher histologic grade [18,19], larger tumor size, positive p53 status, shorter survival [19] and finally with poor prognosis [17,18]. Consequently, the mutually conflicting data have tempered early enthusiasm for maspin as a biomarker for disease progression [20].

Another unclear topic about maspin expression in breast cancer is the relevance of subcellular localization of maspin, as the latter may indicate different functions [3]; therefore, the aforementioned discrepancies may be a consequence of differential cellular distribution of maspin [20]. Still, many authors [19,21] do not dissociate between its nuclear and cytoplasmic staining.

In light of the above controversies, this study aims were:

i. To focus on the subcellular localization of maspin (nuclear – cytoplasmic) in breast cancer, examining the association between the expression of maspin in the two compartments .
ii. To compare the evaluation of maspin immunostaining by a pathologist via LM to the estimation via CIA system; to our knowledge this is the first published effort in the literature.
iii. To examine correlations between maspin expression and several clinicopathological parameters in breast cancer.

Methods

Patients and tissue specimens

Paraffin-embedded tissue blocks were collected from 48 patients with primary breast cancers and their adjacent normal breast tissues. These patients had been operated on from April 2004 to November 2008, either with mastectomy or breast-conserving surgery with axillary lymph node dissection at the 1st Department of Propaedeutic Surgery of Athens Medical School, Hippokration General Hospital. All breast cancers were histologically classified as IDC according to the criteria of the World Health Organisation (WHO). Cases of IDC with a predominant *in situ* component were excluded; moreover, patients who had undergone chemotherapy or radiotherapy prior to surgery were excluded, too. The histologic tumor type and grade were assigned according to the criteria of Elston and Ellis [22]. Cancers were staged according to the International Union against Cancer – TNM classification [23]. They were also classified in 4 categories (luminal A, luminal B, HER2(+) and triple-negative) according to the modern molecular classification [24,25]. The study protocol was approved by the local institutional review board.

Immunohistochemistry

The following antibodies and dilutions were used:

- Maspin Rb Polyclonal (h-130): sc-22762 (Santa Cruz Biotechnology, INC), 1:100.
- Monoclonal mouse CD34 antibody: NCL-L-END (Novocastra), 1:50.
- Monoclonal mouse anti-human ki-67 antigen: Clone MIB-1, Code: M7240 (Dako), 1:50.
- Monoclonal mouse anti-human estrogen receptor α: Clone 1D5, Code: M7047 (Dako), 1:50.
- Monoclonal mouse anti-human progesterone receptor: Clone PgR 636, Code: M3569 (Dako), 1:50.
- Mouse anti-HER2: MSK044 (Zytomed Systems), 1:100.
- Monoclonal mouse anti-human p53 protein: Clone DO-7, Code: IR616 (Dako), 1:50.

Specimens were fixed in 10% neutrally buffered formalin and embedded in paraffin. The slides, 3 μm thick, were heated at 40 °C, deparaffinized and rehydrated through a graded series of ethanol. Then, endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 solution in methanol. Antigen retrieval was achieved when the slides were placed in sodium citrate buffer (10 mM, pH 6.0) in a microwave oven for 10 min. The appropriate primary antibody was diluted to its optimum concentration in phosphate buffered saline (PBS). The slides were then incubated with primary antibodies at 4 °C for 24 h. Thereafter, the slides were thoroughly rinsed with PBS and polymer envision secondary antibody was applied to them for 30 min at room temperature. To reveal the color of antibody

staining, DAB (diaminobenzidine) substrate solution was used as chromogen. In each case, maspin staining was also examined in adjacent normal breast tissue for matching (internal control).

Assessment of immunohistochemical staining by the pathologists

Immunostaining was quantified and graded by LM. At least 3 areas with the highest degree of positive cells were selected and typically 400-500 tumor cells in each field were counted irrespective of immunoreactive status. Thereafter, positive cells were counted and the percentage of positive cells was determined. Each staining result was assessed independently by two pathologists (NK and SS), blind to outcome data and results of CIA. When the evaluations differed, final agreement was reached by consensus.

