Original paper

Survivin in breast lesions: immunohistochemical analysis of 196 cases

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We examined the survivin expression pattern by immunohistochemistry in 43 fibroadenomas and 153 ductal carcinomas of the breast. The subcellular localization of survivin and the intensity of immunoreaction were assessed. We analyzed the differences of survivin expression between fibroadenomas and carcinomas. We also correlated the survivin expression pattern in carcinomas with other clinicomorphological parameters such as the age of patients, the grade and size of primary tumor as well as the lymph node metastasis. Overall, survivin was detected in 107/153 carcinomas (69.9%) and in 26/43 fibroadenomas (60.5%). Statistical analysis confirmed significant correlations between the assessed parameters in fibroadenomas and carcinomas. Grade of carcinomas was significantly related to survivin expression in both subcellular localization and the intensity of immunoreaction. Tumor grade 3 was associated with nuclear positivity and combined nuclear and cytoplasmic localization. Carcinomas larger than 20 mm showed nuclear and combined localization in 81% of cases and higher intensity of survivin immunoreaction was also notably related to larger carcinomas. Statistically significant differences were also observed between subcellular survivin localization and intensity of immunoreaction. Our result suggest that nuclear accumulation of survivin is associated with proliferative fenotype and survivin was shown to be a worse prognostic marker in breast ductal carcinoma.

Key words: breast cancer, survivin, immunohistochemistry, biomarker.

Introduction

Breast carcinoma is still one of the most common malignant tumors in women. It is also one of the leading causes of cancer death in female patients [1]. The balance between cell proliferation and apoptosis controls normal breast development as well as maintains cellular homeostasis of tissues. Furthermore, there is a strong evidence that tumor growth is not just the result of uncontrolled proliferation, but also of reduced apoptosis [2]. Tumor cells can develop resistance to apoptosis by expression of anti-apoptotic proteins (inhibitors of apoptosis proteins – IAP). IAP proteins were first discovered in baculoviruses, where they were shown to be involved in suppressing host cell death response to a viral infection [3, 4]. Human genome encodes eight IAP family members [5]. Survivin, however, has a number of distinct features not

shared with other IAPs. It is the shortest polypeptide consisting of 142 amino acid residues. The expression of survivin is cell cycle-regulated and occurs in the G2/M phase. It is undetectable in most terminally differentiated normal cells, but it is abundant in embryonic and fetal tissues as well as in a majority of human malignancies including breast carcinomas [6]. Survivin is a multifunctional protein that controls cell division and inhibition of apoptosis as well as enhances angiogenesis [7]. It is also one of the chromosome passenger proteins and plays an important role in mitosis and spindle check points [8]. Survivin shuttles between nucleus and the cytoplasm and hence, can be associated with different subcellular compartments [1, 9]. Due to large quantitative difference in the level of survivin expression in malignant tumors on the one hand, and in corresponding normal tissue on the other hand, survivin appears to represent a promising prognostic biomarker [10]. The prognostic value of survivin expression is a matter of discussion. For breast cancer patients, its prognostic role has been reported either nonexistent [9, 11, 12] or associated with an improved [13] or adverse outcome [7, 14, 15].

The aim of this work was to investigate survivin expression in 153 cases of breast ductal carcinoma by using immunohistochemistry and to study the association between its subcellular compartmentalization and clinicomorphological parameters. Furthermore, we studied the relationship of survivin expression pattern between breast carcinomas and 43 cases of fibroadenoma.

Material and methods

Patients

Archival blocks of formalin-fixed paraffin-embedded tissue samples from 43 fibroadenomas and 153 breast carcinomas were enrolled into this study. The study was approved by the Ethics Committee of Jessenius Faculty of Medicine, Comenius University in Martin. All methods were carried out in accordance with the approved guidelines. Pathology reports from all patients were reviewed and their age, grade, size of tumors and lymph node status recorded. Tissue samples were taken from patients in the age interval of 32-84 years with total average age 58.41 ±13.25 years (median 59 years).

