

The significance of Survivin and Nectin-4 expression in the prognosis of breast carcinoma

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Abstract: To investigate the prognostic significance of Survivin and Nectin-4 expression in breast carcinomas. Imprint smears were obtained from 140 breast carcinoma specimens and studied immunocytochemically for the expression of Survivin and Nectin-4. The results were correlated with several clinicopathological parameters, including five-year survival. Increased Survivin staining pattern correlated with increased grade ($p < 0.0001$), increased lymph node invasion ($p < 0.0001$), increased tumor size and reduced survival ($p < 0.0001$). Elevated Nectin-4 expression also correlated significantly with increased grade ($p < 0.0001$), increased tumor size ($p < 0.0001$) and reduced survival ($p < 0.0001$). In addition, Survivin and Nectin-4 staining patterns correlated strongly with one another ($p < 0.0001$). However, on multivariate analysis, neither Survivin nor Nectin-4 expression seemed to have an independent impact on survival in our study cases. The findings of our study suggest that increased expression of Survivin and Nectin-4 may indicate a worse prognosis in breast cancer patients. The exact implications of the expression of these markers in breast cancer prognosis and treatment remain to be clarified. (*Folia Histochemica et Cytobiologica* 2011; Vol. 49, No. 1, pp. 26–33)

Key words: breast cancer, Survivin, Nectin-4, immunocytochemistry, prognosis

Introduction

Breast cancer is a complex and heterogeneous disease at the molecular level. Therefore the emerging molecular classification of breast cancer is based on data obtained from gene and protein expression profiling.

Immunohistochemistry has helped in defining the molecular and prognostic signatures of breast cancer. Identifying new protein markers that help improve patient management is a priority in breast cancer research [1–6].

Survivin has been identified as a novel inhibitor of apoptosis. It blocks common downstream elements of both the mitochondrial pathway and the

death receptor pathway, by directly inhibiting terminal effectors caspases activity [7–10].

Survivin is expressed during embryonic and fetal development, but is undetectable in terminally differentiated normal adult tissue. However, it is re-expressed in human cancer cells at a frequency of 34–100% [11–13]. As a prognostic factor, Survivin expression is significantly associated with a poor clinical outcome in cancers including breast cancer [8, 14–18].

Nectins are cell adhesion molecules involved in the regulation of epithelial physiology. Four Nectins have been described so far. Nectin-4 is a new tumor-associated antigen and a reliable marker for breast carcinoma. Furthermore, it represents a sensitive and complementary marker for the follow-up of patients with metastatic breast carcinoma [19–21].

Our aim was to study the expression pattern of Survivin and Nectin-4 proteins in imprints of invasive breast carcinoma and their clinicopathological and prognostic value, as well as their relation to markers indicative of the tumor phenotype.

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Material and methods

One hundred and forty breast tumors, freshly resected from women who had undergone total mastectomy, were studied. Cytologic imprint smears were obtained by touching the cut surface of breast carcinoma tissues. One hundred and fifteen of the tumors were histologically ductal invasive of no special type, and 25 were lobular invasive carcinomas. The patients were aged from 28 to 85 years with a mean of 55.72 years. The histological diagnosis was determined during routine pathological assessment. The tumors were classified according to the histological typing of breast tumors by the WHO [22] and the tumors were graded according to the Scarff-Bloom Richardson classification as modified by Elston and Ellis [23]. Staging at the time of diagnosis was based on the TNM system [24]. Tumor size (less than 2 cm, 2 to 5 cm, more than 5 cm) and lymph node status were evaluated separately. Additional information was recorded concerning the patient's menopausal status (ERa/PR) as determined immunohistochemically [23] and HER-2 expression. The clinicopathological characteristics of the patients are listed in Table 1. Follow-up was available for all 140 patients every three months post-operatively. The follow-up period was 60 months. At the end of those 60 months, 32 (22.9%) patients had died of their disease and 108 patients had developed recurrence. Mean survival time was 36.71 months (range 5 to 60 months) and median survival time was 37.50 months. Patient outcome was defined as disease overall survival.