For maspin staining quantification, we used an already published system [26,27]; specifically, the intensity of staining (no staining =0; low level of staining =1; medium staining =2; strong staining =3) and the percentage of stained cells (0% =0; <10% =1; 11-50% =2; 51-80% =3; >80% =4) were multiplied and an immunoreactive score ($IRS_{PATHOLOGIST\ SCORE}$) ranging from 0 to 12 was obtained. Separate IRS scores were obtained for nuclear and cytoplasmic maspin.

Tumors that showed <10% of positive cells in p53, ER and PR were interpreted as negative. The ki-67 labelling index was determined as the percentage of positive cells. Finally, scoring of HER2 results was done using the categories 0, 1+, 2+, 3+. Categories 0 & 1+ were considered as negative and category 3+ as positive; cases categorized as 2+ underwent Chromogenic In Situ Hybridization (CISH) to define HER-2 status.

Computerized image analysis system

CIA system was used, as described elsewhere [28,29], for the estimation of total maspin expression and for the estimation of MVD, through counting the total number of intimal blood vessels, which were lined with CD34-positive endothelial cells.

Processing methodology

- In every histological section, 7-10 regions were selected (Hot Spots) and captured as images (Analog SC30 Olympus Camera), using 40x objective lens (BX43 3-Ophthalmic Microscope). All images were stored as TIFF files.
- Image calibration, based on stain and Optical Density [30].
- Image calibration, based on microscope lens, with micrometric scale, in measurements unit microns.
- Positive cells detection and counting.
- In each image, the parameters measured by the CIA system (Image-Pro Plus 6.0 – Media Cybernetics, INC) [30] were the intensity of maspin staining in tumor cells and the percentage of maspin-stained area (% area) in relation to the whole tissue.
- Concerning CD34-positive endothelial cells blood vessels counting and thus MVD estimation, the parameters measured were number of vessels, area, diameter, aspect ratio, and percentage area.
- The areas stained by the antibodies were identified and calculated using the software "histogram-based algorithm".
- Staining intensity levels were measured using arbitrary units on a linear scale ranging from 0 (highest intensity) to 255 (not detectable), for every RGB (Red – Green – Blue) channel (color segmentation). Averaging the quantitative CIA data from the 7-10 images of each tissue section yielded an average staining intensity and an average percentage of extent of staining (% area) (Figures 1d, 2b, 2d, 3b).
- The $IRS_{IMAGE\ ANALYSIS\ SCORE}$ ($IRS_{I.A.SCORE}$) was estimated through the following formula:
- $IRS_{I.A.SCORE}$ = (255 – a) x b%, where:
- a = the measured intensity of maspin staining (range between 0 – 255) and
- b = the percentage of positive cells.
- MVD estimation was expressed as the total number of intimal blood vessels per unit area (No/mm^2).

Statistics

After the calculation of descriptive statistics, the intercorrelations between total maspin ($IRS_{I.A.SCORE}$), nuclear maspin ($IRS_{PATHOLOGIST\ SCORE}$), cytoplasmic maspin ($IRS_{PATHOLOGIST\ SCORE}$), CD34 (No/mm^2) and clinicopathological parameters (molecular classification, grade, stage, tumor size, nodal status, metastasis, p53 expression, ER status, PR status, HER2 status) were evaluated. Given the marked deviation from normality of the scores (as attested by the Shapiro-Wilk test), non-parametric statistical tests were performed; specifically, Spearman's rank correlation coefficient, Mann-Whitney-Wilcoxon (MWW) test for independent samples and Kruskal-Wallis (KW) test were undertaken, as appropriate. The assessment of the intercorrelations between the aforementioned parameters should be deemed exploratory, due to the multiple underlying comparisons-tests.

