

# S100P calcium-binding protein expression is associated with high-risk proliferative lesions of the breast

ANA PAULA TORRES SCHOR<sup>1</sup>, FILOMENA MARINO CARVALHO<sup>1</sup>, CLÁUDIO KEMP<sup>2</sup>,  
ISMAEL D.C.G. SILVA<sup>3</sup> and JOSE RUSSO<sup>4</sup>

<sup>1</sup>Pathology Division of Clinical Hospital, <sup>2</sup>Pathology Department, São Paulo University, Av. Dr Eneas de Carvalho Aguiar 155, 10th floor, São Paulo, SP 05403-000; <sup>3</sup>Molecular Gynecology Laboratory, Federal University of São Paulo, Rua Pedro de Toledo 781, 4th floor, São Paulo, SP 04039-001, Brazil; <sup>4</sup>Breast Cancer Research Laboratory, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA

Received January 3, 2005; Accepted March 4, 2005

**Abstract.** Benign breast diseases represent the vast majority of diagnosis in breast pathology. However, the limited capability in identifying lesions at high risk of breast cancer evolution is an increasing problem in clinical practice. In the present study, we tested the hypothesis that the over-expression of S100P calcium-binding protein, previously identified in the very early stages of breast carcinogenesis, could be used as a marker to differentiate lesions at high risk of malignant evolution. In addition to S100P, the well-known proliferative marker, Ki-67, and estrogen receptor (ER) status were also assessed by immunohistochemistry in 155 samples from patients who submitted to stereotactic vacuum-assisted core biopsy due to breast microcalcifications. Results showed a positive association between ER and S100P overexpression, as well as a clear positive association between S100P overexpression and high-risk lesions. The strong association between S100P and ER expression highlights the hypothesis about the possible role played by S100P in the very early stages of breast carcinogenesis.

## Introduction

Due to the widespread implementation of minimally invasive procedures, a large amount of benign breast diseases have been diagnosed, particularly proliferative lesions (1). Nevertheless, the relationship between benign breast lesions and subsequent risk of breast carcinoma has worried researchers, pathologists and surgeons all over the world. Indeed, according to the Nashville group, the risk of breast cancer increases in

the presence of proliferation, cell atypia, calcification and previous family history of mammary cancer (2,3).

Considering that just a small proportion of proliferative lesions will progress to invasive neoplasia, and classification of these lesions based entirely on morphological criteria might be limited, we consider that the association of histology, immunohistochemistry and biological markers may elucidate a more informative diagnosis in breast biopsies (4).

Breast proliferative lesions are considered to be one of the first events towards malignant transformation (5,6). Many studies have been conducted with typical and atypical ductal hyperplasia, trying to identify the lesions at greater risk of progression concerning the expression of one of the most important prognostic markers of breast cancer, the estrogen receptor (ER). Indeed, ER expression seems to be a very good marker of breast lesions at high risk of breast cancer evolution, acting as a marker in the early stages of breast carcinogenesis and confirming anti-estrogen therapy as a possible prophylactic treatment (7-10).

When activated by estrogen or its toxic metabolites, the ER can mediate the cellular transcription of several genes, including the ones that might be involved in processes that can facilitate or promote malignant transformation (11-15). Silva *et al* have identified another very interesting candidate gene that became overexpressed in the early events of breast carcinogenesis, the S100P calcium-binding protein (16).

This protein was first identified by Emoto *et al* in the human placenta; the gene is located in chromosome 4p16 and has 95 residues with a molecular mass of 10 kDa (17). S100P is a homodimer with two functional EF-hands/ polypeptide chains, and the C-terminal is an ideal candidate for calcium-dependent interactions with cellular targets (18,19). Whereas the specific function of S100P is still unknown, there is a large body of evidence suggesting that this protein is important to cell proliferation in breast and other tissue (20-28).

