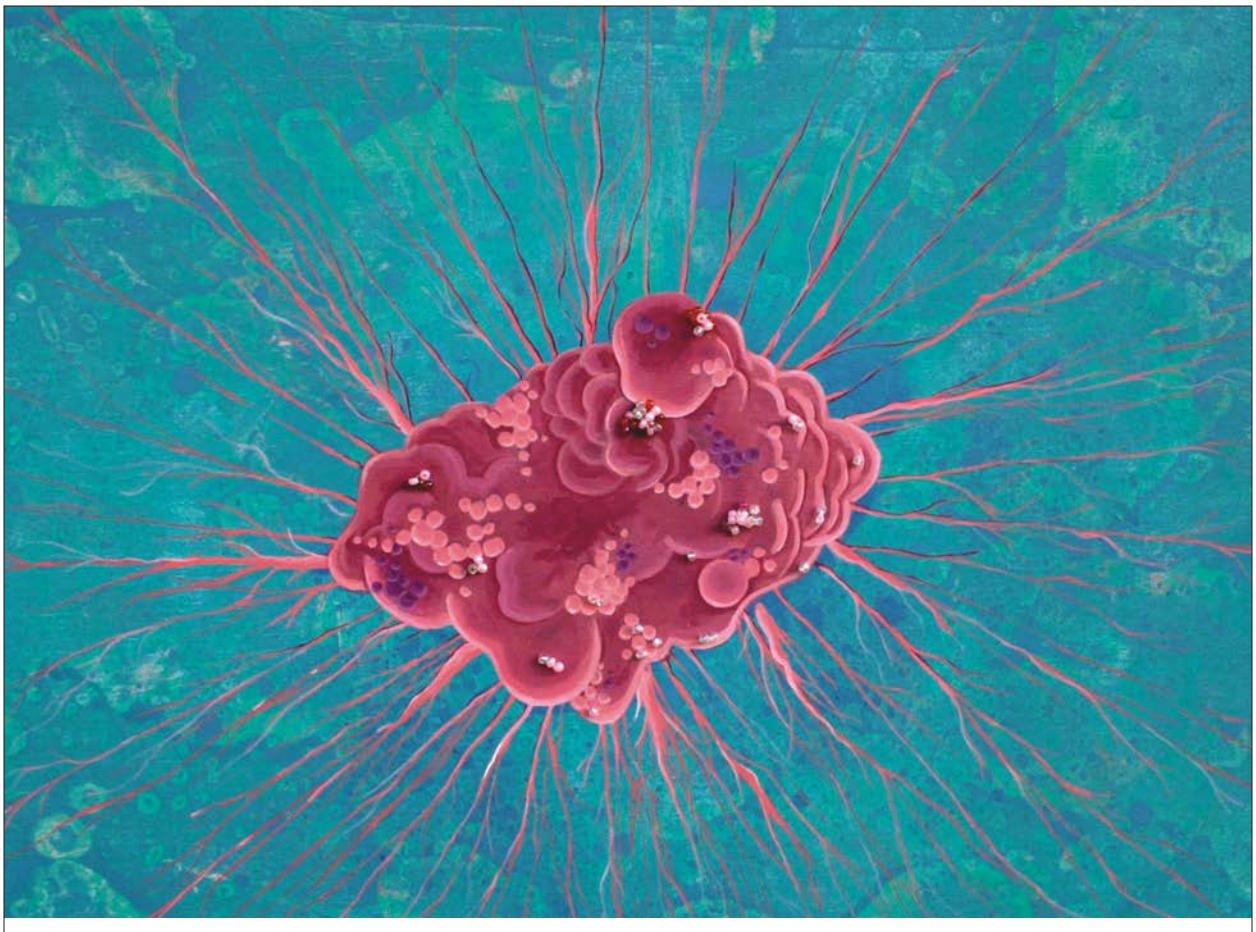

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Research Article
Nuclear and Cytoplasmic Expression of Survivin in Breast Carcinoma: Correlation with Clinicopathological Parameters

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Abstract

Background: Survivin, a member of the inhibitor of apoptotic protein family is involved in cell proliferation and apoptotic suppression. Survivin is highly expressed in many cancers and correlated with more aggressive disease. **Objective:** Assessment of survivin expression in breast carcinoma and its association with clinicopathological factors. **Materials and Methods:** One hundred and eight breast carcinoma and 22 control benign specimens were used for survivin immunohistochemical assessment. Survivin expression was evaluated according to staining intensity and percentage of positive cells. A numerical score was calculated by multiplying them. Cases with scores of ≥1 were considered positive. **Results:** Survivin expression was obviously higher in malignant cases compared to the control cases ($p<0.001$). Among the clinicopathological parameters analyzed, significant correlations were established with the patient's age ($p<0.001$), the size of the tumors ($p = 0.005$) and HER2 status ($p = 0.05$). Cytoplamic staining was detected in all positive cases, either alone (62.0%) or associated with nuclear staining (38.0%). Cytoplasmic staining only was significantly correlated with good prognostic parameters; small sized tumor, grade II, ER-positive and HER2-negative tumors ($p<0.05$). All triple negative cases (100%), 90% of luminal B and 72.2% of HER2 subtype showed survivin positivity, while only 48.7% of luminal A was positive. The association between survivin expression and molecular classification was insignificant ($p = 0.069$). **Conclusion:** Survivin has a potential role in diagnosis of malignancy. Survivin expression is associated with younger age, large tumor size, HER2-positive tumor and triple negative molecular subtype. Cytoplasmic staining was correlated with good prognostic parameters.