In order to overcome the statistical problem of multiple comparisons and ascertain the independence of associations, stepwise multivariate ordinal logistic regression was performed; total maspin IRS score ($IRS_{I.A.SCORE}$) was converted into ordinal variable using a 4-level scale (1: minimum value-25th percentile; 2: 25th percentile – median; 3: median - 75th percentile, 4: 75th percentile-maximum value). The proportionality-of-odds assumption was evaluated by the appropriate likelihood ratio test. The level of statistical significance was set at 0.05. Statistical analyses were performed with STATA 11.1 software (StataCorp, College Station, TX, USA).

Results

Table 1 presents the description of the study sample. The age of women ranged between 33 and 86 years (63.3±12.5 years, mean±SD). Luminal A (33.3%) and luminal B (36.1%) carcinomas were the most prevalent categories. The majority of cases consisted of grade 3 carcinomas (70.8%); nearly half the cases were stage II (II_A: 29.2% and II_B: 20.8%). T2 (54.2%) and N0 (another 54.2%) were the most common categories regarding tumor size and nodal status, respectively.

Table 2 presents the intercorrelations of variables. Total maspin score ($IRS_{I.A.SCORE}$) was positively and closely associated with nuclear maspin ($IRS_{PATHOLOGIST\ SCORE}$) (Spearman's rho=+0.797, p<0.001) and cytoplasmic maspin ($IRS_{PATHOLOGIST\ SCORE}$) (Spearman's rho=+0.752, p<0.001); of note, both components (nuclear and cytoplasmic) were closely associated with each other (Spearman's rho=+0.673, p<0.001).

Table 3 presents the results of the multivariate ordinal logistic regression analysis regarding total maspin (lower panels). Both mutually interwoven components, namely nuclear (OR=1.63, 95%CI: 1.20-2.21, p=0.002) and cytoplasmic maspin (OR=1.95, 95%CI: 1.26-3.02, p=0.003) were independently associated with the total maspin.

Representative figures of immunostaining and CIA are provided in Figures 1, 2 & 3.

Discussion

The present study, through estimating maspin expression separately in the nucleus and cytoplasm, revealed a statistically significant positive correlation between nuclear and cytoplasmic staining of maspin, as estimated by the pathologists via LM. In addition, the two pathologist-evaluated counterparts (nuclear and cytoplasmic) were closely linked to the results of CIA system. Most probably, taking into account the close correlation between nuclear and cytoplasmic maspin, it seemed fairly impossible for the program to distinct and measure separately maspin staining in the two compartments and thus, maspin expression was evaluated as an aggregate, independently integrating both compartments.

Based on the differential expression of maspin in normal epithelial cells and breast carcinoma cell lines, a tumor-suppressive property for maspin has been proposed [10,31]. In vitro studies have revealed that the function of maspin as a tumor suppressor is a combination of increased cell adhesion and apoptosis and decreased mo-

Table 1. Description of the study sample

Continuous variables	*Mean±SD*
Age (years)	63.3±12.5
Total maspin ($IRS_{I.A.SCORE}$)	763±1025
Nuclear maspin ($IRS_{PATHOLOGIST\ SCORE}$)	3.31±2.73
Cytoplasmic maspin ($IRS_{PATHOLOGIST\ SCORE}$)	2.75±2.34
CD34 (No/mm^2)	220±201
Ki-67 (%)	20.5±21.3
Categorical and ordinal variables	*N (%)*
Molecular classification	
Luminal A	12 (33.3)
Luminal B	13 (36.1)
HER-2 overexpressing	6 (16.7)
Triple negative	5 (13.9)
Grade	
1	4 (8.3)
2	10 (20.8)
3	34 (70.8)
Stage	
I_A	9 (18.7)
II_A	14 (29.2)
II_B	10 (20.8)
III_A	3 (6.3)
III_B	7 (14.6)
IV	5 (10.4)
Tumor size	
T1	12 (25.0)
T2	26 (54.2)
T3	8 (16.7)
T4	2 (4.2)
Nodal status	
N0	26 (54.2)
N1	10 (20.8)
N2	5 (10.4)
N3	7 (14.6)
Metastasis	
M0	43 (89.6)
M1	5 (10.4)
p53 expression	
Positive	14 (29.2)
Negative	34 (70.8)
ER status	
Positive	28 (58.3)
Negative	20 (41.7)
PR status	
Positive	22 (45.8)
Negative	26 (54.2)
HER-2 status	
Positive	16 (33.3)
Negative	32 (66.7)