To pursue and confirm these previous findings, we decided to analyze the expression of S100P alone and in combination with ER expression and the proliferative marker, Ki-67, in 155 breast lesions associated with microcalcifications and stratified, according to histological criteria, for cancer risk development.

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*Correspondence to:* Dr Jose Russo, Breast Cancer Research Laboratory, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA  
E-mail: j\_russo@fccc.edu

**Key words:** S100P, breast proliferative lesions, calcium-binding protein, estrogen receptor

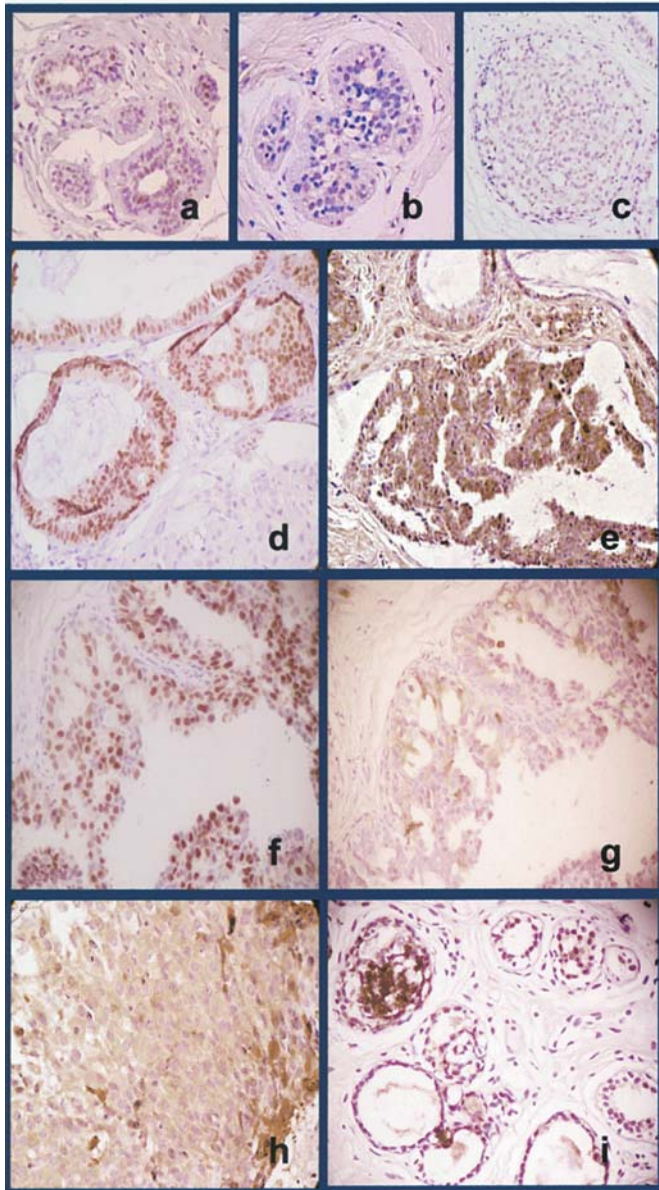


Figure 1. Immunohistochemical localization of S100P in a paraffin section of formalin-fixed tissue. (a and b) Normal breast tissue stained for ER and S100P, respectively. (c) Typical ductal hyperplasia stained for S100P. (d and e) Atypical ductal hyperplasia stained for ER and S100P, respectively. (f) S100P detection in a case of *in situ* carcinoma; and (g) S100P detection in breast microcalcifications.

## Materials and methods

**Specimens.** Human breast tissue was obtained at the Mastology Image Section, Gynecology and Obstetrics Specialized Laboratory in São Paulo, Brazil, according to a protocol approved by the Human Investigations Committee. The patients were 155 women that underwent an 11-gauge (Biopsy, Johnson & Johnson) vacuum-assisted directional biopsy of breast calcifications using stereotactic guidance (Lorad M-IV, Stereoloc-II; Lorad Corporation, CT, USA). We only included cases of calcifications without evidence of mammographic-associated density or mass, classified under the Breast Imaging Reporting and Data System (BI-RADS, 1998) as 3, 4 or 5. Cases with diagnosis of invasive carcinoma

Table I. Frequencies of risk groups and histological lesions.