None of the patients had received radiation or chemotherapy preoperatively. After surgery, the patients received adjuvant therapy, depending on the extent of the disease, including anti-estrogen therapy and radiation therapy when indicated. Premenopausal patients with lymph nodes involvement were treated with four cycles of adjuvant chemotherapy using doxorubicin/cyclophosphamide followed by paclitaxel for four cycles.

Imprint smears were obtained from each patient by touching the cut surface of the freshly resected tumor tissues. We prefer to use imprint smears instead of paraffin-embedded tissue sections and cells (cell blocks), because the latter present difficulties with regard to immunoreactivity. Depending on the thickness of the section, there will always be a number of cells either sliced or overlapped, leading either to false low or to false high immunoreactivity, respectively [25–27]. Furthermore, tissue fixation and, to a lesser degree, tissue processing, are potential causes of variation in the reproducibility of

immunohistochemical staining [28]. Above and beyond that, in cytologic preparations, the cells are whole, a larger surface of the tumor is sampled, and tissue is preserved for subsequent pathological and molecular analyses [27, 29–31]. It is now firmly established that a wide variety of markers can be applied on cytologic preparations and that immunocytochemistry correlates well with immunohistochemistry [32–36].

For immunocytochemistry 4 smears were fixed for 10 minutes in 5% buffered formalin and stored at -70°C until used.

Immunostaining was performed by the Avidin-Biotin Complex immunoperoxidase method [37]. Smears were incubated for 45 minutes with a normal rabbit serum diluted 1:40 in PBS. Then the smears were rinsed in three changes of PBS for five minutes each and incubated overnight in Survivin (0–8 Santa Cruz Biotechnology, USA) at a dilution 1:50 and Nectin-4 antibody (AF2659 Browse by Molecule, USA) at a dilution of 1:100. After washing in PBS, the smears were incubated with rabbit anti-mouse biotinylated immunoglobulins diluted 1:200 followed by the ABC complex/HPR. Visualization was achieved by a final incubation in 3,3'-diaminobenzidine tetrahydrochloride. The smears were counterstained with Mayer's hematoxylin. Known positive controls were also stained simultaneously. Negative controls were stained by omitting the primary antibodies incubation.

The results were interpreted by two independent cytologists by means of light microscopic observation. In cases where staining was heterogeneous in the slide, examined fields included those with the highest and those with the lowest percentage of stained cells. The score was the average of ten distinct high power fields observed at $\times 400$ magnification. Nuclear or cytoplasmic staining for Survivin and cytoplasmic staining for Nectin-4 were scored on a scale of zero to three, as follows: a score of 0 was given when there was no staining; a score of 1 if there was staining in less than 10% of cells; a score of 2 if there was moderate to strong staining in 11–50% of cells and a score of 3 if strong staining was detected in more than 50% of cells. For the purposes of statistical analysis, scores of 1, 2 and 3 were considered positive, and a score of 0 was considered negative (Figures 1, 2).

Statistical analysis of the results was performed using Pearson's χ^2 test. Survival curves were constructed with the use of the Kaplan–Meier method and compared using the log-rank test. Multivariate analysis was performed with the use of Cox's proportional hazard model.

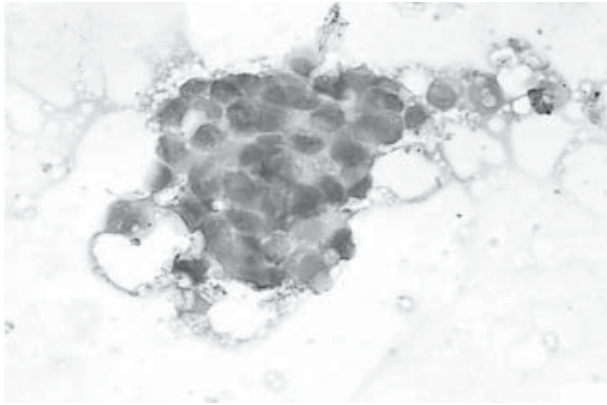


Figure 1. Breast adenocarcinoma cells with strong nuclear and mild cytoplasmic expression for Survivin (magnification $\times 500$)

