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Original Research Article

Survivin Immunophenotyping in Breast Cancer

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Abstract

Background: Survivin, an inhibitor of apoptosis, is a protein known to be over expressed in most tumour cell types while being absent in normal cells. As this may contribute to the resistance of cancer cells to apoptotic stimuli from the environment, the use of antisense Survivin therapy hopes to render cancer cells susceptible to apoptosis by eliminating Survivin expression in the cancer cells.

Methods: This study was carried out on 25 breast carcinomas samples. Survivin was demonstrated immunohistochemically in the malignant epithelial cells of the tissue samples by using the ImmPRESS polymerized reporter enzyme staining system, which has been shown to have very good sensitivity and signal intensity, low background staining and reduced non-specific binding. This system is based on a new method of polymerizing enzymes and attaching these polymers to antibodies. Survivin immunoreactivity was evaluated semiquantitatively according to the percentage of cells demonstrating distinct nuclear and/or diffuse cytoplasmic immunohistochemical reactions. The specimen was considered to be positive if more than 10% of the tumour cells stained. The distribution of staining in the tumour cells was graded as 1 = focal (<10%), 2 = regional (11–50%), or 3 = diffuse (>50%).

Results: Survivin immunostaining was observed in 10 of 25 (40%) cases of breast carcinomas. Among the positive cases, 3/7 (43%), 5/10 (50%) and 2/8 (25%) cases belonged to grades I, II and III respectively. The expression of Survivin gradually increased with increasing histological grades.

Conclusion: As a result of its widespread expression in different tumours, and generally low-level expression in normal tissue, Survivin, is considered to be the prospective newer adjuvant target for apoptosis-based chemotherapy. There is a need for further studies to investigate the contribution of this protein to various cancer phenotypes and to be used as a potential molecular target for their treatment.

Keywords: Survivin, Breast Cancer, Invasive Ductal Carcinoma, ImmPRESS.

Introduction

Survivin, an inhibitor of apoptosis, is a protein known to be highly expressed in most tumour cell types and absent in normal cells, making it a good target for cancer therapy. The exploitation of Survivin's over-active promoter in most cancer cell types allows for the delivery of therapeutics only to cancer cells and not to normal cells. As it is known that Survivin is over-expressed in most cancers and may be contributing to the resistance of cancer cells to apoptotic stimuli from the environment, the use of antisense Survivin therapy hopes to render cancer cells susceptible to apoptosis by eliminating Survivin expression in the cancer cells [1].

Hence, we chose to study the immunophenotyping profile of Survivin in 25 samples of invasive breast cancers and its relation to the histopathological grade of the tumour, thereby attempting to predict tumour behaviour and patient survival.

Materials & Methods

Immunohistochemical Detection of Survivin by Immunoperoxidase Method

The ImmPRESS polymerized reporter enzyme staining system (Vector Laboratories) was used to demonstrate Survivin in an immunohistochemical manner in the malignant epithelial cells of the tissue samples. The primary antibody is first bound to the target antigen in the tissue section using the two-step method for antigen detection, which is then followed by detection and visualisation using an appropriate enzyme-substrate (chromogen) system.

Based on a novel approach to polymerizing enzymes and coupling these polymers to antibodies, the ImmPRESS polymerized reporter enzyme staining system.By forming enzyme "micropolymers" using a novel method, large dextran or other macromolecules are spared from their inherent drawbacks.A secondary antibody can be combined with a special "micropolymer" that contains a high concentration of an extremely active enzyme to create a reagent that overcomes steric interference and improves accessibility to its target. Additionally, according to the manufacturer, this yields exceptional sensitivity and signal intensity, little background staining, and less non-specific binding. [2]

Requisite solutions & reagents

Primary Antibody used for immunohistochemical staining:

- Anti – Survivin – is a rabbit monoclonal antibody (IgG) to Survivin Purified Rabbit ascites; purchased from Biogenex Laboratories Inc., CA, USA.