Risk group <sup>a</sup>	N (%)	Histological lesion <sup>b</sup>	N (%)
No risk	35 (22.6)	DUH	39 (25.2)
Low	52 (33.5)	AH	33 (21.3)
Moderate	44 (28.4)	DCIS	24 (15.5)
High	24 (15.5)	LN	10 (6.5)
		Others	49 (31.5)
Total	155 (100.0)	Total	155 (100.0)

<sup>a</sup>Fitzgibbons *et al*, 1986. <sup>b</sup>DUH, ductal usual hyperplasia; AH, atypical hyperplasia; DCIS, ductal carcinoma *in situ*; LN, lobular neoplasia grades 1 and 2.

Table II. Predictive value of each marker considering the histological risk group.

Risk group	ER (%) (n=153) p<0.001	Ki-67 (%) (n=153) p<0.001	S100P (%) (n=138) p<0.001
No risk	11.4	57.1	0.0
Low	60.8	5.9	33.3
Moderate	65.1	84.1	86.1
High	95.8	39.1	100.0

were excluded. The mean age was 51.9 years (range, 32 to 81 years). The majority of cases presented calcification BI-RADS 4 (84.4%). Those with BI-RADS 3 and 5 corresponded to 13.6 and 2.0%, respectively. On average, 12 (range 7-25) fragments were obtained in each case. Specimens were radiographed for the documentation of microcalcifications. Fragments were immediately dropped in 10% neutral-buffered formalin and embedded in paraffin following standard histological procedures. Histological sections (5 microns each) were stained with hematoxylin and eosin for routine histology. Two independent pathologists reviewed all of the slides in order to confirm diagnosis. The histological lesions were grouped according to breast cancer risk consensus (29) as: no risk, low risk, moderate risk and high risk. For each patient, we related the highest risk lesion directly associated or not with the calcification. Slides containing the most significant and representative histological lesion for each patient were then selected for the immunohistochemistry reaction.

**Immunohistochemistry preparation.** Sections (5  $\mu$ m) were taken from paraffin blocks, deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and milk. The mouse antibodies against the following markers were used: ER (monoclonal, clone 1D5; dilution, 1:100; Dako), Ki-67 (monoclonal, clone MIB-1; dilution, 1:400; Dako) and S100P (monoclonal, clone 16; dilution, 1:150; Transduction

		ER ( $\chi^2=7.958$ , $p=0.019$ )			Ki-67 ( $\chi^2=13.208$ , $p=0.001$ )		
		Zero n (%)	<10 n (%)	>10 n (%)	Zero n (%)	<10 n (%)	>10 n (%)
S100P	Negative	3 (42.9)	18 (34.6)	12 (15.2)	3 (75.0)	13 (15.1)	17 (36.2)
	Positive	4 (57.1)	34 (65.4)	67 (84.8)	1 (25.0)	73 (84.9)	30 (63.8)

Laboratories). The immunoperoxidase staining was performed using a streptavidin biotin-peroxidase method with DAB (Dako). Slides were mounted after hematoxylin counterstaining. Negative and positive controls were carried out.

We analyzed the expression of S100P, considering the presence or not of cytoplasmatic and/or nuclear expression. The ER was considered positive only when the intensity of the reaction in the nuclei was at least moderate. The proportion of positive cells was scored subjectively by two independent observers using a cut-off value of 10%. For Ki-67, we also scored subjectively in a semi-quantitative manner considering 0%, <10%, and >10% since few cases presented higher scores.

The data were analyzed using Pearson's correlated coefficient contingent analysis (2) and multinomial logistic regression and discriminate analysis using SPSS software. All tests were considered significant at a level of 5% ( $\alpha$ ).