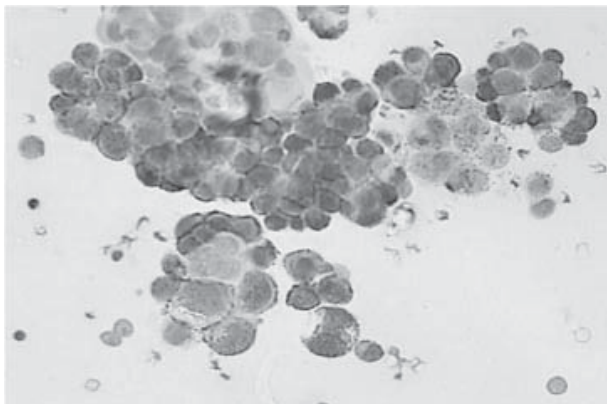


Figure 2. Clusters of breast adenocarcinoma cells with positive membrane and cytoplasmic expression for Nectin-4 (magnification $\times 500$)

Results

Table 1 also provides the distribution of clinicopathological characteristics in the 140 breast tumors studied. Thirty eight women were premenopausal and 102 postmenopausal. Thirty five tumors were histological grade I, 40 grade II and 65 grade III. Tumor sizes were as follows: 37 were ≤ 2 cm, 78 were 2–5 cm, and 25 were ≥ 5 cm. According to the number of positive nodes, four groups were formed (0 = 35 patients; 1–3 = 59 patients; 4–9 = 37 patients; and >10 = 9 patients) with positive lymph nodes. Of the 140 tumors, 90 (64.3%) were ER and 80 (57.1%) PR positive. HER-2 positivity was 31.4% as determined immunohistochemically.

Table 2 shows the distribution of Survivin and Nectin-4 staining pattern in all cases of breast carcinomas. Survivin was expressed in 65.7% and Nectin-4 in 64.3% of all tumors.

Survivin immunoreactivity was observed both in the cytoplasm and nuclear compartment of malig-

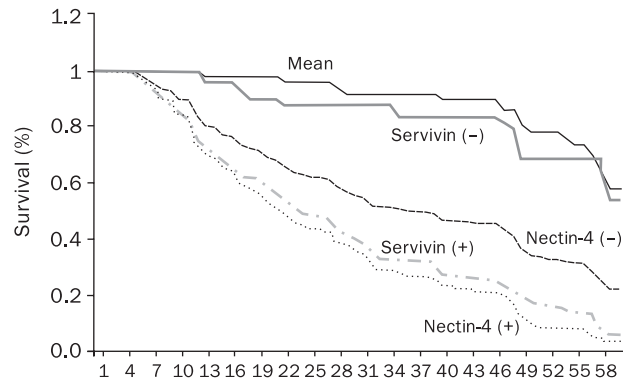


Figure 3. Overall survival curves according to Survivin and Nectin-4 expression

Table 1. Clinicopathological characteristics of patients

Variables	Number	%
Menopausal status		
Premenopausal	38	27.1
Postmenopausal	102	72.9
Tumor size (cm)		
< 2	37	26.4
2–5	78	55.7
> 5	25	17.9
Histological type		
Ductal	115	82.1
Lobular	25	17.9
Histological grade		
I	35	25.0
II	40	28.6
III	65	46.4
Lymph nodes status		
0	35	25.0
1–3	59	42.1
4–9	37	26.4
≥ 10	9	6.4
ERa immunostatus		
Negative	50	35.7
Positive	90	64.3
PR immunostatus		
Negative	60	42.9
Positive	80	57.1
HER-2		
Negative	96	68.6
Positive	44	31.4

nant cells or only in the cytoplasm or nucleus. Cytoplasmic Survivin immunoreaction was observed in 75/92 (81.5%) cases, while nuclear was observed in 26/92 (28%) cases. Twenty two (23.9%) cases showed

Table 2. Distribution of Survivin and Nectin-4 staining expression in 140 tumors of breast carcinoma

Staining pattern	Frequency (No)	Percentage (%)
Survivin		
Negative	48	34.3
Positive	92	65.7
Nectin-4		
Negative	50	35.7
Positive	90	64.3

Survivin staining both nuclear and cytoplasmic compartment.