Key words: Survivin, immunohistochemistry, breast carcinoma, tumor grade, prognosis, molecular types, cell proliferation, anti-apoptic gene

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among women in 140 of 184 countries worldwide with nearly 1.7 million new cases (11.9%) diagnosed in 2012. This represents about 12% of all new cancer cases and 25% of all cancers in women[1]. Since the 2008 estimates, breast cancer incidence has increased by more than 20%, while mortality has increased by 14%[2]. In the National Cancer Institute (NCI), Cairo University, the largest multidisciplinary cancer center in Egypt (about 500 beds) to which patients are referred from different provinces of Egypt, breast cancer ranked first among cancers in females representing 38.8% of all newly diagnosed cancers and occupied the second rank among cancers of both sexes (15.4%) following liver cancer (23.8%). The median age is 46 years, one decade younger than the corresponding age in Europe and North America. Most patients are premenopausal (60.5%) with a female to male ratio of 44:1[3].

Breast cancer is by far one of the leading causes of cancer death in women all over the world with continuous rise in incidence[4]. The main reasons consist of high tendency to spread at an early stage and acquired resistance to a wide range of anticancer agents[5]. Once the cancer has spread beyond the breast and loco-regional lymph nodes, it is seemed to be incurable. In such cases, chemotherapy or radiotherapy considered to be the main treatment, but accompanied by various adverse effects[6]. This fact emphasizes the importance of selecting sensitive diagnostic and prognostic markers in the early stage and more efficient targeted therapy in order to improve survival outcome of breast cancer patients. Accordingly, a basic standard is to screen those markers which are highly associated with breast cancer progression and prognosis[7]. Expression status of protein which has anti-apoptotic potential is believed to renovate tumor cells resistance to programmed cell death and overexpressions of these proteins lead to chemotherapy resistance and malignantly inclined biologic features of tumor cells.

In this context, survivin is the most extensively studied molecule in the family of the inhibitor of apoptosis protein (IAP). It is a 16.5 kDa protein that is located on chromosome 17q25, which contains a baculovirus inhibitor of apoptosis repeat (BIR) protein domain, via this domain, it inhibits apoptosis either directly or indirectly by interfering with the function of caspases-3, 7 and 9[8]. Survivin also controls the G2/M phase of the cell cycle via combining with mitotic spindle microtubules. Survivin can be expressed as cytoplasmic and nuclear protein in various embryonic tissues as well as most human tumors of the lung, colon, breast, stomach, liver, cervix and prostate[9]. In contrast; it is either undetectable or expressed at a very low level in differentiated adult tissues. Therefore, it has been suggested that survivin could be an indicator for tumor progression and prognosis[4]. Survivin is also found in approximately 50% of high-grade non-Hodgkin's lymphomas but not in low grade ones[10]. The incidence of survivin expression in cancer is reported to be from 30% up to 100%. Despite its role in mitosis, it is clear that overexpression of survivin in cancer does not simply reflect the presence of a higher number of proliferating cells. In melanoma, survivin expression is indistinguishable in cases with low or high mitotic index[11]. In addition, the fact that survivin is typically observed in nearly all tumor cells and not just in the mitotic fraction, suggests that expression of the survivin gene is deregulated in cancer, although still retaining cell-cycle periodicity in mitosis[12]. Survivin is thought to guard tumor cells from the physiological process of cell death and to promote tumor cell proliferation[13]. Cancer patients with tumors expressing survivin exhibited shortened survival, associated with unfavorable markers of disease progression, accelerated rates of recurrence and increased resistance to therapy[14]. The relationship between more aggressive behavior and parameters of poor prognosis in side and survivin expression in the other side has been shown in colorectal and gastric carcinoma and in neuroblastoma[12,15]. Although relationship between survivin expression in breast cancer patients and clinicopathological parameters has been previously reported, controversy still remains and the prognostic value failed to reach consensus[8].

The aim of present study was to assess the association between immunohistochemical expression of survivin in breast cancer specimens and clinicopathological parameters of the studied cases to investigate subcellular localization of survivin and its relation with clinicopatholgical parameters, finally to show survivin expression by different molecular breast carcinoma subtypes using the readily available data regarding ER, PR and HER2 immunohistochemical expression.