Table 2. The intercorrelations of CD34 (No/mm2), maspin scores (total; nuclear; cytoplasmic) and clinicopathological parameters in the study sample. Spearman's rank correlation coefficients (p-values in parentheses) are provided, unless denoted otherwise. Bold cells denote correlations with p<0.05

	CD34 (No/mm²)	*Total maspin ($IRS_{I.A.Score}$)*	*Nuclear maspin ($IRS_{Pathologist\ Score}$)*	*Cytoplasmic maspin ($IRS_{Pathologist\ Score}$)*
CD34 (No/mm²)				
Total maspin ($IRS_{I.A.Score}$)	+0.028 (p=0.852)			
Nuclear maspin ($IRS_{Pathologist\ Score}$)	+0.113 (p=0.444)	**+0.797 (p=<0.001)**		
Cytoplasmic maspin ($IRS_{Pathologist\ Score}$)	+0.075 (p=0.614)	**+0.752 (p=<0.001)**	**+0.673 (p=<0.001)**	
Age	-0.113 (p=0.443)	+0.123 (p=0.407)	+0.006 (p=0.966)	+0.071 (p=0.631)
Stage	-0.008 (p=0.959)	-0.185 (p=0.209)	-0.070 (p=0.639)	-0.167 (p=0.256)
T status	+0.085 (p=0.566)	-0.115 (p=0.437)	-0.017 (p=0.908)	-0.054 (p=0.718)
N status	+0.093 (p=0.531)	-0.131 (p=0.376)	-0.057 (p=0.700)	-0.114 (p=0.441)
M status§	-0.540 (p=0.589)	-0.861 (p=0.389)	-0.258 (p=0.796)	-0.693 (p=0.489)
Grade	-0.032 (p=0.827)	-0.011 (p=0.941)	+0.113 (p=0.443)	+0.028 (p=0.849)
Molecular classification†	2.411 (p=0.492)	3.570 (p=0.312)	3.521 (p=0.318)	0.950 (p=0.813)
Ki-67 (%)	-0.109 (p=0.528)	+0.144 (p=0.402)	+0.220 (p=0.197)	+0.127 (p=0.460)
ER positivity§	-0.241 (p=0.810)	+0.167 (p=0.867)	-0.726 (p=0.468)	+0.976 (p=0.329)
PR positivity §	-0.631 (p=0.528)	-1.552 (p=0.121)	**-1.891 (p=0.059)**	-0.287 (p=0.774)
HER-2 positivity §	+0.722 (p=0.470)	-0.853 (p=0.394)	-0.045 (p=0.964)	-0.415 (p=0.678)
p53 positivity §	-0.049 (p=0.961)	-0.281 (p=0.779)	-0.062 (p=0.950)	+0.100 (p=0.920)

§ Mann-Whitney-Wilcoxon (MWW) test for independent samples was performed, as these variables were binary; MWW z-values and p-values (in parentheses) are provided.

† Kruskal-Wallis (KW) test was performed, as this variable included more than two groups; KW chi-square values (3 degrees of freedom) and p-values (in parentheses) are provided.

tility, angiogenesis and pericellular proteolysis [5,12,14,32,33]. Indeed, a shotgun proteomic approach has indicated that restoring the expression of maspin in invasive carcinoma cells alters the expression of proteins regulating cell death, cytoskeletal architecture and protein turnover, resulting in increased rate of spontaneous apoptosis, more prominent actin cytoskeleton, reduced invasive capacity and altered proteasome function [34]. Maspin has been reported to induce apoptosis by reducing cell surface-associated prosurvival uPA – uPA receptor complex [35,36]. Maspin may function in the inhibition of cell invasion either through interactions with collagens [37] or regulation of integrins [14]. Sharma et al. [38] observed a significant association between loss of maspin expression and cytoplasmic accumulation of VEGF-A, underlining the role of maspin in angiogenesis.