1. Bovine serum albumin- used in a concentration of 1.5 % to suppress non- specific binding of the primary antibody.
2. Phosphate buffer saline – stock (pH 7.4) -
   Solution a 0.15M NaH2PO4. 2 H2O 23.4g/l
   Solution b 0.15M Na2HPO4 21.2g/l
   A working solution was prepared by adding 18ml of solution 'a' and 82ml of solution 'b' and this was stored at 4° C. This was used to wash the slides after each staining step.
3. Trisodium citrate buffer (pH 6- 6.2) –
   0.001M solution - 2.941g trisodium citrate dihydrate was dissolved in 1000ml of deionised water.
4. DAB (3, 3' Diaminobenzidine Tetrahydrochloride) substrate buffer: consists of Tris buffer, peroxide, DAB and stabilizers.
5. The ImmPRESS™ polymerized reporter enzyme staining system ready- to- use kit: The kit comprises the following reagents:

- ImmPRESS Universal reagent - contains a "micropolymer" of a very active peroxidase coupled to a mixture of affinity – purified anti-mouse IgG (H+L) and anti – rabbit IgG (H+L) secondary antibodies.
- 2.5 % normal horse serum blocking solution – universal protein blocking solution to reduce non- specific staining.
- Peroxide block – 3% hydrogen peroxide in deionised water, to quench the endogenous peroxidase.

6. APES (3- aminopropyl triethoxysilane) – permanent section adhesive.
7. Lillie – Mayer's haematoxylin –used as counterstain.
8. DPX- permanent mounting medium.

Procedure for immunohistochemical staining of Survivin: (Table 1)

Representative samples of human breast carcinomas were cut into sections of 3 µm thickness, which were then deparaffinized in xylene, hydrated in graduated alcohols, and cleaned in water. After that, sections spent 10 minutes in citrate buffer (pH 6). Slides were pressure cooked for 10 minutes for heat-induced epitope retrieval (antigen unmasking).The slides were then immediately taken out of the pressure cooker and allowed to cool for 20 minutes at room temperature in the same citrate buffer. After being placed in Phosphate Buffered Saline (PBS), the slides were incubated for 10 minutes. Slides were treated with a 3% hydrogen peroxide solution for 5 minutes while they were laid flat in a humidifying chamber.

After a quick (5 minutes) immersion in PBS, the sections were treated for 10 minutes with a blocking solution made up of 2.5% normal horse serum. After draining the excess blocking solution and carefully wiping it off the sections, the sections were incubated with the primary antibody.

The primary antibody was incubated on the sections for one hour at room temperature and then overnight at 4°C. PBS was used to thoroughly wash the sections for 5 minutes. The sections were then incubated with ImmPRESS Universal Reagent for an additional 30 minutes. Freshly prepared DAB chromogen solution was applied to the sections and incubated for 10 minutes after washing the slides in PBS solution for 5 minutes. Sections were then moved to a PBS solution for 5 minutes, washed with tap water for 5 minutes, and then dried. Sections were then washed with tap water after being counterstained for 5 seconds with Lillie-Mayer's hematoxylin. Finally, after thorough dehydration and section clearing, slides were mounted with DPX. The sections weren't allowed to dry during the staining process.

The slides were carefully cleaned around the sections without harming them after PBS washes. Along with the test cases, positive and negative controls were used to check the staining's quality and gauge how well the reagents adhered to the specimen in general. The finished product was identified by brown staining in the nucleus and/or cytoplasm of the cells.

Table 1: Protocol For Survivin IHC Staining

Marker	Technique	Primary antibody dilution	Primary antibody incubation time	Micropolymerised enzyme - attached secondary antibody	Secondary antibody time	Chromogen	Haematoxylin
Survivin Monoclonal	Immunoperoxidase method	1 : 50	1 hour at 37°c + overnight at 4°c	Peroxidase enzyme attached to anti-mouseigg and anti – rabbitigg sec-ondary antibodies	30 minutes	Dab	Lillie - mayer's haematoxylin

Interpretation of the Results

Two observers independently used standardised techniques to gauge the stained sections' level of expression. According to the percentage of cells exhibiting distinct nuclear and/or diffuse cytoplasmic immunohistochemical reactions, Survivin immunoreactivity was assessed semiquantitatively. At least five high-power fields at a magnification of x40 were used to assess the immunoreactivities of nuclear and cytoplasmic tumour cells separately. [3] By measuring the proportion of stained tumour cells and the staining intensity semi-quantitatively, the expression of Survivin in the nucleus and in the cytoplasm was identified. The percentage of positive cells was rated as follows: 1. 1-10% positive cells; 2. 11-50%; 3. 51-80%; and 4. >80% positive cells and staining intensity was scored as 1 = weak; 2 = moderate, and 3 = intensive. Scores for expression intensities were multiplied to calculate an immunoreactive score (IRS) 0-2 = no staining; 3-4 = weak staining; 6-8 = moderate staining; 9-12 = strong or intense staining.[4]