MATERIALS AND METHODS

Tissue samples selection: This retrospective study was conducted at the Pathology Department, National Cancer Institute (NCI), Cairo University, between November, 2014 and March, 2015. The present study included 108 specimens from 108 patients presented with breast lump. They had undergone modified radical mastectomy at the Surgical Department, NCI. They did not receive any form of treatment prior to surgery. Specimens of the study were selected from those received in the Pathology Department and were fixed in formalin and

embedded in paraffin. All sections were stained with routine hematoxylin-eosin staining and diagnosed according to the criteria of the World Health Organization (WHO)[5] and graded according to the modified Scarff-bloom and Richardson method[16].

The patient's clinical and histopathological reports were reviewed from the computer-based data to determine age of patients, tumor type, size (greatest dimension), grade, lymph node involvement, Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2 status.

Inclusion criteria:

- Pathologically diagnosed breast carcinoma cases, with available tissue material for immunophenotyping
- Absence of neoadjuvant therapy
- Availability of complete clinico-pathological data

Once the cases have been chosen, the corresponding standard hematoxylin and eosin stained slides as well as immunostained slides of ER, PR, HER2 were retrieved from the archives of the Pathology Department. All slides were reviewed and re-evaluated by pathologists to confirm diagnosis and assure tumor content in the archival paraffin blocks.

The immunohistochemistry (IHC) results for ER, PR and HER2 status were used as surrogate assay for expression profiling, various breast cancer subtypes were detected according to Cheang *et al.*[17] classification:

- Luminal A (ER-positive and/or PR-positive, HER2-negative)
- Luminal B (ER-positive and/or PR-positive, HER2-positive)
- Triple negative/basal-like (ER-negative, PR-negative, HER2-negative)
- HER2 type (ER-negative, PR-negative, HER2-positive)

Immunohistochemical staining: The most representative blocks, containing the main bulk of tumor and the adjacent normal breast tissue were collected and used for IHC staining. One serial section of four microns was prepared from each block and placed on electrostatically charged coated slides. Immunostaining for survivin was done for all cases using BenchMark XT autostainer (a product of Ventana Medical Systems) according to the manufacturer's recommendation. The followingsteps occurred automatically: deparaffinization, cell conditioning (standard conditioning for 80 min), application of 100 µ of ready to use survivin mouse monoclonal anti-human antibody (clone 12C4, DAKO Japan) at a dilution of 1:15 under incubation temperature at 42°C for 32 min, application of DAB, counter stain with hematoxylin for 8 min and post counter stain with bluing reagent for 4 min. Slides were washed in tap water and soap for 5 min, dehydrated in the ascending grades of alcohol for 5 min ineach container, cleared in xylene and then cover slips were applied.

Histologic sections of urinary bladder carcinoma of known positive survivin reactivity were used as positive control and a negative control was used by substituting Phosphate Buffer Saline (PBS) for the primary antibody. All controls yielded appropriate results, thus it makes sure that the procedure was optimized and all reagents were working properly therefore any negative or positive results were valid.

Assessment of immunoreactivity: The stained slides were viewed independently by the two pathologists, who were blinded to the patient's clinicopathological data, using Olympus bright field binocular light microscope. In discrepant cases, a final decision was made based on consensus by the study pathologists. Survivin immunoreactivity was observed in the cytoplasm and/or nucleus of cancer cells. Immunostaining for survivin was recorded according to staining intensity and percentage of cancerous cells that stained positively. The protein expression was quantified in the various samples using scoring method utilized previously[18]. A mean percentage of positive tumor cells was determined and assigned to one of five following categories: 0: <5%, category 1: 5-20%, category 2: 21-50%; category 3: 51-75% category 4 and category 5: >75%. The immunostaining intensity was scored as follows: Weak 1^+, moderate 2^+ and intense 3^+. The percentage of positive cells and the staining intensity were multiplied to produce a weighted score for each case ranging from 0 (<5% positive tumor cells) to 12 (>75% of tumor cells with intense staining). Cases with a weighted survivin score <1 were considered to be negative and those with scores of $\geq$1 were considered positive. For tumors with heterogeneous staining, the predominant pattern was taken into account for scoring. Survivin expression was correlated with the clinicopathological parameters.

Statistical methods: Data management and statistical analysis were performed using Statistical Package for Social Sciences (SPSS) version 21. Numerical data were summarized using means and standard deviations or medians and ranges. Categorical data were summarized as percentages. Comparisons between the 2 groups with respect to normally distributed numeric variables were done using the t-test. For categorical variables, differences were analyzed with χ^2

(chi square) tests and Fisher's exact test when appropriate. All p-values are two-sided. A p-value <0.05 were considered significant.

RESULTS

This retrospective study included 108 female cases with primary breast carcinoma. The detailed clinicopathological characteristics of the studied cases were summarized in Table 1. All the studied cases were female. The mean age was 52.9±11.5 with range between 29 and 76 years. The median age was 48 years. The control group included 22 female with a mean of age 49.1 and range between 30 and 57 years.