There seems to be no association between cytoplasmic and nuclear Survivin staining. However 7/12 (58.3%) cases with very strong cytoplasmic intensity also showed nuclear staining. This was in contrast to only 17/63 (26.9%) cases with lighter cytoplasmic staining ($p = 0.002$). We failed to find any relationship between positivity rate of cytoplasmic or nuclear staining with all clinicopathological parameters analyzed. Moreover, no relationship was observed between cytoplasmic or nuclear Survivin staining with overall survival.

Therefore, a tumor was assessed as Survivin positive if the staining was positive cytoplasmic, or positive nuclear, or both. The results of χ^2 analysis of the studied variables are summarized in Table 3.

Survivin positive staining was significantly correlated with increased grade (III) ($p < 0.0001$), increased number of lymph nodes invasion ($p < 0.0001$) increased tumor size (> 2 cm), negative ERa/PR status ($p < 0.0001$ respectively) and positive expression of HER-2 ($p < 0.0001$).

Similar associations were found for Nectin-4. Positive Nectin-4 expression was significantly associated with increased grade (II and III) ($p < 0.0001$), increased tumor size ($p < 0.0001$), negative ERa and PR status ($p < 0.0001$) as well as with positive HER-2 expression ($p < 0.0001$).

In addition, Survivin and Nectin-4 staining patterns correlated strongly to one another ($p < 0.0001$).

We evaluated the association between Survivin and Nectin-4 expression and overall survival (Figure 3). Positive staining for Survivin and Nectin-4 was associated with lower five-year survival rates compared to negative staining (6.5% vs. 54.2% and 3.3% vs. 58.0%) ($p < 0.0001$). A shorter survival was also strongly associated with higher grade of the tumor ($p < 0.0001$), increased number of lymph nodes invasion ($p < 0.0001$), positive expression of HER-2 ($p < 0.0001$), and increased size of the tumor > 2 cm

($p < 0.0001$) as well as with a negative ERa and PR status ($p < 0.0001$). Further associations were demonstrated between Survivin and Nectin-4 staining respectively and the remaining clinical and pathological parameters examined.

The independent relationship of Survivin and Nectin-4 was studied with Cox multivariate regression analysis. We thus could establish whether the prognostic value of Survivin and Nectin-4 as found in univariate analyses could be attributed to its relationship with other clinicopathological factors, or whether Survivin and Nectin-4 contribute independently to prognosis. A multivariate analysis was performed, including age, menopausal status, nodal status, tumor size, histological type, ERa/PR status and HER-2. For overall survival, age ($p < 0.0001$), grade ($p < 0.0001$) and lymph nodes status ($p < 0.014$) were significant (Table 4). Therefore, neither Survivin nor Nectin-4 expression seemed to have an independent impact on survival in our studied cases.

Discussion

Our study detected positive expression for Survivin in 65.7% of all breast carcinoma specimens. This percentage is within the range of previously published reports, in the majority of which positive Survivin expression is demonstrated with the use of immunohistochemistry [8].

The sub-cellular distribution of Survivin seems to be altered during the growth and progression of breast carcinoma, under the influence of yet unknown molecular mechanisms. However, the exact prognostic and clinical implications of the immunohistochemical localization (nuclear or cytoplasmic) of Survivin in different types remain controversial.

Our study found Survivin to be predominantly detected in the cytoplasmic compartment of breast cancer cells, confirming previous observations. Fortugno et al. showed that cytoplasmic and nuclear Survivin are immunocytochemically different, and this might partly explain the conflicting data on Survivin localization in solid tumors [38].