Immunohistochemical staining

Each representative paraffin block was cut into 4mm-thick sections subjected to immunohistochemical staining. Silanized slides (DAKO, Denmark) baked for 2 hours in an oven at 56°C were used for a better adherence of tissue sections to glass slides. The slides were then treated in a PT Link System (Dako). The endogenous peroxidase activity was quenched with 3% hydrogen peroxide for ten minutes. Immunohistochemical staining was performed using monoclonal mouse anti-survivin antibody (DAKO, Denmark, Clone12C4, dilution 1 : 50). After a one hour incubation with a primary antibody and Linker/Mouse treatment for 20 minutes, survivin was visualized by means of the EnVision™ Flex / HRP System using 3, 3´-diaminobenzidine chromogen as substrate, according to the manufacturer´s instructions. All sections were counterstained with Mayer´s hematoxylin (Dako). Negative controls were obtained by omitting the primary antibody.

In all cases, both the subcellular localization of survivin (nucleus – N, cytoplasm – C, or both – NC) and the intensity of immunoreaction (weak +, moderate ++, and strong +++) were assessed.

To achieve good reproducibility, the above-mentioned parameters were evaluated semiquantitatively by two experienced observers separately (MA, SD). The well-known three scale scoring system was used to assess the intensity of immunoreaction for survivin [16, 17]. The age of patients, grade, size of the tumor and lymph node metastasis were designated to represent clinicomorphological parameters.

Statistical analysis

Statistical analysis was performed with Microsoft Excel with XLSTAT software package. χ^2 test was used to demonstrate the differences of survivin expression in fibroadenoma and carcinoma cases, and to evaluate the correlation between survivin expression pattern and the clinicomorphological parameters of carcinomas. Moreover, Cochran-Armitage trend test Monte Carlo method was used to evaluate whether the intensity of survivin immunoreaction correlates with the survivin subcellular localization. P-value less than 0.05 was considered to indicate a statistical significance.

Results

Evaluation of immunohistochemical staining

In the group of 43 cases of breast fibroadenoma (Fig. 1), survivin was detected in 26/43 cases (60.5%). Mostly cytoplasmic localization (55.8%) (Fig. 3) of survivin was detected among these positive cases. Combined cytoplasmic and nuclear localization was found in only two cases (4.7%). All positive cases demonstrated a weak or moderate intensity of immunoreaction.

In our panel of 153 carcinoma cases (Fig. 2), survivin was expressed in 107/153 cases (69.9%). The positive cases showed variable subcellular localization. Solely nuclear positivity (Fig. 4) was observed in 35/153 cases (22.9%), while cytoplasmic staining

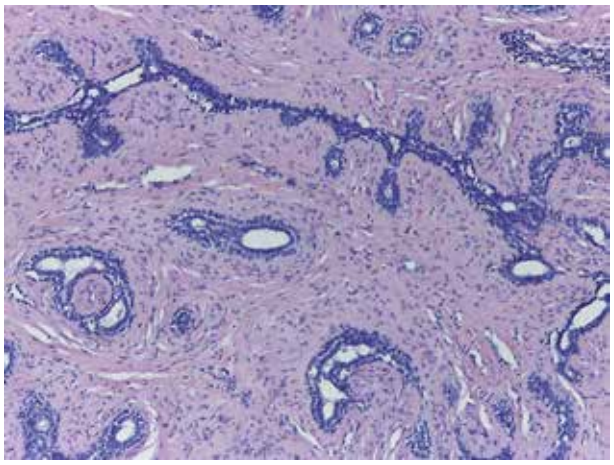

Fig. 1. Breast fibroadenoma, HE (hematoxylin-eosin) (scale bar = 200 μm)