Although original observations pointed to the association of reduced maspin with cancer progression, ensuing studies have revealed this correlation to be far more complex than originally concluded. Factors contributing to this complexity include, but are not limited to, genetic background, type of cancer, the organ where tumorigenesis originated, the expression of maspin (or lack of it) in the original corresponding normal tissue, subcellular distribution of maspin and use of cytotoxic drugs for cancer therapy. It is also imperative to consider that both methylation and demethylation processes could, at least in part, determine the "presence or absence" of maspin in the tumor [3]. Maspin has been located in many normal epithelial tissues, i.e. breast, prostate, placenta, small intestine, colon, uterus, kidney, thymus, testis [39,40], being paradoxically either down-regulated or up-regulated therein. Down-regulation of maspin expression correlated with progression and metastatic status in prostate, colon, gastric and oral cancers [39,41-43], while maspin expression was up-regulated in other malignancies such as pancreatic, lung, thyroid, ovarian and endometrial cancers [6-9].

Unfortunately, contradictory results have also been reported concerning the role of maspin in breast cancer. Some crucial points are discussed below:

i. *Grade:* A significant correlation of maspin with low grade cases has been suggested [44], while, elsewhere [16-19,21] high maspin expression was demonstrated in high-grade cases.

ii. *Lymph node positivity:* Hojo et al. [44] and Maass et al. [26] found a lower frequency of regional lymph node metastasis in the maspin-positive group. Conversely, Tsoli et al. [21] showed a positive association between high maspin expression and lymph node positivity.

iii. *Maspin expression changes during disease progression:* A significant stepwise decrease in maspin expression occurred in the sequence DCIS – invasive cancer – lymph node metastasis, in an early study [26]. Similarly, other studies showed that maspin mRNA is reduced in primary tumors and undetectable in metastases [45,46]. In contrast, Umekita et al. [16] demonstrated a stepwise increase in the frequency of maspin expression from DCIS to invasive breast ductal carcinoma.

iv. *p53:* Sharma et al. [38] recently showed that p53 immunopositivity seems to contribute to the loss of maspin expression, whereas positive p53 status has been found elsewhere [17] to correlate significantly with maspin expression.