Immunohistochemical expression of survivin in the studied cases: Of the 108 studied malignant cases and 22 benign control cases, survivin immunoreactivity was observed in 79 malignant cases (73.1%) and 4 benign control cases (18.2%). Survivin expression was obviously higher in malignant cases compared to the control cases (p<0.001). No expression of survivin was observed in the adjacent normal breast tissue.

The intensity of survivin staining was usually homogenous within a given case and the number of positive tumor cells varied between 10 and 95%. The distributions of weighted survivin score were detected in Table 2.

Relationship between expression of survivin and clinicopathological factors: Table 3 shows the association between expression of survivin and clinicopathological characteristics. Among the clinicopathological parameters analyzed, significant association were established only with the patient's age (p<0.001), the size of the tumors (p = 0.005) and HER2 status (p = 0.05). Thirty six cases (92.3%) of younger patients, 30 cases (90.9%) of the large sized tumors, as well as 43 cases (82.7%) of HER2 positive cases showed positive staining for survivin. The remaining clinicopathological parameters showed no significant relationship with survivin expression (p>0.05). Although the numbers of non-ductal carcinoma cases were small and were considered as statistically unrepresentative samples, 66.7 and 100% of invasive lobular carcinoma and mixed ductal and lobular carcinoma were survivin negative, respectively whereas 78.6% of invasive ductal carcinoma were positive.

Subcellular localization of survivin: It is interesting to note that all the studied malignant cases demonstrated survivin staining in the cytoplasm of the cancer cells either alone (Fig. 1a, b), in 49 out of 79 positive specimens (62.0%) or combined with nuclear staining (Fig. 2a, b) in 30 cases (38.0%). No cases demonstrated nuclear staining only.

There was a statistically significant relationship between the subcellular localization of survivin and tumor size, histological grade, estrogen receptor and HER2 status (p<0.05). It was observed that survivin cytoplasmic staining only was significantly correlated with the small sized tumor, histological grade II, estrogen receptor positive and HER2-negative tumors. The presence of the nuclear staining with the cytoplasm was found to be higher in the larger tumor size, the higher grade, estrogen receptor negative and HER2-positive tumors (Table 4).

The subcellular localization of survivin was independent of the age, histological types, lymph node status and progesterone receptor status (p>0.05). It was observed that 70.7% of lymph node negative cases revealed cytoplasmic

Table 1: Clinicopathological characteristics of the breast carcinoma cases

Clinicopathological characteristics	No.	%
Age		
≤50	39	36.1
>50	69	63.9
Size		
<5 cm	75	69.4
>5 cm	33	30.6
Histologic types		
IDC[1]	98	90.7
ILC[2]	6	5.6
Mixed duct lobular carcinoma	4	3.7
Grade		
II	83	76.9
III	25	23.1
Lymph nodes status		
N0	56	51.9
N1	20	18.5
N2	20	18.5
N3	12	11.1
Estrogen receptor		
Negative	68	63.0
Positive	40	37.0
Progesterone receptor		
Negative	47	43.5
Positive	61	56.5
HER2[3]		
Negative	56	51.9
Positive	52	48.1

[1]IDC: Invasive ductal carcinoma, [2]ILC: Invasive lobular carcinoma and [3]HER2: Human epidermal growth factor receptor

Table 2: Distribution of the weighted survivin scores of the studied cases

Survivin score	No.	%
0	29	26.9
1-3	5	4.6
4-8	24	22.2
9-12	50	46.3
Total	108	100.0

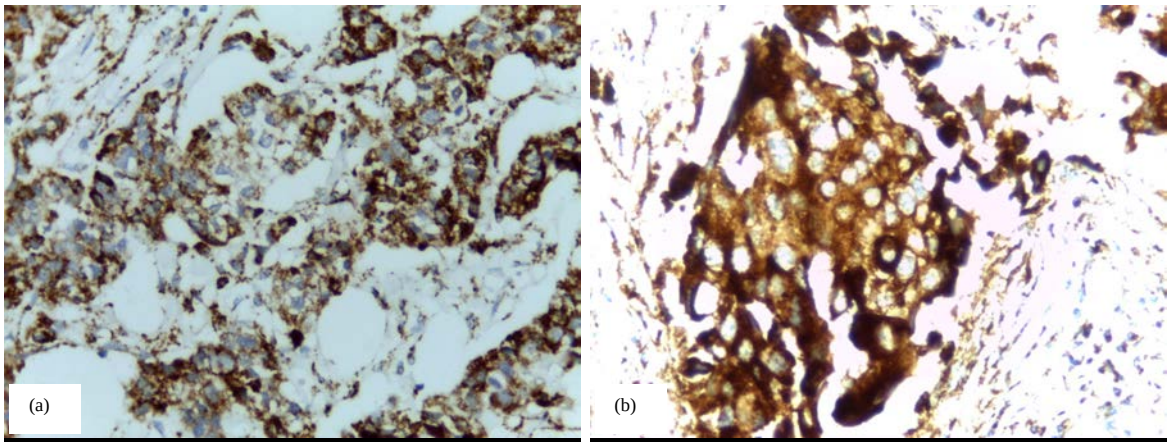

Fig. 1(a-b): Immunohistochemical staining of survivin in paraffin-embedded tissue specimens of invasive duct carcinoma, (a) IDC grade II with cytoplasmic survivin expression (x200) and (b) IDC grade II score (9-12) survivin expression (x400)