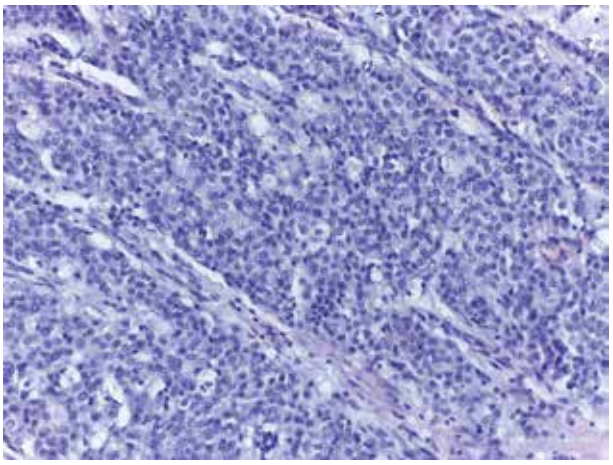

Fig. 2. Breast ductal carcinoma, HE (scale bar = 100 μm)

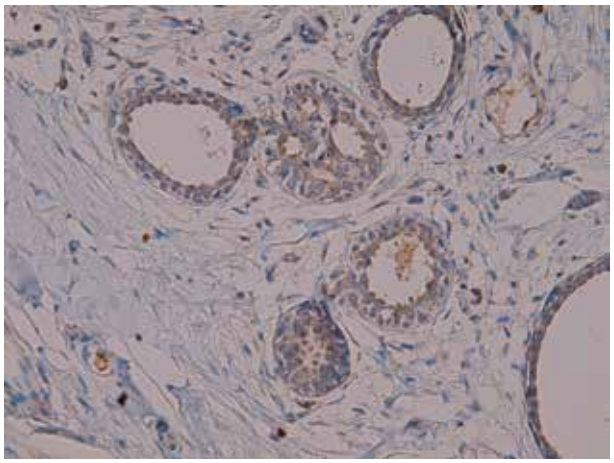

Fig. 3. Weak cytoplasmic survivin positivity in fibroadenoma cells (scale bar = 50 μm)

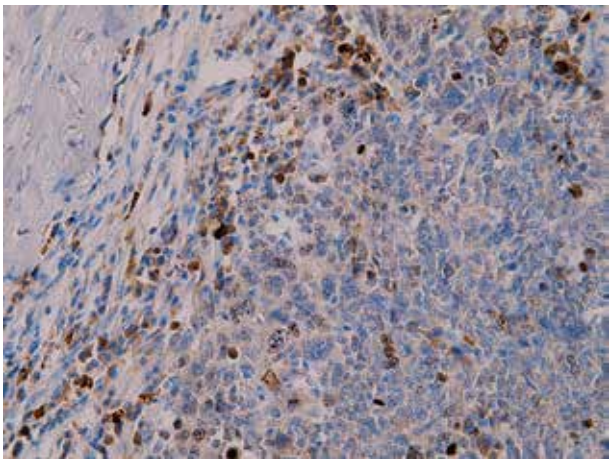

Fig. 4. Nuclear survivin positivity in carcinoma cells (scale bar = 50 μm)

was found in 13/153 cases (8.4%). Combined nuclear and cytoplasmic survivin expression (Fig. 5) was detected in 59/153 cases (38.6%). The intensity of immunoreactivity varied from weak to strong. Weak intensity of immunostaining was detected in 51/153 cases (33.3%), while moderate to strong intensity was found in 56/153 (36.6%).

The results of all expression profiles are summarized in Table I.

Statistical analysis results

Statistically, the chi-square test revealed significant differences in the subcellular localization of survivin expression in breast fibroadenomas and breast carcinomas ($p < 0.001$) (Table I).

Statistical analysis also confirmed significant differences in the intensity of survivin immunoreactivity between breast fibroadenomas and breast carcinomas ($p < 0.001$) (Table I).

Age, grade, size and lymph node metastasis of carcinomas were all analyzed in relation to survivin expression – subcellular localization and immunoreactivity intensity (Table II). Cases with absent survivin in expression were included in the statistical analysis as well.