Results

Our study material included 25 cases of breast carcinoma from women belonging to different age groups and they were categorised based on their histomorphology in haematoxylin and eosin-stained sections. Among the 25 cases of breast carcinoma (invasive ductal carcinoma not otherwise specified), 7 cases belonged to grade I, 10 cases to grade II cases, and 8 cases to grade III.

Evaluation of Survivin Expression in Breast Carcinoma: (Table 2)

Survivin immunostaining was observed in 10 of 25 (40%) cases of breast carcinoma. The intensity of Survivin staining was generally homogenous, but the number of positive tumour cells ranged from 10% to 100%. On grouping the positive cases, 3/7 (43%), 5/10 (50%), and 2/8 (25%), belonged to grades I, II, and III, respectively. (Figure 1)

On computing the immunoreactivity scores, our observations were as follows: IRS score 1 = nil, IRS score 2 = 8%, IRS score 3 = 24% and IRS score 4 = 4% for the cytoplasmic Survivin immunoreactivity and IRS score 1 = 8%, IRS score 2 = nil, IRS score 3 = 12% and IRS score 4 = 12% for the nuclear immunoreactivity scores. (Figures 2,3)

Among the grade I cases positive for Survivin, 2/3 (67%) cases showed predominantly cytoplasmic expression and the level of staining was weak to moderate in these cases. In one case (33%), Survivin was detected in both cytoplasmic and nuclear locations. Moderate cytoplasmic and weak nuclear staining were observed in it. There was no case with only nuclear staining. (Figure 4)

Dual location staining (both nuclear and cytoplasmic) for Survivin was seen in 4/10 (80%) cases of the grade II group. The predominant pattern of Survivin expression was moderately to intensely cytoplasmic in quality. 1/5 (20%) cases demonstrated moderate nuclear Survivin expression. (Figure 5)

In the advanced grade tumours (III), 2/8 (25%) depicted dual positivity. The levels of expression in these two cases were moderate nuclear and intense nuclear and cytoplasmic respectively.(Figure 6)
Interestingly, in a few cases, the benign breast ducts adjacent to the tumour, showed Survivin staining in their luminal cytoplasm. Also, peritumoural lymphocytes showed immunoreactivity for Survivin. (Figure 7)

Table 2: Evaluation of Survivin Expression

S.no.	Grade	Survivin expression score	
		Cytoplasmic	Nuclear
1.	I	6	2
2.	I	3	-
3.	I	4	-
4.	I	-	-
5.	I	-	-
6.	I	-	-
7.	I	-	-
8.	II	-	8

9.	II	8	12
10.	II	8	12
11.	II	8	6
12.	II	-	-
13.	II	-	-
14.	II	6	2
15.	II	-	-
16.	II	-	-
17.	II	-	-
18.	III	-	-
19.	III	-	-
20.	III	-	-
21.	III	8	6
22.	III	-	-
23.	III	-	-
24.	III	-	-
25.	III	12	9

