Correlations of survivin expression with clinicomorphological parameters and hormonal receptor status in breast ductal carcinoma

M. ADAMKOV[1]*, K. KAJO[2], D. VYBOHOVA[3], J. KRAJCOVIC[4], F. STULLER[4], J. RAJCANI[5]

[1]Department of Histology and Embryology, Jessenius Faculty of Medicine Martin, Comenius University, Malá Hora 4, 03601 Martin, Slovakia; [2]Department of Pathology, BB Biocyt Diagnostic Center, L. Svobodu 1, 974 01 Banská Bystrica, Slovakia; [3]Department of Anatomy, Jessenius Faculty of Medicine Martin, Comenius University, Martin, Slovakia; [4]Department of Forensic Medicine and Medical Expertises, Jessenius Faculty of Medicine Martin, Comenius University, Martin, Slovakia; [5]Laboratory of Pathological anatomy, Alpha medical, a.s., Hodžova 1, 03601 Martin, Slovakia

*Correspondence: adamkov@jfmed.uniba.sk

The antiapoptotic protein survivin is widely expressed in most human cancers, including carcinomas of the breast. It is rarely detected in corresponding normal adult tissues. Therefore, survivin comes into the limelight as a promising diagnostic biomarker and prognostic parameter. Immunohistochemically, we examined the expression of this protein in 126 cases of ductal breast carcinoma to determine the association with clinicomorphological parameters such as age of patients, grade, stage and size of the primary tumor, lymph node metastasis, vascular invasion as well as estrogen and progesterone status. In each section, the subcellular location of survivin antigen, the intensity of staining and the percentage of labeled cells were assessed. Overall, survivin was expressed in 111 cases (88.1%). The statistical analysis revealed a significant correlation between the nuclear location of survivin and tumor grade 3. Furthermore, a significant relation was also found between vascular invasion and nuclear and combined nuclear and cytoplasmic survivin expression, together with a higher intensity of immunoreaction. However, no significant correlations were shown with other clinicomorphological parameters, such as stage and size of the tumor, lymph node metastasis, estrogen and progesterone receptors and age. Our findings revealed that survivin was frequently overexpressed in carcinoma cells, where it was present in different subcellular compartments. The nuclear positivity of survivin or combined nuclear and cytoplasmic expression was shown to be a poor prognostic parameter in ductal breast carcinoma.

Key words: breast, carcinoma, survivin, clinicomorphological parameters

Survivin is a unique member of the inhibitor of apoptosis protein (IAP) family. So far eight human IAP family members were identified: C-IAP1, C-IAP2, NAIP, ILP-2, XIAP, apollon, ML-IAP/livin and survivin [1,2]. Multifunctional survivin possesses a number of distinct features not shared with other IAP members: it regulates cell division, inhibits apoptosis (programmed cell death) and enhances angiogenesis. Except for these features, survivin is not detectable in the majority of normal terminally differentiated adult tissues. Nevertheless, it is frequently expressed in embryonic and fetal organs as well as in developed human malignancies [3,4,5]. Survivin can be detected in different subcellular compartments. Therefore, it is currently undergoing intense investigation as a potential tumor marker and prognostic factor [4,6,7,8]. Conflicting data have been published about the prognostic value of this protein in question in different types of malignant tumors, including breast cancers [9,10,11,12,13]. Review of literature shows that the location of survivin expression is still a matter of discussion [13]. As described previously, there are three patterns of survivin immunohistochemical positivity in malignant cells: nuclear, cytoplasmic and combined (nucleus and cytoplasm) [14,15,16,17]. However, the reason for subcellular compartmentalization of survivin in neoplastic tissues is not yet fully understood. There is a growing evidence that several oncogenic pathways are potentially involved in the up-regulation of survivin protein in malignant lesions [18]. Certain novel studies strictly differentiate the location of survivin in malignant cells [19,20]. In spite of some disagreement, several research groups found that nuclear expression of survivin in breast cancer correlates with poor prognosis [19,21,22]. Many

other morphological parameters, such as histological type, grading, tumor size, mitotic rate, vascular invasion and lymph node metastasis, were regarded predictive for the prognosis of malignant breast tumor [23,24,25,26,27,28,29,30]. Furthermore, the prognosis may be influenced by a number of other significant variables, including hormonal receptor status, race, age and family history [23,24,25,26,27].