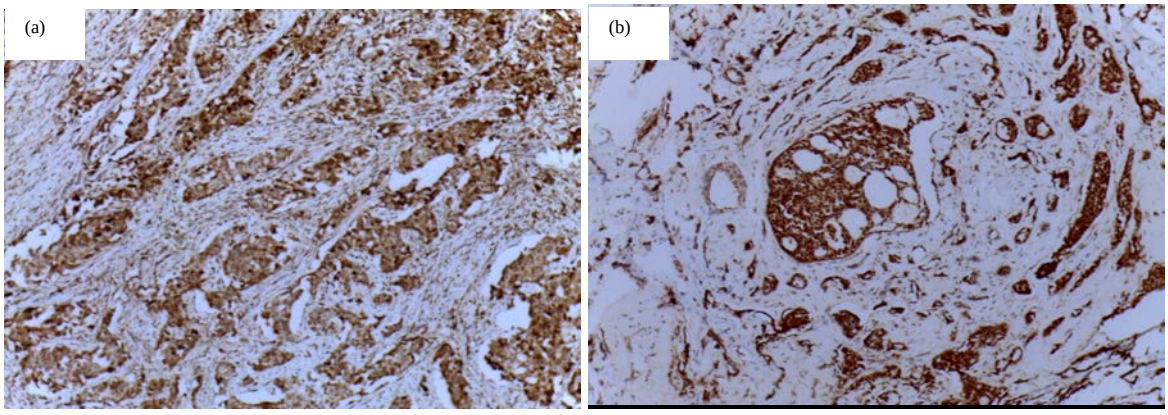

Fig. 2(a-b): (a) IDC grade II with both nuclear and cytoplasmic survivin expression (x100) and (b) Immunohistochemical staining of survivin in paraffin-embedded tissue specimen of IDC grade II with both cytoplasmic and nuclear survivin expression (x200)

staining only in comparison to 52.6% of node positive cases that showed both nuclear and cytoplasmic staining. It was also observed that 71.1% of negative PR status revealed cytoplasmic staining only, while 40.7% of cases showed both nuclear and cytoplasmic staining. Nevertheless, there was no statistically significant correlation between lymph node status or PR status and subcellular localization ($p>0.05$). Similarly, it is also noted that 63.6% of invasive duct carcinoma cases demonstrated survivin positivity in only the cytoplasm of the tumor cells. However, no significant correlation was detected.

Among the control cases, survivin was mainly immunolocalized in the cytoplasm of epithelial cells. However, 25% (1/4) of benign cases showed distinct nuclear expression along with cytoplasmic expression.

Survivin expression by different molecular subtype of breast carcinoma: The survivin expression in different breast cancer molecular subtypes is summarized in Table 5. All triple negative cases (100%), 90% of luminal B and 72.2% of HER2 subtype showed survivin positivity; while only 48.7% of luminal A was positive. On comparing the combined luminal subtypes (Luminal A and luminal B) with the other two subtypes, regarding survivin positivity, the association was considered to be statistically insignificant ($p = 0.069$).

DISCUSSION

Breast cancer is a major cause of morbidity and mortality in women all over the world. Despite the tremendous therapeutic efforts that have been reached, breast cancer is still has a poor outcome[3]. The outcome of breast cancer depends mainly on established clinicopathological factors that can place patients with breast cancer into good and poor prognostic groups. However, it is found that tumors within the

Table 3: Association between clinicopathological characteristics and expression of survivin in the studied 108 cases

Clinicopathological characteristics	No. of studied cases (n = 108)	Survivin IHC: Negative (n = 29) No.	%	Positive (n = 79) No.	%	p-value
Age						
≤50	39	3	7.7	36	92.3	<0.00
>50	69	26	37.7	43	62.3	
Size						
<5 cm	75	26	34.7	49	65.3	0.005
>5 cm	33	3	9.1	30	90.9	
Histologic types						
IDC	98	21	21.4	77	78.6	NA*
ILC	6	4	66.7	2	33.3	
Mixed D and L carcinoma	4	4	100	0	0	
Grade						
II	83	21	25.3	62	74.7	0.608
III	25	8	32	17	68	
Lymph node status						
N0	56	15	26.8	41	73.2	1.000
N1	20	9	45	11	55	
N2	20	5	25	15	75	
N3	12	0	0	12	100	
Estrogen receptor						
Negative	68	17	25	51	75	0.655
Positive	40	12	30	28	70	
Progesterone receptor						
Negative	47	9	19.1	38	80.9	0.129
Positive	61	20	32.8	41	67.2	
HER2						
Negative	56	20	35.7	36	64.3	0.05
Positive	52	9	17.3	43	82.7	

NA*: Not applicable

same group can behave in different manners so the response to treatment can vary. This is because breast cancer is a malignant disease with many factors involved in and affects tumor growth and progression and hence they potentially limit clinicopathological factors prognostic effect[1]. Therefore, it is important to detect other prognostic biomarkers that will predict the prognosis and later targeting them with their respective antagonists[19]. Survivin, a member of the inhibitor of apoptosis protein family is commonly overexpressed in cancer cells but not in normal differentiated tissues[20].