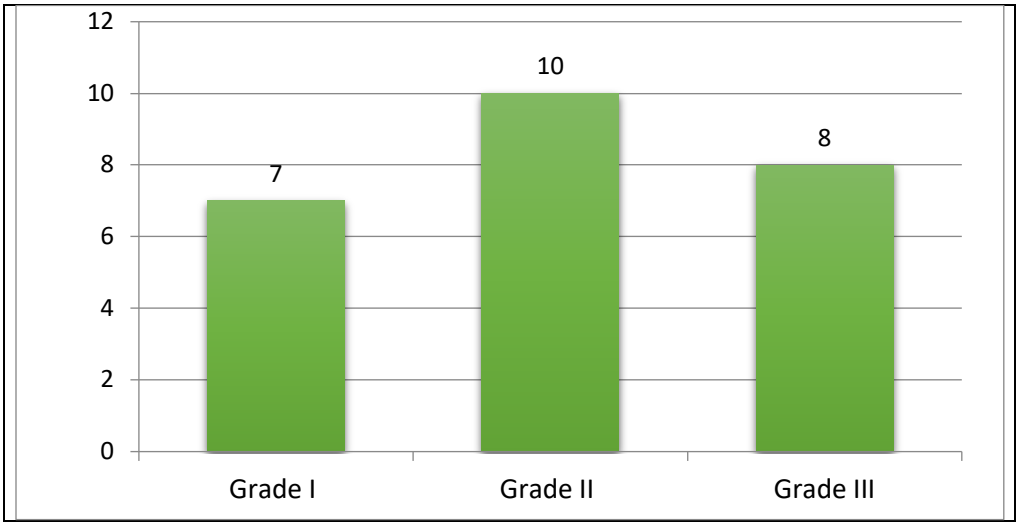

Figure 1: Breast Carcinoma - Histological Grade Wise Distribution of Cases

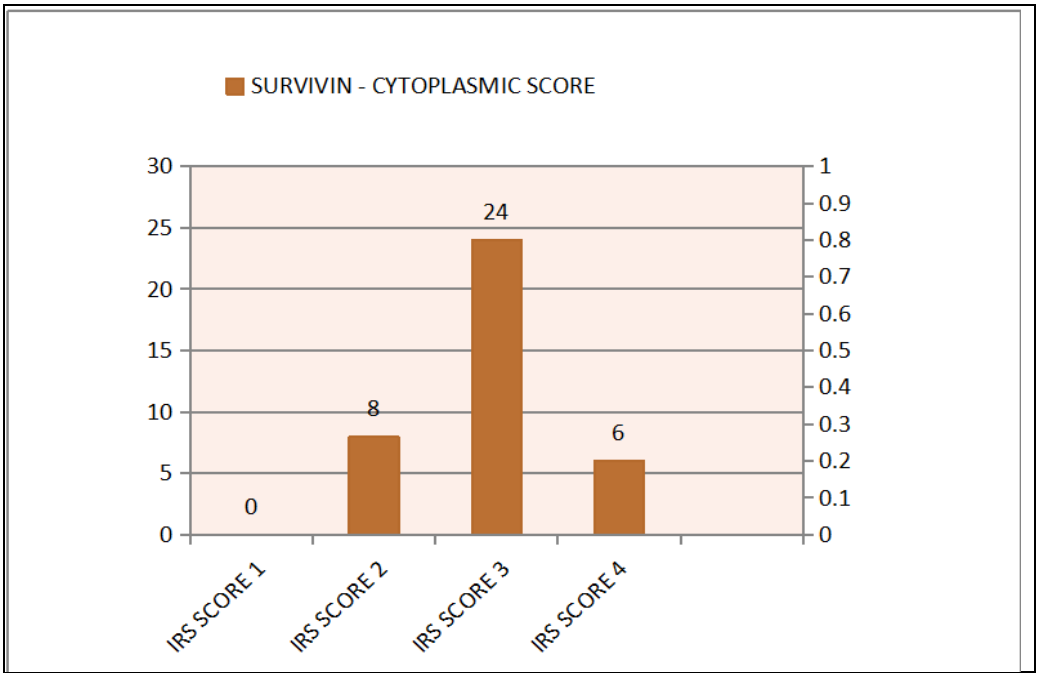

Figure 2: Survivin-Cytoplasmic Immunoreactivity Score (IRS)