The purpose of this study is to evaluate survivin expression levels in ductal breast carcinoma tissue by using immunohistochemistry and to determine the association between survivin cellular location and histomorphological parameters. The hormonal receptor status is also studied in respect to the expression of protein in question.

Material and methods

Formalin-fixed paraffin-embedded tissue samples from 126 cases of invasive ductal carcinoma were included in the present study. Hematoxylin and eosin stained slides from all cases were reviewed by two pathologists (KK, MA) to confirm the diagnosis. Each representative paraffin block was cut into four micrometer sections subjected to immunohistochemical staining where three sections from each one have been stained for survivin protein. For greater adherence of tissue sections to glass slides, silanized slides (DAKO, Denmark) were used, which had been baked for two hours in an oven at 56°C. The sections were then deparaffinized in xylene for 20 minutes, rehydrated at decreasing ethanol concentrations and washed with phosphate-buffered saline (PBS). The endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 minutes. Antigen unmasking was achieved by heating the sections, which had been previously immersed into target solution (DAKO) within a hot water bath (96°C, for 45 minutes). Immunohistochemical staining was performed using monoclonal mouse anti-survivin antibody (DAKO, Clone12C4, dilution 1:50). Following an overnight incubation, the immunodetection was completed using the LSAB Vizualization System (DAKO) utilizing 3, 3´- di-aminobenzidine chromogen as substrate, according to the manufacturer´s instructions. All sections were counterstained with hematoxylin (DAKO). Negative controls were obtained by omitting the primary antibody.

In each case, the following parameters were assessed:

1) the intensity of staining, 2) the relative number of positively stained cells and 3) the subcellular localization of survivin antigen.

To achieve good reproducibility, the above mentioned parameters were evaluated semiquantitatively by two observers separately (MA, JR), who scored them using unified and clear cut-off criteria.

The age of patients, grade, stage and size of the tumor, lymph node metastasis, vascular invasion, estrogen and progesterone receptors status were designated as clinicomorphological parameters.

Statistical analysis. Statistical evaluation was performed with Microsoft Excel software package. Chi – square (χ^2) test was used to demonstrate the correlation between survivin expression and clinicopathological parameters of invasive ductal cancer as well as between histological grade and clinicopathological parameters. P value less than 0.05 was considered to indicate a statistical significance.

Results

Evaluation of immunohistochemical staining. In our group of 126 ductal carcinoma cases, survivin was expressed in 111 cases (88.1%). The positive cases showed a variable subcellular localization. Cytoplasmic staining was detected in 18 out of 126 cases (14.3%), while solely nuclear positivity was observed in 22 out of 126 cases (17.5%). Combined (nuclear as well as cytoplasmic) expression of survivin was demonstrated in 71 out of 126 cases (56.3%). In those cells with combined expression, the survivin localization was found predominantly cytoplasmic. Furthermore, the majority of cells in these cases expressed cytoplasmic immunoreaction (Fig. 1B). At higher magnification, nuclear staining exhibited a punctuate pattern (Fig. 1A) and also the nucleoli often showed survivin immunopositivity. The pattern of cytoplasmic staining was either granular, diffuse or both (Fig. 1C). Due to the fact that carcinoma cells frequently expressed heterogenic survivin immunoreactivity, this dominant pattern was used for scoring.

Statistical analysis results. Clinicopathological findings in the invasive ductal cancer cases (age, histological grade, tumor size and stage, lymph node metastasis, vascular invasion and expression of estrogen and progesterone receptors) were confronted with the immunohistochemical characterization of survivin expression – i.e. its subcellular localization, the intensity of immunostaining and the percentage of survivin positive cells (Table 1). The cases with absent survivin expression were also included in the statistical analysis of all immunohistochemical characteristics of survivin.

The statistical analyses revealed a significant correlation between the tumor grade and the subcellular localization of survivin (p<0.05). Grade 3 was significantly associated with nuclear localization of survivin. It was demonstrated in only 6.3% of cases with grade 1 and 5.8% with grade 2, while in 30% of cases with grade 3.