Survivin plays an important role in the initiation and progression of breast cancer but its prognostic and therapeutic relevance has long been a matter of debate[21]. Many anticancer therapies, including radiation therapy and some chemotherapeutic agents, kill cancer cells through apoptosis. By inhibiting apoptosis, survivin may play a role in cancer drug resistance[9]. Recent studies have pointed out the importance of survivin assessment and reporting that decrease survivin expression was found to increase sensitivity to etoposide and 5-fluorouracil, so using survivin antisense oligonucleotide can specifically inhibit the expression of survivin in breast cancer cell line and increase chemotherapy sensitivity[22]. During the last years, many investigations have been made to develop antagonists of survivin as targeted therapy agents, aiming at eliminating tumor cells and sparing normal tissues. The first clinical trial of YM155 (survivin antagonist) showed potential for the management of various breast cancer subtypes regardless of the expression of ER, HER2 and caspase-3[23,24]. To gain further data about the expression of survivin and its role as biologic marker in breast carcinoma, it was investigated survivin protein expression and its correlation with some clinicopathological characteristics.

In the present study, staining for survivin was detected in 73.1% of the studied malignant cases. This results were comparable with previous published data where the survivin detection range was 60-81%[25-27]. The determinant factors for such a wide range may be attributed to differences in number of studied cases, different scoring systems used with different cutoff value, different antibodies used whether poly or monoclonal with variable sensitivity and specificity, different

Table 4: Subcellular localization of surviving staining among the studied 79 positive malignant cases

		Survivin sublocalization				
		Cytoplasmic only (n = 49)		Cytoplasmic and nuclear (n = 30)		
Clinicopathological characteristics	No. of positive cases (n = 79)	No.	%	No.	%	p-value
Age						
≤50	36	21	58.3	15	41.7	0.643
>50	43	28	65.1	15	34.9	
Size						
<5 cm	49	39	79.6	10	20.4	<0.001
>5 cm	30	10	33.3	20	66.7	
Histologic types						
IDC	77	49	63.6	28	35.4	0.141
ILC	2	0	0	2	100	
Grade						
II	62	48	77.4	14	22.6	<0.001
III	17	1	5.9	16	94.1	
Lymph node status						
N0	41	29	70.7	12	29.3	0.111
N1	11	8	72.7	3	27.3	
N2	15	9	60.0	6	40.0	
N3	12	3	25.0	9	75.0	
Estrogen receptor						
Negative	51	24	47.1	27	52.9	0.001
Positive	28	25	89.3	3	10.7	
Progesterone receptor						
Negative	38	27	71.1	11	40.7	0.164
Positive	41	22	53.7	19	46.3	
HER2						
Negative	36	36	100	0	0	<0.001
Positive	43	13	30.2	30	69.8	

Table 5: Survivin expression in different molecular subtype of breast carcinoma

		Survivin staining		Subcellular localization	
Molecular subtype	No.	Negative	Positive	C**	C and N*
Luminal A					
(ER-positive and/or PR-positive, HER2-negative)	39	20 (51.3%)	19 (48.7%)	19	0
Luminal B					
(ER-positive and/or PR-positive, HER2-positive)	30	3 (10%)	27 (90%)	7	20
Triple negative/basal-like					
(ER-negative, PR-negative, HER2-negative)	17	0	17 (100%)	17	0
HER2 type					
(ER-negative, PR-negative, HER2-positive)	22	6 (27.3%)	16 (72.7%)	6	10
Total	108	29	79	49	30

*C and N: Cytoplasmic and nuclear staining, **C: Cytoplasmic staining only

methods of quantitative assessment (subjective assessment versus computer-assisted image analysis) or may be related to patient's heterogeneity. Survivin expression was detected in 18.2% of our benign control cases. The adjacent normal tissue did not express survivin. Similar results were reported in a previous report where 12.8% of their benign diseases expressed survivin and no expression was detected in normal tissue[18]. Higher expressions 42.7 and 41.3% were reported by Zhang *et al.*[28] and Jha *et al.*[24] with no expression was detected in their normal tissue. They explained the survivin expression in benign cases by the relation to the dysplastic transformation of the breast epithelium. In the present study, survivin expression was significantly higher in breast cancer cases as compared to control cases (p<0.001). These findings were in agreement with that of others[27,29,30]. Thus survivin can be considered as diagnostic marker or at least can use to confirm malignancy as well as it can be used as suitable target for tumor therapy.