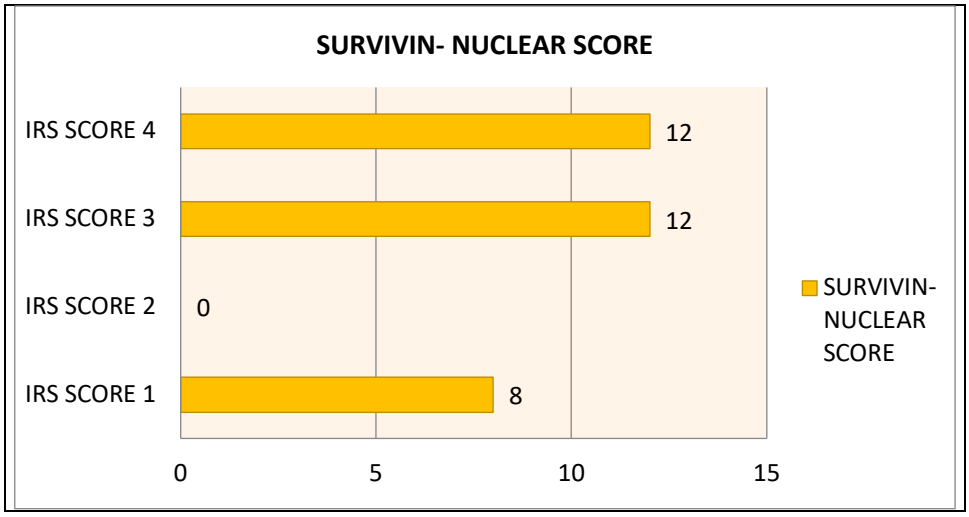

Figure 3: Survivin-Nuclear Immunoreactivity Score (IRS)

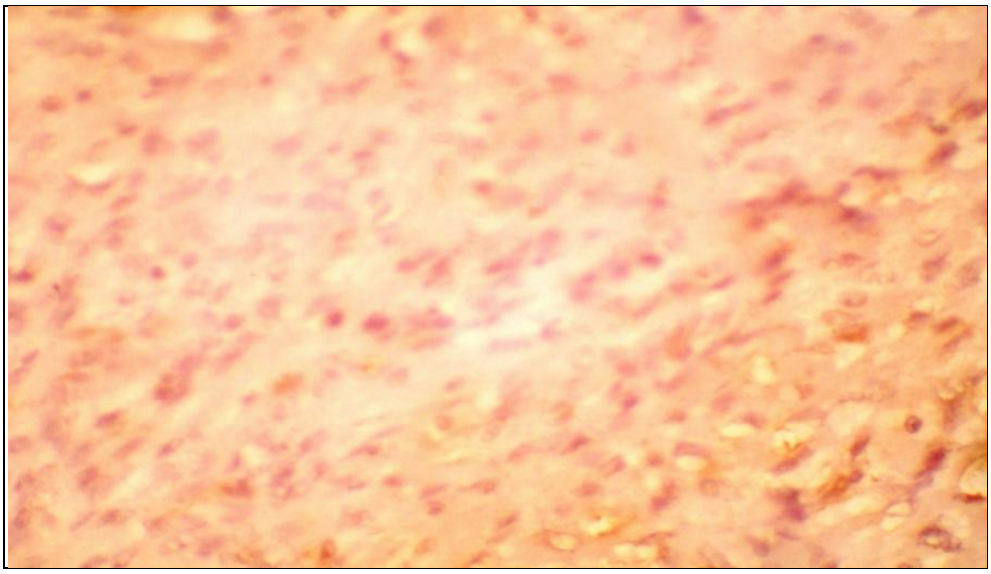

Figure 4: Invasive Ductal Carcinoma (NOS), Histological Grade I, Cells Showing Diffuse Weak Cytoplasmic Survivin Positivity, x 400 Magnification

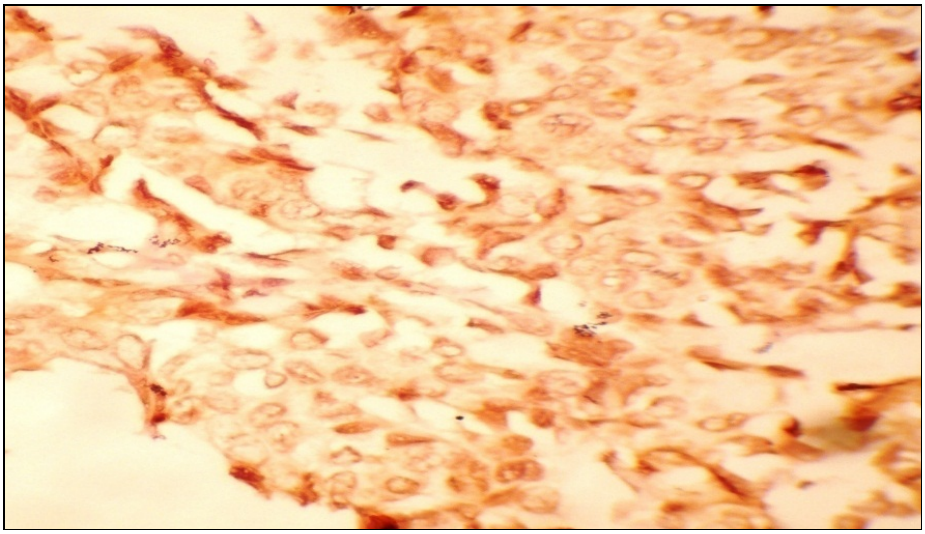

Figure 5: Invasive Ductal Carcinoma (NOS), Histological Grade II, Cells Showing Diffuse Moderate Cytoplasmic and Diffuse Intense Nuclear Survivin Positivity, x400 Magnification