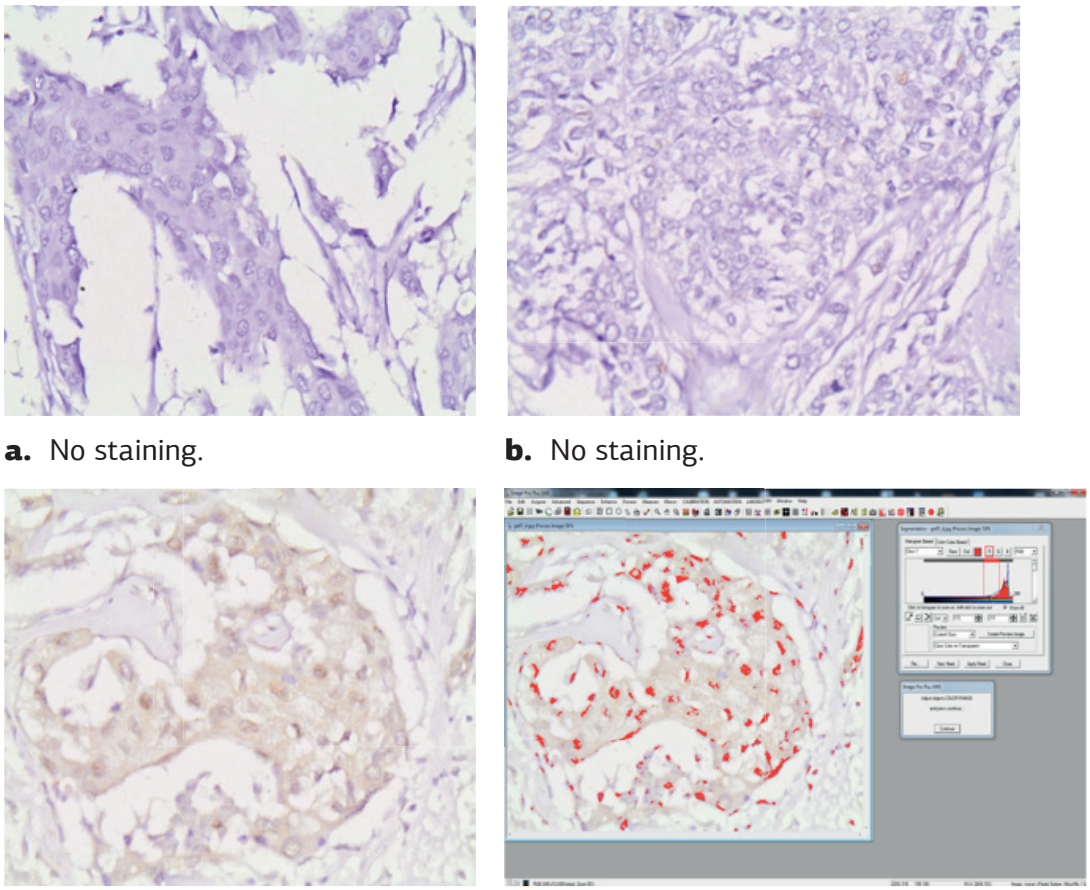
v. *Maspin as a prognostic indicator:* Maass et al. [26] portrayed maspin as a useful prognostic indicator, since its up-regulation predicted favorable prognosis. However, many other researchers have presented maspin as a poor prognostic factor. Specifically, Umekita et al. [16,17,47] suggested that the expression of maspin predicted an aggressive phenotype; similar suggestions came from Tsoli et al. [21] who considered the possibility that maspin overexpression is associated with a high risk for clinically undetectable disease spread and subsequent recurrence in patients with lymph node negative disease.

The other point that remains elusive and is probably strongly related to the contradictory results concerning the role of maspin in breast cancer pertains to its subcellular localization. Since maspin is related to the serpin family proteins, its

Table 3. Results of the multivariate ordinal logistic regression analysis for total maspin levels (IRS score)

Variables	*Category or Increment*	*OR (95% CI)*	*p-value*
Nuclear maspin ($IRS_{Pathologist\ Score}$)	1 unit increase	1.63 (1.20-2.21)	0.002
Cytoplasmic maspin ($IRS_{Pathologist\ Score}$)	1 unit increase	1.95 (1.26-3.02)	0.003

OR: odds ratio, CI: confidence interval



a. No staining.

b. No staining.

c. Faint staining.

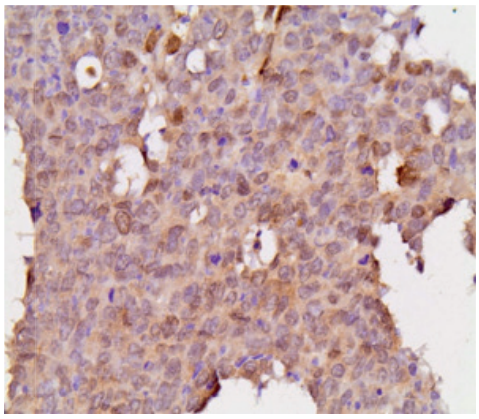
d. Faint staining – Computerized image analysis.