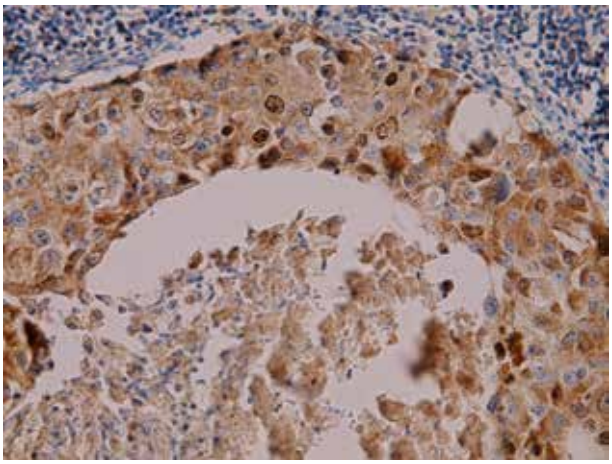

Fig. 5. Combined nuclear and cytoplasmic survivin positivity in carcinoma cells (scale bar = 50 μm)

Table I. Results of the immunohistochemical staining of survivin in fibroadenomas and carcinomas

Survivin expression	A	Subcellular localization			Intensity of staining	
		C	N	NC	+	++ +++
Fibroadenoma (n = 43)	17	24	0	2	25	1
Carcinoma (n = 153)	46	13	35	59	51	56
P value			0.001		0.001	

A – absent; C – cytoplasmic; N – nuclear; NC – combined nuclear and cytoplasmic localization

Table II. Relationship between survivin expression and clinicomorphological parameters in carcinomas

Survivin expression	A	Subcellular localization			Intensity of staining	
		C	N	NC	+	++ +++
Age (n = 153)						
< 40	5	0	6	5	9	2
41-50	8	2	9	15	12	14
51-60	9	3	8	20	13	18
61-70	9	4	7	9	9	11
> 70	15	4	5	10	8	11
p-value			0.413		0.208	
A cases included						
Grade (n = 153)						
1	21	6	4	13	11	12
2	15	4	5	16	14	11
3	10	3	26	30	26	33
p-value			0.0001		0.002	
A cases included						
Size (n = 130)						
< 11 mm	17	5	3	9	9	8
11-20 mm	13	3	7	14	11	13
> 20 mm	9	2	20	28	19	31
p-value			0.002		0.007	
A cases included						
LN metastases (n = 109)						
positive	6	2	12	20	11	23
negative	12	9	16	32	16	41
p-value			0.524		0.863	
A cases included						

A – absent; C – cytoplasmic; N – nuclear; NC – combined nuclear and cytoplasmic localization

The statistical analysis did not reveal any kind of significant relation between the age and subcellular localization of survivin and the intensity of immunoreactivity. However, the grade of carcinomas was substantially related to survivin expression in both subcellular localization and the intensity of immunoreactivity. Carcinomas with grade 1 were survivin negative in 48% of cases, grade 2 in 33% and grade 3 only in 14% of cases. On the contrary, grade 3 was associated mainly with nuclear positivity (in 38% of cases) and combined nuclear and cytoplasmic localization of survivin (in 43% of cases). Similarly, the intensity of survivin immunoreactivity was associated with the grade of carcinoma (Table II).

A statistically significant relationship was also confirmed between survivin expression and the size of carcinoma (Table II). Specimens smaller than 11 mm demonstrated absent survivin expression in 50% of cases, while specimens larger than 20 mm only in 15% of cases. Carcinomas larger than 20 mm showed nuclear and combined (both nuclear and cytoplasmic) expression of survivin in 81% of cases, while a solely cytoplasmic expression of survivin was detected only in 3% of cases and absent survivin expression in 15% of cases. Furthermore, a higher intensity of immunoreactivity of survivin was statistically significantly related to larger carcinomas.

The positivity of lymph node metastasis did not show any relevant correlation with the immunohistochemical characteristics of survivin expression (Table II).