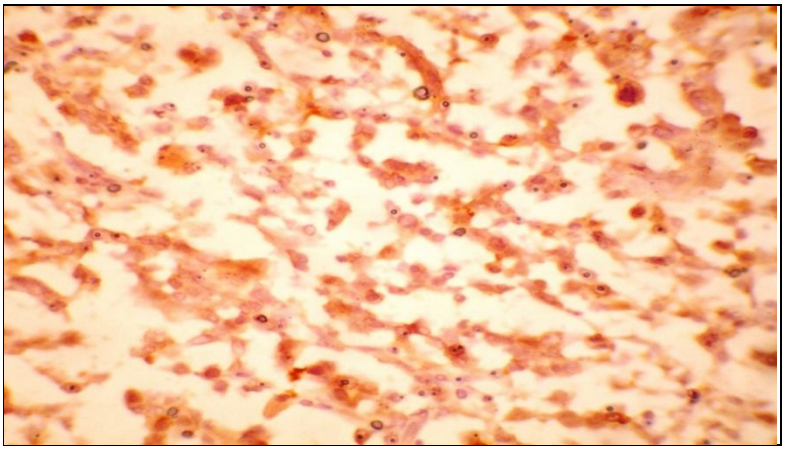

Figure 6: Invasive Ductal Carcinoma (NOS), Histological Grade III, Cells Showing Diffuse Intense Cytoplasmic and Nuclear Survivin Positivity, x 400 Magnification.

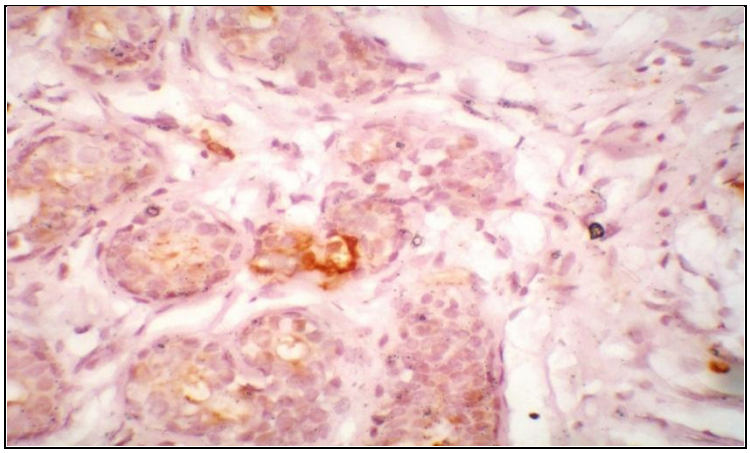

Figure 7: Benign Breast Ductal Cells Showing Diffuse Weak to Moderate Cytoplasmic Positivity, x 400 Magnification.

Discussion

Survivin has been a target of attention in recent years for cancer immunotherapy, as it is an antigen that is expressed mostly in cancer cells and absent in normal cells. This is because Survivin is deemed to be a crucial player in tumour survival. There has been much evidence accumulated over the years that shows Survivin as a strong T-cell-activating antigen and clinical trials have already been initiated to prove its usefulness in the clinic.[5]

Survivin regulates cell division and inhibits apoptosis.[6] It is a member of the Inhibitor of Apoptosis (IAP) family, which has been shown to inhibit activated caspases, the cell death proteases, either directly or indirectly. [7-11] In the majority of cancers studied to date, Survivin is associated with a poor prognosis. Survivin is overexpressed in most human cancers including bladder, blood, colon, liver, brain, lung, pancreas, prostate, and kidney etc.[12-22]

Although most immunohistochemical studies show Survivin predominantly located in the cytoplasm, in some tumours Survivin may have a mainly nuclear cellular location by immunohistochemistry [23], and its expression in the nucleus may be associated with a more favourable outcome (Okada et al., 2001). Clinicopathological investigations on the role of Survivin in breast cancer, focusing on its importance as a prognostic factor have been limited.