The relationship between immunocytochemical detected cytoplasmic and nuclear localization of Survivin has been not investigated. Although we found no relationship between cytoplasmic and nuclear staining, a high percentage of cases with very strong cytoplasmic intensity also showed nuclear Survivin immunoreaction [15, 39, 40]. In the literature, cytoplasmic expression of Survivin is reported to be a poor prognostic parameter in colorectal neuroblastoma, squamous cell and urothelial carcinoma. On the other hand, in studies of pancreatic, gastric,

Table 3. Results of χ^2 analysis showing the correlations between Survivin and Nectin-4 staining patterns and several clinicopathological parameters in 140 patients with breast carcinoma

Variable	Total	Staining pattern					
		Survivin		p	Nectin-4		p
		(-)	(+)		(-)	(+)	
Histology	140						
Ductal		41 (25.7%)	74 (64.3%)	0.465	42 (36.5%)	73 (63.5%)	0.669
Lobular		7 (28.0%)	18 (72.0%)		8 (32.0%)	17 (68.0%)	
Grade	140						
I		27 (77.1%)	8 (22.9%)	< 0.0001	33 (94.3%)	2 (5.7%)	< 0.0001
II		20 (50.0%)	20 (50.0%)		16 (40.0%)	24 (60.0%)	
III		1 (1.5%)	64 (98.5%)		1 (1.5%)	64 (98.5%)	
Tumor size [cm]	140						
< 2		27 (73.0%)	10 (27.0%)	< 0.0001	28 (75.7%)	9 (24.3%)	< 0.0001
2-5		21 (26.9%)	57 (73.1%)		22 (28.2%)	56 (71.8%)	
> 5		0 (0%)	25 (100%)		0 (0%)	25 (100%)	
Lymph nodes status	140						
0		27 (77.1%)	8 (22.9%)	< 0.0001	33 (94.3%)	2 (5.7%)	< 0.0001
1-3		20 (33.9%)	39 (66.1%)		17 (28.8%)	42 (71.2%)	
4-9		1 (2.7%)	36 (97.3%)		0 (0%)	37 (100%)	
> 10		0 (0%)	9 (100%)		0 (0%)	9 (100%)	
ER α status	140						
Negative		2 (4.0%)	48 (96.0%)	< 0.0001	7 (14.0%)	43 (86.0%)	< 0.0001
Positive		46 (51.1%)	44 (48.9%)		43 (47.8%)	47 (52.2%)	
PR status	140						
Negative		2 (3.7%)	58 (96.7%)	< 0.0001	9 (15.0%)	51 (85.0%)	< 0.0001
Positive		46 (57.5%)	34 (42.5%)		41 (51.3%)	39 (48.8%)	
HER-2 Expression	140						
Negative		48 (50.0%)	48 (50.0%)	< 0.0001	47 (49.0%)	49 (51.0%)	< 0.0001
Positive		0 (0%)	44 (100%)		3 (6.8%)	41 (93.2%)	
Menopausal status	140						
Premenopausal		13 (34.2%)	25 (65.8%)	< 0.991	17 (44.7%)	21 (55.3%)	0.174
Postmenopausal		35 (34.3%)	67 (65.7%)		33 (32.4%)	69 (67.6%)	

Table 4. Cox's regression analysis of the prognostic value of the assessed variables with regard to overall survival

	Variables in the equation							
	B	SE	Wald	df	p	Exp (B)	95% CI for Exp (B)	
							Lower	Upper
Age	1.051	247	18.064	1	0.0001	2.861	1.762	4.646
Hist grade	1.570	0.262	35.938	1	0.0001	4.806	2.876	8.029
LN status	0.519	0.210	6.096	1	0.014	1.681	1.113	2.538

SE — standard error; B — coefficient

urothelial and esophageal carcinomas, no association has been found between cytoplasmic Survivin and patient survival [7, 9, 41, 42].