Furthermore, another statistically significant correlation was confirmed also between the vascular invasion in tumor and subcellular localization of survivin (p<0.01). Specimens with positive vascular invasion showed nuclear and combined (both nuclear and cytoplasmic) expression of survivin in 86.7% of cases. A higher intensity of survivin immunoreactivity was also statisticaly related to the appearance of vascular tumor invasion.

The other clinicopathological parameters (such as age, tumor size and stage, lymph node metastasis, estrogen and progester-
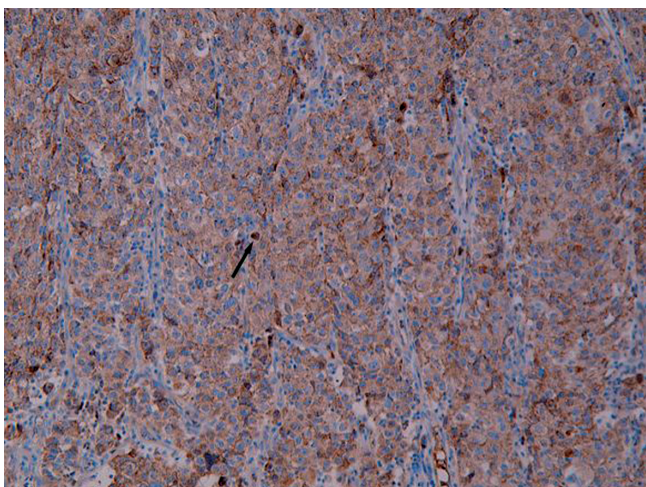
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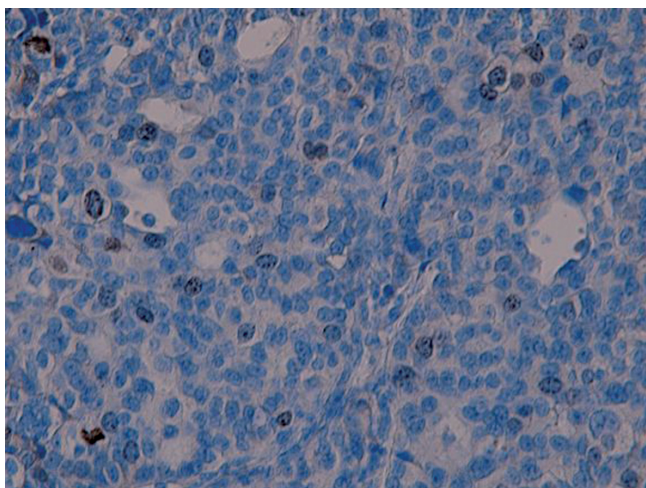
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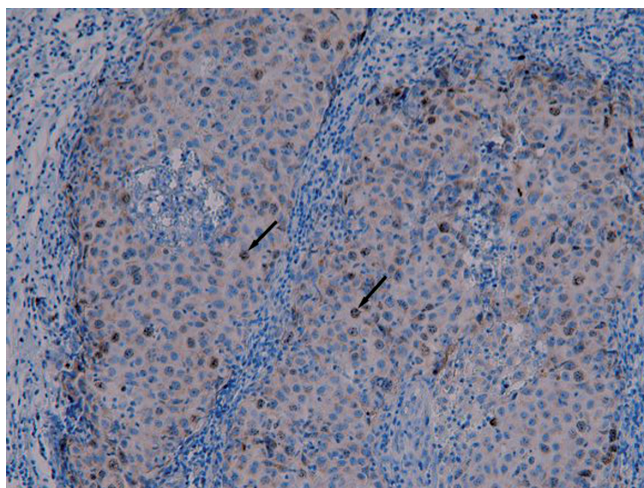
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Figure 1. Expression of survivin in breast ductal carcinoma.
A. **Solely nuclear staining showing punctuate pattern in carcinoma cells (original magnification: x400)**
B. **Combined nuclear (arrows) and cytoplasmic positivity in carcinoma cells (original magnification: x200)**
C. **Cytoplasmic granular and diffuse reaction in carcinoma cells; single carcinoma cell with nuclear positivity (arrow) (original magnification: x200)**

one receptors) did not correlate with the immunohistochemical characterization of survivin expression (p>0.05; Table 1).