Figure 1. Invasive ductal carcinomas showing no immunostaining for maspin in both the nucleus and cytoplasm of cancer cells **(a & b).** Invasive ductal carcinomas showing faint immunostaining **(c)** and the respective snapshot from computerized image analysis **(d).**

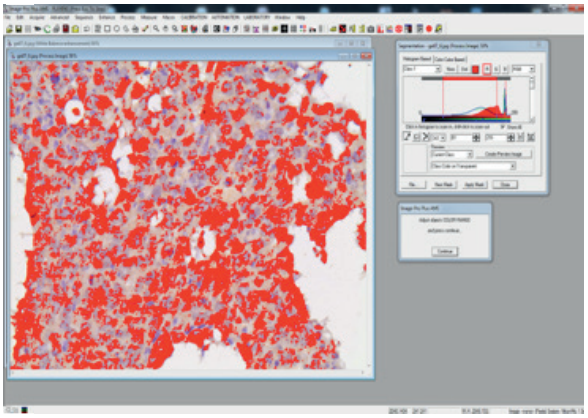
expression was always regarded to be limited to the cytoplasmic compartment of the cell. However, Pemberton et al. [48] first demonstrated that, by western blotting and immunohistochemistry, maspin can be detected in the nucleus of cancer cells. More recently, other investigators have documented the presence of nuclear maspin in breast, prostate and pancreatic tumors [6,26,49].

Studies that have shown a correlation of maspin with poor prognosis and poor prognostic clinicopathological parameters have estimated its staining mainly irrespective of its subcellular localization. Umekita et al. [16,17,47], Lee et al. [19] and Tsoli et al. [21] who have found significant correlations between maspin expression and larger tumor size and higher histological grade, have measured maspin's staining without separating between nuclear and cytoplasmic maspin.

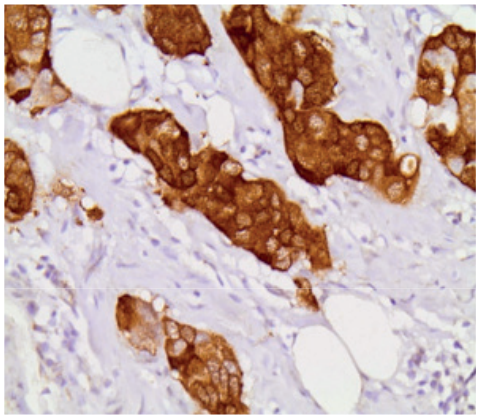
In contrast, researchers who estimated the correlation between breast cancer and especially the nuclear fraction of maspin, have obtained exactly the opposite results, as a rule. Mohsin et al., in an early large clinicopathological study [4], were the first investigators to estimate the impact of nuclear maspin on breast cancer separately of cytoplasmic maspin. In their study, maspin nuclear staining was significantly associated to good prognostic factors (ER, PR positivity), while cytoplasmic staining was associated to poor prognostic ones (ER, PR negativity). Thus, they suggested that the presence of maspin in two different compartments of the cell may have different biological and clinical implications. Joensuu et al. [50] found that especially cytoplasmic expression of maspin was significantly higher in the primary tumors of the early metastasizing breast cancers and also in their metastases compared to late metastasizing cancers. Moreover, the expression of p53 (poor prognostic marker) correlated significantly with cytoplasmic maspin. In contrast, tumors with late recurrence displayed significantly increased staining for nuclear maspin [50]. It is



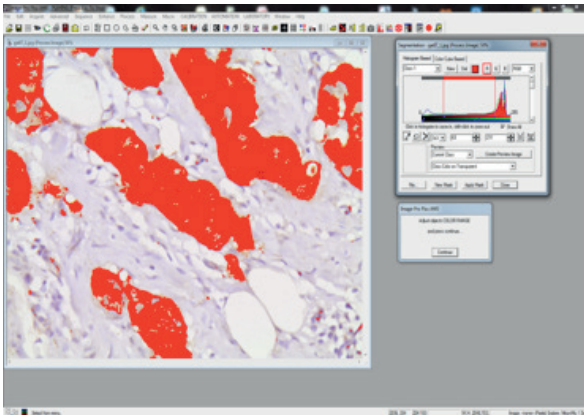
a. Moderate staining.



b. Moderate staining – Computerized image analysis.