The differential nuclear and cytoplasmic localization of Survivin is shown to be due to differences in the amino-acid sequence of its carboxy-terminal do-

main [43]. Moon and Tornawski suggest the cytoplasmic localization of Survivin in non-malignant cells suppresses apoptosis, while its nuclear translocation may be important in the regulation of proliferation and differentiation [44].

In other studies of different types of cancers, the overexpression of Survivin correlates with increased proliferation, although the expression was cytoplasmic [43–46]. We believe that further studies designed to clarify the relationship between cytoplasmic and nuclear Survivin are needed in order to determine the role of Survivin detected immunocytochemically in cancer.

Our study found that Survivin positivity correlated with positive expression of HER-2 and lack of PR and ER α status. This is in agreement with other studies which have indicated that the Survivin protein positivity detected by immunohistochemical staining might be correlated with HER-2 expression but not with hormone receptors [47–49].

Furthermore, Survivin staining pattern correlated strongly with increased grade, increased tumor size, increased number of lymph nodes involvement, and reduced survival rates affecting the clinical outcome but as an independent parameter. In some studies it failed to demonstrate any association between Survivin positivity rate and the clinicopathological prognostic parameters in some solid tumors [43].

The possibility that the biological role of Survivin expression might also be influenced by tissue specificity cannot be ruled out and requires further attention.

In a study by Al-Joudi et al. it was found that negative and low expression of Survivin correlated significantly with favorable outcomes [8]. Conversely, high expression correlated with unfavorable outcomes [8, 17].

Also, experimental studies of different kinds of tumors have reported that overall survival is significantly decreased in patients with neoplasms expressing Survivin compared to those with no expression [41, 43, 50, 51].

Nectin-4 belongs to the family of cell adhesion molecules that regulate the formation of adherence functions in epithelial cells through the AF-6 afa-din scaffold molecule [51–53]. Nectin-4 is an antigen mainly expressed during embryogenesis and highly expressed (68%) in tumors of breast origin [20, 21]. Nectin-4 staining has been described as prevalent cytoplasmic and discrete membrane stain [54–56].

In the present study, we detected positive expression for Nectin-4 in 63.5% of all breast carcinoma

specimens. This percentage is within the range of previously published results [20]. Fabre Lafay et al. reported positive expression of Nectin-4 in 62% of ductal type and 6% of lobular type carcinomas, thus strongly correlating with histological type. Our study failed to reveal any difference in Nectin-4 expression rates among histological types of breast carcinoma. Moreover, Nectin-4 staining has been described as prevalent in the cytoplasm and membrane of malignant cells [20].

The same study found that ER α /PR negative tumors expressed Nectin-4. Furthermore, serum Nectin-4 is a marker of disease progression, metastatic status and clinical outcome of patients. No correlation was found between Nectin-4 and tumor size, tumor grade or lymph nodes status.

Nevertheless, univariate statistical analysis of our data showed a strong correlation between increased Nectin-4 staining pattern and the following adverse clinicopathological parameters: increased grade (II, III), increased tumor size, increased lymph nodes infiltration and reduced survival. These findings suggest that Nectin-4 overexpression may be a marker for a more aggressive clinical behavior and worse prognosis in breast carcinoma. However, the exact mechanism by which Nectin-4 contributes to cancer progression remains unclear.

We also found that the expression levels of Survivin and Nectin-4 correlated strongly to one another. The association of Survivin and Nectin-4 with unfavorable prognostic indicators, and with one another, as demonstrated in our study, suggests that these proteins may also interact in breast carcinoma in order to exert their adverse effect.

In conclusion, Survivin and Nectin-4 expression demonstrates a strong independent association with poor prognosis. It is possible that Survivin and Nectin-4 inhibitors may be of value in the treatment of breast cancer patients. For this reason, additional studies are needed to further define the prognostic importance of these markers. In addition, further molecular studies should determine the interactions that take place between Survivin and Nectin-4 during breast carcinogenesis, as well as their exact effect on the clinical outcome of patients.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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