The comparison of histological grade and clinicopathological parameters (age, tumor size and stage, lymph node metastasis, vascular invasion and expression of estrogen and progesterone receptors) is summarized in Table 2. The χ^2 test confirmed that in all our invasive ductal carcinoma cases, the histological grade was significantly correlated with the tumor size and stage, lymph node metastasis, vascular invasion and expression of the estrogen and progesterone receptors (p<0.01).

Tumors over 20 mm in size were with a probability of 55.4% grade 3 tumors, but only with 3.3% probability grade 1 tumors. All grade 1 ductal cancers were at stage T1, whereas 22.2% of grade 3 cases were at stage T4.

Lymph nodes were positive for metastases in only 11.1% of the grade 1 tumors and 12.1% of the grade 2 tumors, while

in 45.7% of grade 3 tumors. Vascular invasion was detected in 30.0% of tumors at histological grade 1, but in 68.5% of cases at grade 3. Expression of the estrogen and progesterone receptors in ductal carcinomas grade 1 and 2 was found out in 123 cases out of 124 total (>99%). However, only 56.7% of the grade 3 tumors expressed estrogen receptors and 50.0% of them showed progesterone receptors.

Noteworthy, the histological grade was not related to the age of patients (p>0.05).

Discussion

Breast carcinoma is still one of the most common malignant tumors in female patients. It also belongs to the leading group of lethal cancers in women. Therefore, several histomorphological and clinical parameters as well as tumor markers were

Table 1. Clinical and pathological parameters of survivin expression

Survivin expression	A	Subcellular localization			Intensity of immuno-reactivity		Percentage of labelled cells		
		C	N	NC	+	++ +++	<10 %	11–50 %	>50 %
Age (n=126)									
<40	0	0	0	1	0	1	0	1	0
41-50	2	4	4	17	11	14	3	4	18
51-60	3	5	5	21	12	19	4	8	19
61-70	3	5	5	14	7	17	1	8	15
>70	7	4	8	18	13	17	2	12	16
p-value			0.950			0.766		0.555	
Grade (n=126)									
1	6	5	2	**19**	10	16	2	6	18
2	4	7	2	**21**	16	14	3	7	20
3	5	6	18	**31**	17	38	5	20	30
p-value			0.025			0.175		0.587	
Tumour size (n=120)									
<11 mm	5	3	2	16	9	12	2	6	13
11-20 mm	5	11	9	28	20	28	4	15	29
>20 mm	5	3	11	22	12	24	4	12	20
p-value			0.239			0.682		0.927	
Stage (n=121)									
T1	8	14	11	46	28	43	6	20	45
T2	4	2	5	14	7	14	1	9	11
T3	1	0	1	0	0	1	0	1	0
T4	2	1	4	8	5	8	2	3	8
p-value			0.440			0.691		0.607	
LN metastasis (n=99)									
positive	2	3	6	17	8	18	3	5	18
negative	10	10	12	39	25	36	6	23	32
p-value			0.725			0.418		0.275	
Vascular invasion (n=115)									
positive	4	4	**15**	**37**	17	39	7	16	33
negative	10	12	6	27	21	24	2	17	26
p-value			0.008			0.042		0.111	
ER (n=124)									
positive	11	17	15	54	36	50	7	24	55
negative	3	1	7	16	7	17	3	9	12
p-value			0.248			0.529		0.666	
PR (n=124)									
positive	13	13	14	53	33	47	6	23	51
negative	2	4	8	17	9	20	4	10	15
p-value			0.450			0.330		0.397	

survivin expression: A – absent, C-cytoplasmic, N-nuclear, NC-combined (N+C)
LN – lymph node
ER – estrogen receptors
PR – progesterone receptors
Note: the significant relationship is shown in **bold** type

reported to have prognostic significance in these patients [11,31,32,33]. From these, the histological grade of tumor, number of metastatic lymph nodes, tumor size, vascular invasion and tumor stage were found to be the most important histomorphological prognostic parameters widely accepted and used in daily practice. Estrogen and progesterone receptor status and Her-2/neu should also belong to the routinely performed markers for the assessment of prognosis and therapeutic management of breast carcinoma patients [34]. Among these tumor markers, the antiapoptotic protein survivin was