Specific staining for Survivin was observed in tumour cells in 176 (60%) tumours by Kennedy, O'Driscoll et al. (2003). Survivin was detected in 260 (68.1%) study cases by Al-Joudi, Iskandar et al. (2007).[24] In our study, immunostaining for Survivin was observed in 10 of 25 (40%) cases of breast carcinoma.

Histological grade III predominated with the highest incidence among the invasive ductal carcinoma of the breast cancer patients (47.1%, n = 80) in the study by Al-Joudi, Iskandar et al. (2007) compared to histological grade II (10/25, 40%) predominance in our study.

One of the studies by Sohn, Kim et al. (2006)[25] showed only nuclear staining, only cytoplasmic staining and both nuclear and cytoplasmic staining in 11.3%, 31.3% and 22.5% of the cases respectively. In a study by Al-Joudi, Iskandar et al. (2007)

nuclear expression of Survivin was detected in 16.5 percent of the study cases, cytoplasmic expression was detected in 24.1%, and 27.5% of the cases expressed Survivin in both nuclear and cytoplasmic locations simultaneously. In comparison with our study, where parallel results are seen for cytoplasmic expression (20%) and nuclear expression (10%) but dual or both cytoplasmic and nuclear staining (70%) prevailed over the others.

In our study, among the grade I cases that were positive for Survivin, 2/3 (67%) showed predominantly weak to moderate cytoplasmic expression. In one case (33%), Survivin was detected with moderate cytoplasmic and weak nuclear staining. Both nuclear and cytoplasmic staining for Survivin was seen in 4/10 (80%) cases of grade II tumours. Survivin expression was moderate to intense cytoplasmic and moderate to intense nuclear in quality in 2/8 (25%) cases of grade III tumours.

The breakdown of the distribution of Survivin in tumours was as follows: 38.7% score 1; 26.3% score 2; 25% score 3; and 10% score 4 (Kennedy, O'Driscoll et al., 2003). As compared to our study, where IRS score 1 = nil, IRS score 2 = 8%, IRS score 3 = 24% and IRS score 4 = 4% were observed in the cytoplasmic Surviving immunoreactivity. Nuclear immunoreactivity scores were IRS score 1 = 8%, IRS score 2 = nil, IRS score 3 = 12% and IRS score 4 = 12%.

Significant correlations were found with the clinicopathological factor, tumour histological grade in our study, similar to the study in hepatocellular carcinomas by Fields, Cotsonis et al. (2004)[26] and in contrast to the studies on breast cancers by Al-Joudi, Iskandar et al. (2007) and Sohn, Kim et al. (2006).

Interestingly, in a few cases, the benign breast ducts adjacent to the tumour, showed Survivin staining in their luminal cytoplasm and also in peritumoural lymphocytes but not in stromal cells. This was similar to the observation by Kennedy, O'Driscoll et al. (2003).

Both cytoplasmic and nuclear expression of Survivin detected by immunohistochemistry was considered an independent prognostic factor for leiomyosarcomas as seen in a study by Helge Taubert et al. (2010).[27] Recently, the export of nuclear Survivin to the cytoplasm could be shown to be causal for the Survivin mediated protection against chemo- or radiotherapy-induced apoptosis. In our study with breast carcinomas, the expression of cytoplasmic and nuclear Survivin was common and this was in resemblance to the study by Sohn, Kim et al. (2006) and Helge Taubert et al. (2010). This protein could be both a useful diagnostic marker and an important source of prognostic information.

In the literature, cytoplasmic expression of Survivin by immunohistochemistry is reported to be a poor prognostic parameter in neuroblastoma[28], laryngeal squamous cell [29] colorectal [30] and urothelial carcinomas.[31] On the other hand, for pancreatic, gastric (Okada, Murai et al., 2001; Ikeguchi, Kaibara et al., 2001), esophageal (Kato et al., 2001) [32] and urothelial (Nakanishi, Tominaga et al., 2002) carcinomas, no association was found between cytoplasmic Survivin and patient survival. The translocation of Survivin from cytoplasmic in the normal to cytoplasmic and nuclear in high-grade dysplasia and squamous cell carcinoma is noted in the esophagus.[33]

The differential nuclear and cytoplasmic localization of Survivin is shown to be due to differences in the amino-acid sequence of its carboxy-terminal domain. In hepatocellular carcinoma, the predominant function of Survivin is its cell cycle nuclear distribution and not its cytoplasmic caspase-3-dependent antiapoptotic effect.