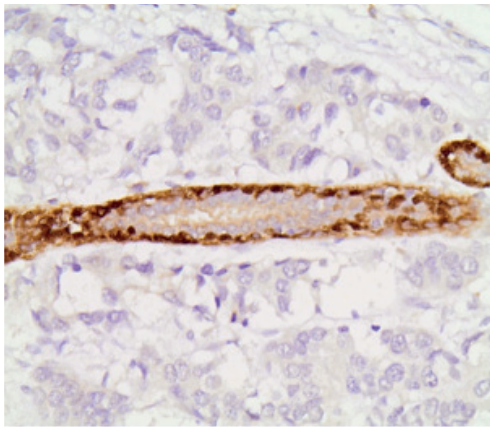


c. Strong staining.

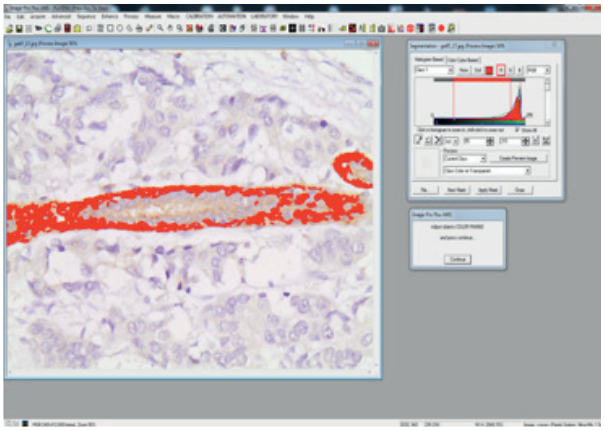


d. Strong staining – Computerized image analysis.

Figure 2. Invasive ductal carcinomas showing moderate **(a)** and strong **(c)** positive immunostaining for maspin in both the nucleus and cytoplasm of cancer cells and the respective snapshots from computerized image analysis **(b & d).**



a. Normal tissue



b. Normal tissue – Computerized image analysis.

Figure 3. Normal, non-neoplastic mammary duct, showing strong positive immunostaining for maspin in myoepithelial cells, but not in epithelial mammary cells **(a)** and the respective snapshot from computerized image analysis **(b)**. This normal duct exists inside an invasive neoplastic tissue area.

worth mentioning that nuclear maspin correlates with better clinicopathological parameters and survival also in other cancer types such as lung [51].

Consequently, it looks obvious that nuclear localization of maspin is required and necessary for its tumor and metastasis suppressor function *in vivo* [20,52]. Taking into consideration that nuclear staining assessment, generally speaking in pathology, is less time-consuming, less strenuous, demanding and ambiguous and more objective and unbiased, the separate estimation of the maspin nuclear fraction seems to be particularly valuable. Regarding pathophysiological mechanisms, maspin possibly exerts its role in the nucleus at the level of gene or chromatin regulation and thus indirectly affects the cell-matrix interaction or differentiation state [20,52,53].

Concerning the intercorrelation of maspin with clinicopathological parameters we found no statistically significant correlations, probably due to the small sample. The only factor that showed an inverse borderline correlation (p=0.059) with nuclear maspin was PR status; on the contrary, there was no correlation between PR positivity and cytoplasmic maspin, a result that contributes to the idea of the different roles by subcellular maspin fractions. Once again, data from the relevant literature is contradictory [4,17,19] and further investigation is needed for safer conclusions.

In conclusion, the cytoplasmic and nuclear fractions of maspin seem to be closely interwoven. Evidently, both mutually intertwined counterparts are reflected upon the total maspin levels measured by CIA. Future studies should ideally encompass all three approaches (nuclear, cytoplasmic, total) adopted herein and should report the intercorrelations regarding the expression of maspin in the subcellular compartments, so as to further validate the present results.

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