## Results

**Immunohistochemical studies.** Normal breast tissue stained for ER up to 10% and negative for S100P (Fig. 1a and b). In addition, the absence of S100P expression can also be observed in typical ductal hyperplasia (Fig. 1c). In contrast, atypical ductal hyperplasia and *in situ* carcinoma stained for ER and S100P showed very strong expression of both proteins (Fig. 1d-h). The majority of cases showed a low proliferative rate, <10% of the cells. All cases of ductal carcinoma *in situ* were positive for S100P and ER in >10% of the cells and this positivity decreased gradually from atypical to typical ductal hyperplasia.

**Correlation between immunohistochemistry and histopathology.** Patients (155) were evaluated and stratified in groups based on their histological lesion and risk of developing invasive breast cancer (Table I). The predictive values of S100P and ER were quite similar concerning positivity in the low, moderate and high risk groups (Table II). Age was strongly associated with ER expression mainly among patients under 50 years of age ( $p=0.002$ ).

We performed the same analysis concerning histological diagnosis and found an association of ER and S100P with hyperplastic lesions and ductal *in situ* carcinoma, but not with lobular neoplasia ( $p<0.001$ ). The Chi-square test showed a strong association between S100P and ER ( $p=0.019$ ), and between S100P and Ki-67 ( $p=0.001$ ) (Table III). We could not find an association between ER and Ki-67 expression ( $p=0.596$ ).

## Discussion

Silva *et al* previously demonstrated that S100P protein was absent in normal breast tissue and detected in typical and atypical hyperplasia, and *in situ* and invasive ductal carcinoma, indicating that this protein exhibits a strong link with tumor progression (16).

In the present study, we found a strong positive correlation among S100P and high risk breast lesions. The significant positive correlation between ER and S100P strongly suggests that S100P might represent a good marker for differentiating between high and low risk of cancer evolution. This is, in a certain way, confirmed by the findings that breast lesions negative for S100P expression are at extremely low risk of cancer progression.

Arumugan *et al* demonstrated that S100P stimulates cell proliferation and survival via the receptor for activated glycation end products (RAGE) (30). In addition, they observed that expression of S100P led to the release or secretion of this molecule into the culture media and conferred significant proliferation and survival benefits to NIH3T3-transfected cells. These data confirmed the previous observation of Silva *et al* who reported the accumulation of S100P in the apical supra-nuclear portion of breast epithelium, secreted to the extracellular compartment (16).

In the present study, we further confirm these results by detecting S100P around the microcalcifications of breast tissue. It seems that S100P would be able to transport the calcium out of the cell with two consequences: reducing the intracytoplasmic calcium concentration and leading to calcium precipitation in the extracellular environment. This was also described by Russo and Russo who showed a correlation between the expression of S100P during the process of cell immortalization and the ability of these cells to pump out  $Ca^{++}$  more efficiently, and implicated by the formation of microcalcifications (31). Since increments in intracellular calcium concentration are responsible for mechanisms that can lead to senescence and programmed cell death in breast epithelial cells (32), apoptosis would be blocked by S100P (33). We also found an important positive correlation between the proliferation marker, Ki-67, and S100P expression in our clinical samples, suggesting that S100P can stimulate proliferation.

As expected, a strong positive correlation was also detected between ER expression and high risk lesions for breast cancer. However, when a specific lesion is analyzed from the point of view of both ER and S100P expression, it seems that the risk of breast cancer is negligible in the absence of S100P

expression. Therefore, it is tempting to speculate an eventual participation of S100P in ER action. Indeed, it is already known that S100A10 (p11) is the regulatory subunit of annexin II, the major cytoplasmic avian sarcoma virus (src) transforming protein kinases substrate. Since the src1 family is considered to be an ER co-regulator, we can speculate that the eventual action of S100P indicates its role as another co-regulator of ER (16).

In conclusion, despite the few S100P studies addressing its mechanism of action, we were able to demonstrate a strong association between S100P overexpression and breast lesions at high risk of cancer progression in the present study.

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