Detection of Survivin by immunohistochemistry enables differentiation between Survivin expression in the two subcellular pools (cytoplasmic and nuclear). Survivin expression in the cytoplasm could be associated with its control function of cell survival (inhibitor of apoptosis) whereas nuclear staining may rather promote cell proliferation. In multivariate analysis done in other studies, the presence of Survivin protein in invasive breast cancers is a strong independent prognostic indicator of 5-year recurrence free survival and overall survival.

Surprisingly, different patterns of Survivin localisation are seen in different tumour types, which may partly explain the different prognostic implications of cytoplasmic and nuclear Survivin. Fortugno et al. (2002),[34] in a study using a novel panel of monoclonal and polyclonal antibodies, have shown that there are different subcellular pools of Survivin. A nuclear pool that segregates with nucleoplasmic proteins was identified. A distinct and predominant cytosolic pool associates with interphase microtubules, centrosomes, spindle poles, and mitotic spindle microtubules at metaphase and anaphase. These two Survivins are immunochemically distinct, independently modulated during cell cycle progression, and only the cytosolic Survivin associates with p34cdc2. Phosphorylation of Survivin by p34cdc2–cyclin B has been identified as a requisite for apoptosis inhibition.

The possible explanation for these findings was that separate post-translational modifications could differentially affect epitope accessibility of nuclear vs. cytosolic microtubule bound Survivin in vivo. If nuclear Survivin cannot associate with p34, an essential step in apoptosis inhibition, it may actually induce apoptosis. This may explain why Survivin may be only effective in blocking apoptosis when located in the cytosol where caspases are predominantly located. Nuclear Survivin must be phosphorylated for binding to processed caspase-9. A non-

phosphorylatable alanine (T34A) mutant of Survivin has been described, which disrupts cell division and induces apoptosis, probably by substrate competition.

Recently, splice variants of Survivin with different antiapoptotic properties have been identified[35,36]. One of these variants, Survivin-2B, has reduced antiapoptotic potential and may act as a naturally occurring antagonist of Survivin. The nuclear form is most common and is an independent prognostic indicator of a good outcome. The intracellular location of Survivin may have an important physiologic role in the cell cycle and have different prognostic implications, as in the case of cyclin D2. The nuclear localisation of cyclin D2 has been reported to have good prognostic value. It is associated with well-differentiated tumours, a lower depth of cancer invasion, fewer lymph node metastases, and less vessel invasion. In contrast, the cytoplasmic location of cyclin D2 is associated with a poor prognosis.

The absence of Survivin in node-negative breast cancer patients may herald a higher risk of relapse and a shorter survival. Further studies on breast cancer, when selective antibodies become available [37], may elucidate the role of Survivin, including its location and possibly antagonistic roles of splice variants in apoptosis inhibition and cell cycle control in breast cancer.

Conclusion

Being a heterogeneous group of tumours, breast cancers vary in morphology, clinical presentation and behaviour. The morbidity and mortality from breast cancer remain high despite significant advances in our understanding and management over the last several decades.

Gene expression profiling studies of individual tumours reveal a gamut of molecular alterations or markers based on which diagnostic classifications and subclassifications are designed. These would help in devising personalised treatment plans. With this standpoint, we embarked on a novel marker, Survivin, the propitious contender in breast cancer prognostication and targeted therapy.

We have showed, through our study, the significance of expression patterns of Survivin, a promising prognosticator and its implications in treatment of these perplexing group of tumours. Our results showed that the expression of Survivin gradually increased with increasing histological grades.

As a result of its widespread expressions in different tumours, and generally low-level expression in normal tissue, Survivin is considered to be the prospective newer adjuvant target for apoptosis-based chemotherapy. There is a need for further studies to be carried out to further investigate the contribution of these proteins to various cancer phenotypes and to be used as a potential molecular target for their treatment.

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