Survivin positive carcinoma samples were also evaluated with regard to the relation between subcellular localization and intensity of immunoreactivity of survivin expression. Samples with solely cytoplasmic localization of survivin demonstrated a weak intensity of immunoreactivity in 77% of cases and mild/strong intensity only in 23% of cases. Carcinomas with nuclear and combined (both nuclear and cytoplasmic) localization of survivin showed a weak intensity of immunoreactivity in 44% of cases and mild/strong intensity in 56% of cases.

χ^2 test and Cochran-Armitage trend test Monte Carlo method revealed significant relation between the subcelluar localization and intensity (Table III).

Table III. Relationship between the subcellular localization and intensity of immunoreactivity of survivin in carcinomas

Surviving expression	Intensity of immunoreactivity	
Subcellular localization	+	++ / +++
C	10	3
N, NC	41	53
χ^2-test	p = 0.024	
Cochran-Armitage trend test	p = 0.013	
Monte Carlo method		

C – cytoplasmic; N – nuclear; NC – combined nuclear and cytoplasmic

Discussion

Our present work summarizes the expression and discusses the functions of the most important IAP family member, survivin, in breast lesions. We assessed the survivin expression in 43 fibroadenomas and 153 carcinomas. While, the evaluation of survivin expression in various types of cancers has been reported in numerous studies [3, 18], very little has been published about the relationship of survivin with benign and malignant tumors of the breast.

In our panel of 43 fibroadenomas, we detected survivin expression in 26 cases (60.5%), whereas in 17 cases (39.5%) survivin expression was absent. Our results are consistent with our previous investigation [19]. A solely cytoplasmic localization was present in 24 cases (55.8%), while a combined nuclear and cytoplasmic immunoreaction was detected in two cases (4.7%). Only a small number of studies was focused on survivin expression in benign breast tumors. Survivin expression was reported by Ranade *et al*. [20] in 17/32 fibroadenoma cases (53%). In their study, immunopositivity was observed mainly in cytoplasm. In contrast to our results, the authors found a solely nuclear expression in only one case of fibroadenoma. On the other hand, our results are almost consistent with the observation of Ryan *et al.* [21], who detected 67.7% survivin positivity in fibroadenomas (21/31 cases). Survivin expression in fibroadenomas is likely to result from the proliferation and/or dysplastic transformation of luminal epithelial cells [20]. It is a well-known fact that survivin promotes cell proliferation and angiogenesis, and inhibits apoptosis. In the process of cell proliferation, survivin contributes to make the sister chromatid segregation and the stabilization of mitotic spindle components during late mitosis more accurate [22]. Moreover, survivin associates with the microtubules of mitotic spindle. A disruption of this interaction causes loss of its function and activation of caspases 3 and 7 [23].

In our uniform group of 153 breast ductal carcinomas, we revealed three patterns of immunohistochemical positivity (only nuclear, only cytoplasmic, and combined nuclear and cytoplasmic) in malignant cells. Survivin was expressed in 107 cases (69.9%). Nuclear and combined nuclear and cytoplasmic localization of survivin was found in 94 cases (61.5%), while cytoplasmic localization was detected in 13 cases (8.4%). Nassar *et al.* [24] described nuclear localization in 84% of breast carcinoma cases. In line with our findings, other research groups also demon-

strated three patterns of survivin staining in breast carcinoma cells. Kennedy *et al.* [13] detected nuclear reaction only in 31% of carcinomas, cytoplasmic positivity in 13%, and combined nuclear and cytoplasmic staining in 16% of these cases. Al-Joudi *et al.* [18] found nuclear survivin positivity in 16.5% of carcinomas, cytoplasmic positivity in 24.1% of cases, and 27.5% of the study cases showed both nuclear and cytoplasmic localization simultaneously.