It was next focused on the correlation between survivin expression and clinicopathological parameters. In the present

study, significant correlations were established only with the patient's age ($p<0.001$), the size of the tumors ($p = 0.005$) and HER2 status ($p = 0.05$). Its expression was significantly higher in younger age, larger tumor size and HER2-positive tumors. This study revealed no correlation with histological grade, lymph node, ER and PR status.

Similar to this findings, a statistically significant relationship with large tumor size was previously reported[26,28,30]. But they also concluded significant relations with nodal status in their studies. This results did not support their concept about significant relation between measuring survivin expression and predicting individual nodal metastatic behavior. Furthermore, this results are incompatible with others who observed no significant association with age of the patients[26,31,32] but they confirmed a correlation with histologic types[31]. Others reported a significant relation with younger age, histologic grade, ductal type and negative hormonal receptor status on the molecular level[33].

Hormonal receptors as well as HER2 status are widely accepted as prognostic and predictive indicators for breast cancer. When it was related their status with survivin expression, this study reported significant correlation with HER2 status and lack correlation with ER and PR. These were in line with other studies[31,32,33]. Youssef *et al.*[9] confirmed the opposite, no association with HER2 with a significant correlation with ER and PR. Athanassiadou *et al.*[25] and Jha *et al.*[24] showed significant correlation of survivin protein expression with the three statuses. Others observed a significant correlation with PR status but the expression was independent of ER status[34].

In an earlier study, survivin protein expression measuring by enzyme-linked immunosorbent assay (ELISA) in primary breast cancer tissue was significantly correlated with all studied clinicopathological prognostic parameters examined in their studies[33]. Others have shown no significant correlation with all parameters[25,27,34]. This results were consistent with Nassar *et al.*[20] who failed to confirm a correlation with tumor grade. On the other hand, many studies had demonstrated significant correlation with increase histological grades[18,29,33,35]. Others observed slight decrease in expression of survivin as the histological grade increased[30]. They tried to explain this contrary result by the up-regulation of proapoptotic mechanisms in high grade tumors that may overcome survivin's inhibitory properties and could reduce its expression slightly. However, the exact mechanism for this phenomenon is still unknown. In a previous study, researchers reported that survivin has been proven to regulate metastatic behavior in both mouse model and human by activating various signal pathways. Survivin has been induced angiogenesis by interaction with vascular endothelial growth factor, angiopoietin and basic fibroblastic growth factor. Thus tumors expressing high level of survivin might be have prominent lymph node and distant metastasis[9].

By IHC analysis, survivin is localized in two subcellular locations (cytoplasmic and nuclear). This is related to its function in the regulation of either cell viability or cell division. One possibility is that the nuclear localization of survivin is involved in cell mitosis whereas the cytoplasmic location participates in controlling apoptosis[36]. A second explanation for subcellular localization suggests that there are different splice variants of survivin with different antiapoptotic properties which may differ in their localization. Survivin-2B variant has reduced antiapoptotic potential of other variants, like survivin-ΔEx3 and survivin-2α and may act as a naturally occurring antagonist of survivin. The anti-survivin antibodies recognize them all due to the existence of an identical amino terminal peptide in all survivin variants[12]. These may explain why different localization are seen in different tumor and may partly explain the different prognostic effect of cytoplasmic and nuclear survivin. A third explanation was reported by Skagias *et al.*[37] in their study on urothelial carcinoma cases. They concluded that cytoplasmic stain was generated as a result of using polyclonal antibodies. By using monoclonal antibody, the cytoplasmic staining of their studied cases was focal and weak and not correlated with the clinicoppathological variable.

In the contrary to this explanation, in the present study, using survivin monoclonal antibody revealed prominent cytoplamic staining in all positive cases either alone in 62.0% or associated with nuclear staining in 38.0% of cases. These results suggested that the survivin protein is mainly localized in the cytoplasm rather than in the nucleus. This study results confirmed previous other's similar observations[20,38,39]. On the other side, some authors concluded that the staining pattern of survivin in breast cancer was predominantly nuclear[19,40] or predominantly nuclear and cytoplasmic together[18,25].

In the present investigation, it was later investigated the relationship between subcellular localization of survivin and clinicopathological parameters. It was observed that survivin only cytoplasmic staining was significantly correlated with good prognostic factors; the small sized tumor, histological grade II, ER-positive and HER2-negative tumors. Whereas the presence of the nuclear staining in association with the cytoplasm was found to be higher in the larger tumor size, the higher grade, ER-negative and HER2-positive tumors. Present reports in this study area, however were inconsistent and proposed opposing conclusions. Nearly similar observations were established in other types of carcinoma where nuclear

survivin localization was associated with poor prognosis in non-small-cell lung cancer[41] and esophageal squamous cell cancer[42]. Certain previous contradictory studies have shown that the nuclear staining of survivin is associated with a favorable prognosis in breast carcinoma[9,40], osteosarcoma[7] and gastric carcinoma[14]. At variance to our report, Athanassiadou *et al.*[25] failed to find any relation between positivity rates of cytoplasmic or nuclear staining with all clinicopathological parameters analyzed. Others showed a trend towards association between cytoplasmic staining and bad prognosis but this was not statistically significant[43]. Kim and Hong[15] on their study on intracellular localization of survivin in cervical squamous cell lesion concluded that cytoplasmic survivin may be important for malignant progression and suggested that the inhibition of the cytoplasmic localization of survivin may present a novel strategy for cervical cancer treatment. However, the reason for these different prognostic significance in the subcellular localization of survivin in different cancers remains unclear[8,15].