Table 2. Histological grading as compared with clinical and pathological parameters

Clinicopathological parameters	Histological grade		
	1	2	3
Age (n=126)			
<40	0	0	1
41-50	5	8	14
51-60	7	9	18
61-70	6	12	9
>70	14	5	18
p-value	0.182		
Tumour size (n=120)			
<11 mm	14	9	3
11-20 mm	15	16	22
>20mm	1	9	31
p-value	1.32E-06		
Stage(n=121)			
T1	32	26	21
T2	0	4	21
T3	0	0	2
T4	0	3	12
p-value	6.61 E-07		
LN metastasis (n=99)			
positive	3	4	21
negative	24	23	24
p-value	0.001		
Vascular invasion (n=115)			
positive	9	14	37
negative	21	17	17
p-value	0. 002		
ER (n=124)			
positive	30	33	34
negative	1	0	26
p-value	1.23E-07		
PR (n=124)			
positive	31	33	30
negative	0	0	30
p-value	6.82E-10		

LN – lymph node
ER – estrogen receptors
PR – progesterone receptors

found to be representing an attractive diagnostic marker as well as a novel prognostic factor [14]. The mRNA for antiapoptotic protein survivin is the fourth most frequently expressed transcript in common cancers [14]. Despite this, much more papers deal with the expression of other proteins in malignant tumors, such as p53 and Bcl-2, rather than with survivin, though the search for survivin has certainly opened new research directions. In this study, we investigated the subcellular expression of survivin and possible correlations of its different subcellular pools with the above mentioned clinicomorphological parameters and hormonal receptor status.

Currently, there is a heated discussion within literature concerning the relationship of survivin with several his-

topathological and clinical parameters in breast carcinomas. Al-Joudi *et al.* [35] found a significant correlation between survivin expression and the tumor size and lymph node involvement. However, no significant correlations were shown with other data, such as the tumor histological grade, and estrogen and progesterone receptors. Sohn *et al.* [36] reported that cytoplasmic survivin expression was correlated with the stage, histological grade and lymph node metastasis. On the other hand, survivin nuclear expression correlated significantly with the histological grade and tumor stage, and also tended to correlate with estrogen receptor (P=0.050) in the study of Nassar *et al.* [32]. According to others, no significant correlations were found between survivin and the tumor size, tumor grade, nodal status, histology type and hormone receptor status [31,37]. Furthermore, Ryan *et al.* [37] studied survivin expression and its splice variants survivin-2B and survivin-ΔEx3 in breast carcinomas, but no significant correlation was revealed between any of the investigated survivin forms and the above mentioned clinicopathological parameters. In our group of 126 invasive ductal carcinomas, we demonstrated a positive correlation between nuclear survivin localization and tumor histological grade 3. Furthermore, nuclear, combined nuclear and cytoplasmic survivin expression, and its higher intensity of immunohistochemical reaction, were significantly correlated with lymphovascular invasion. Both histological grade 3 and lymphovascular invasion are considered to be poor prognostic parameters.

The histological grade of a tumor is one of the most important prognostic factors and together with the age and menopausal status of the patient, the stage of disease, the ER and PR status of the tumor, the proliferative capacity of the tumor and HER2/neu gene amplification may influence therapy selection [38]. Malignant tumors, in contrast to their benign counterparts, demand a much more comprehensive and detailed description. One of the major tasks is to grade the tumor according to its differentiation. Malignant tumors are usually graded as either well (grade 1), moderately (grade 2) or poorly (grade 3) differentiated. In general, well differentiated tumors are less aggressive than their poorly differentiated counterparts. In our panel of 126 ductal carcinomas, 60 cases (47.6%) were diagnosed as grade 3, 34 cases (27.0%) as grade 2 and 32 cases (25.4%) as grade 1. In the group of grade 3 carcinomas, combined NC immunopositivity (31 cases/51.7%) was the most frequent. However, overall nuclear reaction (N, NC) in this group was found in 49 cases (81.7%). Our findings in ductal breast carcinomas showed that nuclear and combined nuclear as well as cytoplasmic survivin expression may be associated with histological grade 3.