The importance and prognostic role of different subcellular survivin expression in breast cancer remains controversial. Individual parts of research focusing on the evaluation of survivin expression in tumors lead to conflicting results. When analyzed retrospectively, cancer patients with survivin overexpression in tumor cells exhibited shortened survival, association with unfavorable markers of disease progression, accelerated rates of recurrence and increased resistance to therapy [25, 26]. The relationship between such an aggressive behavior and survivin has been described in a wide spectrum of malignancies, e.g. in colorectal and gastric carcinoma, in neuroblastoma as well as in prostatic carcinoma [2, 27, 28]. However, the association of survivin with prognosis in breast cancer patients has always been ambiguous [14]. Previous studies reported survivin expression to be either prognostically irrelevant [12], associated with poor prognosis [11], or associated with good prognosis in breast cancer patients [13]. Taking into consideration the key position of survivin in the inhibition of apoptosis, in the promotion of cell proliferation and in the induction of angiogenesis, there is an important argument that the survivin overexpression may be an indicator of a worse prognosis [29, 30]. Surprisingly, many recent studies acknowledge the subcellular localization of survivin in respect to the prognosis of breast carcinoma. For example, Brennan *et al.* [31] and Oh *et al*. [32] concluded that different prognostic information is associated with nuclear and cytoplasmic survivin localization. While nuclear expression correlated with an unfavorable overall survival, cytoplasmic survivin expression was associated with an improved overall survival. Our previous studies [33, 34] also demonstrated that nuclear and combined nuclear and cytoplasmic survivin reaction is associated with worse prognostic parameters. However, this is not a case of solely cytoplasmic positivity. Based on these result, it seems that different survivin localization in tumor cells is associated with distinct functions. Survivin in cytoplasm may be related to the apoptotic process and may be involved in the inhibition of cell death [6, 29, 35] and promoting carcinogenesis [36]. Survivin in nucleus may participate in the regulation of cell proliferation and may lead to a proliferative aggressive phenotype [6, 31, 37]. The importance of nuclear – cytoplasmic shuttling of survivin is still under discussion. Studies of Knauer *et al*. [38] and Knauer *et al*. [39] showed that survivin movement is controlled by an active nuclear export signal, which is very necessary for its anti-apoptotic function. The inhibition of this nuclear export signal may cause the cancer cells to become increasingly susceptible to apoptosis induced by chemotherapy or radiotherapy [39].

According to our results, paying attention to the intensity of immunoreaction seems to be rather important. In the carcinoma group, the intensity of immunoreactivity varies from weak to strong. Moderate to strong positivity of immunoreaction dominated among positive cases (36.6%). On the other hand, predominantly weak intensity was detected in fibroadenomas. Therefore, we suppose that stronger intensity of immunoreaction can be associated with a higher accumulation of survivin in cancer cells. Suga *et al*. [40] demonstrated that the transcription levels of survivin were significantly higher in the tumor tissue samples, and, contrarily, significantly lower in the normal tissue samples.

An ideal diagnostic biomarker should be absent in normal tissue or benign tumors. On the contrary, it should be expressed in malignant tumor cells, including even their early or small lesions [41]. Statistically, we confirmed significant differences ($p < 0.05$) between the assessed parameters of immunohistochemical staining (intensity of staining and subcellular localization of survivin) between fibroadenomas and carcinomas. In our previous observation [19], we came to very similar results. Our results, combined with the literature review, demonstrate that survivin could certainly extend the panel of plausible biomarkers for breast carcinoma [42, 43].

In addition, we studied the relationship between survivin expression pattern and clinicomorphological parameters such as the age of patients, grade and size of tumor as well as lymph node metastases. The above mentioned parameters belong to a group of validated patient metrics and tumor-associated characteristics.

Age is a well known risk factor for breast carcinoma [44]. Rates of breast carcinoma are rather low in women under 40, when they begin to increase and become highest in women around the age of 70. Nearly half of all cases are diagnosed in women aged 60 and higher. In our cohort of patients, 62/153 cases are over the age of 60 (40.5%). However, statistically, there is no significant relation between age and subcellular survivin compartmentalization and the intensity of immunoreaction.