In an order to quantify subcellular localization of survivin, others previously applied automated quantitative algorithms using automated image analyzer to analyze survivin IHC data and demonstrated that a high cytoplasmic-to-nuclear ratio (CNR) of 5 was associated with low grade, hormone receptor positivity and improved survival[44], confirming this results between nuclear survivin and poor outcome and support an important role for nuclear-cytoplasmic transport of survivin in tumourigenesis and disease progression. Nucleo-cytoplasmic shuttling of survivin is controlled by an active and evolutionarily conserved Crm1-dependent Nuclear Export Signal (NES). Inhibition of this signal cancels the anti-apoptotic effect of survivin, while maintaining its mitotic effect activity, suggesting that increased levels of nuclear survivin could lead to a proliferative aggressive phenotype[45].

Among our control benign cases, one positive case showed both nuclear and cytoplasmic staining of survivin; whereas in the remaining 3 cases, cytoplasmic staining was observed. In a previous follow up study ranging from 4-5 years, researchers reported that survivin expression in benign breast tumor was unlikely to be indicative of progression of malignant transformation whether nuclear or cytoplasmic[27].

Molecular classification of breast cancer is an important factor to detect prognosis and clinical outcome[46]. In a previous study studying the 5 and 10 year survival of 4046 invasive breast cancers using IHC molecular classification, HER2 type had the worst outcome while the luminal subtypes had the best prognosis[17]. Triple negative subtype was also proved to have poor prognosis[43,47]. In the present study, it was assessed whether survivin expression was related to the molecular breast subtypes of breast carcinoma. To our Knowledge, few researches have investigated survivin protein expression among different molecular breast carcinoma subtype[9,48]. It was depend on criteria for molecular classification that were defined by Cheang *et al.*[17].

Among the examined cases, luminal A was the most common subtype (36.1%) followed by luminal B (27.8%). The HER2 and triple negative subtype represented 20.4 and 15.7%, respectively. All triple negative cases (100%), 90% of luminal B and 72.2% of HER2 subtype showed survivin positivity; while only 48.7% of luminal A was positive. When this findings were viewed in the context of Cheang *et al.*[17] results, it was found that luminal A subtype, that had the best 5 and 10 year, breast cancer survival outcome showed the lowest survivin positivity (48.7%), while triple negative subtype that had poor outcome showed 100% positivity. These results can be also confirmed that survivin is associated with poor prognosis. The reason for the higher expression in luminal B compared to HER2 might be attributed to the relatively small number of studied cases. Further studies with larger number of cases are required to exactly determined such association. Youssef *et al.*[9] reported an obvious trend of increasing the percentage of cases expressing survivin among luminal A, luminal B, HER2 and triple negative subtype, 64, 78.3, 100 and 100%, respectively. They concluded that survivin is a marker of an aggressive behavior in breast cancer due to low survivin expression in luminal profile tumors and positivity in all HER2 and triple negative cases.

In this study on comparing the combined luminal subtypes (Luminal A and luminal B) with the other two subtypes regarding survivin positivity, the association was considered to be statistically insignificant ($p = 0.069$). This result was incontrast to that of Youssef *et al.*[9], where they reported a significant correlation ($p = 0.01$).

CONCLUSION

Survivin is frequently overexpressed in breast carcinoma as compared to benign lesions, suggests that it has a potential role in diagnosis of malignancy especially in suspicious cases. Its expression is significantly associated with parameters of poor prognosis as younger age, large tumor size, HER2 positive tumor and triple negative molecular subtype. Cytoplasmic only survivin staining is the most frequent intracellular localization and is correlated with good prognostic parameters. Therefore, subcellular localization of survivin has different prognostic implications and it must be precisely clarified in the report to accurately define prognosis.

SIGNIFICANT STATEMENTS

Since the 2008 estimates, breast cancer incidence has increased by more than 20%, while mortality has increased by 14%. Survivin is the most studied molecule in the family of the inhibitor of apoptosis protein (IAP). In the present study, it was assess the correlation between survivin expression and the clinicopathological parameters of the studied cases as well as survivin expression in each subtype of breast cancer using the readily available data regarding ER, PR and HER2 immunohistochemical expression.

Determination of new effective low cost and noninvasive biomarkers may be more valuable for the early diagnosis, prognosis and staging of the disease and can support clinicians in their daily routine. However, analyses tools need to be standardized and simplified in order to be useful, reliable and widely available.

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