The lymphovascular and venous drainages of the breast play an important role in the spread of primary carcinoma. Approximately three quarters of the lymphatics reach the axillary lymph nodes. There are free communications between lymph nodes above and below clavicle as well as between axillary and cervical nodes. Other connections also lead to the contralateral breast tissue. Moreover, the connections of the vertebral plexus

with intercostals veins permit the development of metastases in the nervous system and bones [39]. Therefore, histological assessment of the lymphovascular and vascular invasion represents an important source of prognostic information. In general, conflicting data were reported on survivin expression and its association with the lymphovascular invasion. Some authors concluded that survivin expression correlated with the invasion of lymphatics [40], while other papers described no correlation [20,31]. Our results showed a significant relationship between the vascular invasion and subcellular compartmentalization of survivin and a higher intensity of immunoreaction of this protein. Cases with vascular invasion showed nuclear and combined (both nuclear and cytoplasmic) survivin reaction in 86.7%. Molecular mechanisms of metastatic dissemination have been elucidated in recent study of Mehrotra *et al.* [41]. They pointed out at intermolecular cooperation between extracellular and intracellular constituents. Interaction of two IAP proteins, survivin and XIAP, triggers cascade reaction involving NFκB (Nuclear Factor κB), which leads to increased fibronectin gene expression, signaling by β1 integrins, and activation of cell motility kinases, FAK (Focal Adhesion Kinase) and Src (proto-oncogenic tyrosine kinase). Thus, both of the above mentioned IAP proteins are required for metastatic dissemination. Therefore, these IAPs are considered as direct metastatic genes.

The significance of subcellular survivin expression in breast carcinoma is still controversial, since the research on the role of survivin as a prognostic factor leads to conflicting results [11,19]. Taking into account 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 acceptable argument that the overexpression of this protein in question may be an indicator of a worse prognosis [14,42]. Indeed, multiple clinical studies showed the association of survivin expression with unfavorable prognosis, diminished survival, increased rates of relapse and chemoresistance [43,44]. Furthermore, many recent papers recognize the subcellular location of survivin in respect to the prognosis of breast carcinoma. The detailed study of Brennan *et al.* [19] concluded that different prognostic information is associated with nuclear and cytoplasmic survivin location, nuclear survivin expression being a poor prognostic marker. A multivariate analysis of 157 breast carcinomas by Oh *et al.* [33] revealed that cytoplasmic survivin expression was associated with an improved overall survival, while nuclear expression was correlated with an unfavorable overall survival. Similarly, a high cytoplasmic to nuclear ratio of survivin was associated with an improved overall survival and, conversely, an increased nuclear to cytoplasmic survivin ratio was correlated with an unfavorable overall survival. Both of our previous studies [16,17] and recent results also demonstrated that nuclear and combined nuclear and cytoplasmic survivin reaction are associated with worse prognostic parameters, it is not a case of solely cytoplasmic positivity. It follows that different subcellular survivin compartmentalization has distinct functions. Generally, survivin is a nuclear shuttle protein and its dynamic localization plays a key role in cell function and regulation. Recently, it has been accepted that nuclear survivin may participate in the regulation of cell proliferation and may lead to a proliferative aggressive phenotype [13,19,45]. On the other hand, cytoplasmic survivin may be related to the apoptotic process and may be involved in the inhibition of cell death [13,14,46] and promoting carcinogenesis [47]. Data obtained by Jin *et al.* [47] suggest, that cytosolic survivin expression was induced even in early carcinogenesis.

In contrast to the above mentioned correlations, we also studied the relationship between the histological grade and remaining histomorphological parameters. The statistical evaluation confirmed that tumor grade is one of the key histomorphological and prognostic factors in the assessment of breast carcinoma.

In conclusion, we indicate that different subcellular survivin expression may influence the biological behavior of the ductal breast carcinoma. We point out that only nuclear and combined nuclear and cytoplasmic positivity together with a higher intensity of survivin immunoreactivity (proliferative phenotype) are associated with poor prognostic parameters, such as tumor grade 3 and vascular invasion. Both of them are considered to be powerful prognostic factors. Based on the above mentioned findings, we can conclude that the antiapoptotic protein survivin may represent a poor prognostic marker in the ductal breast carcinoma.

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