Histological grading is a widely used system, which helps to stratify breast cancer patients into favorable and unfavorable outcome groups. Numerous studies validated and confirmed the prognostic importance of the grading system [45, 46]. Many research groups have demonstrated that grading provides valuable clinical information in breast carcino-

ma. The assessment of pathological grading is a significant determinant for breast cancer prognostic and is also associated with tumor biology [45]. Schwartz *et al.* [47] studied 161708 cases of breast cancer and concluded that histological grading remains a well-accepted prognostic marker despite changes in tumor size and involvement of lymph nodes.

Basically, malignant tumors are usually graded as grade 1, grade 2 and grade 3 (well, moderately and poorly differentiated). As a general rule, well-differentiated tumors (G1) more closely resemble the parent tissue and are less aggressive than their poorly differentiated counterparts (G3). Taking into account the subcellular localization of survivin, we demonstrated a positive correlation of nuclear and combined nuclear and cytoplasmic locations with more aggressive G3 tumors. Interestingly, above mentioned subcellular compartmentalizations were also significantly associated with a higher intensity of immunohistochemical reaction.

The size of primary tumor is very valuable traditional prognostic parameter [48]. A couple of well-known facts have been established over the years with regard to size, e.g. the larger size of tumor, the worse outcome; the larger diameter of the tumor, the more axillary lymph nodes are affected by metastases [49]. The analysis of 1038 patients by Largillier *et al.* [50] indicated that tumor size ($>$ 20 mm vs. $\leq$ 20 mm) is a significant and independent parameter associated with overall survival. The size of tumor was also determined to be a factor related to an aggressive metastatic breast cancer [51]. In our recent study, we have found a nuclear and combined survivin expression in 81% of carcinomas larger than 20 mm. Similarly, a higher intensity of immunohistochemical reaction significantly correlated with a larger carcinoma.

The status of lymph nodes represents a powerful and reliable prognostic feature. Numerous studies have proven a strong correlation between the involvement of the lymph node and the size of tumor. A very well known study of Carter *et al*. [52] used the data of 24740 breast cancer cases to analyze the survival of patients. Lymph node status and diameter of tumor were both indicated as independent prognostic factors. Moreover, these authors also demonstrated that while the lymph node involvement increased, the survival decreased, regardless of the size of tumor; and, on the contrary, as size of tumor increased, the survival status decreased, regardless of the lymph node involvement. However, the relation between the size of tumor and the lymph nodes in breast cancer was beyond the scope of our study. Instead, we were interested in the relation between the lymph node status and the subcellular localization of survivin and, as a result, we did not confirm any significant correlation.

Both the subcellular survivin localization and the intensity of immunohistochemical reaction represent major determinants for correlations with other clinicomorphological variables. Using the Cochran-Armitage trend test Monte Carlo method and χ^2-test, we also tried to elucidate the relationship between them. Interestingly enough, we proved this correlation to be statistically significant. Moderate and strong expression was related to N and NC subcellular localization. Overall, our study suggests that the amount of survivin expressed by cells may be an indicator of tumor progression in breast lesions. In scientific literature, the intensity of survivin expression is very rarely studied [53, 54].

To summarize our observations, we have confirmed an increasing survivin expression starting from benign lesions to its overexpression in a majority of primary ductal carcinomas. Furthermore, nuclear and combined nuclear and cytoplasmic pattern as well as higher intensity of immunoreactions both dominated in carcinoma cases. The subcellular compartmentalization of survivin and the intensity of reaction between benign and malignant tumors revealed significant differences ($p < 0.001$). As already mentioned above, our results suggest that survivin may be considered as a potential biomarker for ductal breast carcinoma. In addition, our analysis revealed that nuclear and combined nuclear and cytoplasmic survivin localization as well as moderate and strong intensity of immunoreaction were associated with grade 3 tumors, and tumor size more than 20 mm in diameter. With regard to our previous results [19, 55, 56] and recent data, we can conclude that different subcellular compartmentalizations of the multifunctional protein survivin possess distinct functions, and that the accumulation of nuclear survivin is related to a proliferative fenotype. Therefore, we consider survivin to be a worse prognostic parameter in breast carcinoma.

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The authors declare no conflict of interest.

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