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Research Article

Prognostic Significance of Survivin and Livin Expression in the Primary Breast Cancer and Their Lymph Node Metastases

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ABSTRACT

Aim: To assess the prognostic significance of Survivin and Livin expression in invasive breast cancer and their lymph node metastases.

Materials and Methods: The present series consists of archival samples from 78 women with invasive breast cancer diagnosed and treated during 2010-2014 at National Cancer Institute, Misurata, Libya. Tumor biopsies were analysed for expression of Survivin and Livin by immunohistochemistry, and different grading systems were tested for their expression.

Results: In the cancer samples, a significant correlation was established between Survivin expression and site of the tumor (p=0.021), tumor recurrence (p=0.036), and unifocal tumor (p=0.001). Moreover, Her-2 negative tumors had higher Survivin expression than Her-2 positive tumors (p= 0.047). There were no associations between Survivin expression and histological grade, histological type, lymph node status, tumor stage, TNM classification, estrogen and progesterone receptors, distant metastases, chemotherapy, radiotherapy, hormone replacement, vascular invasion, surgical margin, positive family history. Livin expression in primary breast cancer showed a significant correlation (p=0.025) with positive family history, but no significant association with other clinicopathological parameters. In addition, we found that primary tumors showed higher Survivin expression (82%) compared with the lymph node metastases (34%), whereas Livin expression did not differ between the primary (71%) tumors and their metastases (84%).

Conclusion: Survivin expression in primary breast cancer is significantly associated with several characteristics of favourable prognosis. Livin expression in primary breast cancer is significantly associated only with a positive family history of breast cancer.

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Introduction

Breast carcinoma is the most common malignant tumor and the leading cause of cancer mortality among women, with more than 1000000 annual new cases occurring worldwide [1]. During the past two decades, marked progress has been made in defining some of the critical processes associated with the development and progression of breast cancer. It is now generally accepted that malignant transformation involves genetic and epigenetic changes that derail common regulatory mechanisms and result in uncontrolled cellular proliferation and/or aberrant programmed cell death or apoptosis [2]. The most common molecular alterations include: i) growth receptor overexpression (such as *HER2*/*neu* amplification in 20-25% of cases, *EGFR* overexpression in 3%, *FGFR1* or *FGFR2* overexpression in 10-12%); ii) growth factor overexpression (such as *FGF1*/*FGF4* in 20-30%); iii) alterations in intracellular signaling molecules: *HRAS* mutation in 5-10%); cell cycle regulator alterations (such as *TP53* mutation in 20-60%, *RB* inactivation in 20%, *CCND1* gene amplification in 13-21%); adhesion molecule alterations (such as reduced expression of E-cadherin in 60-70%, reduced expression of P-cadherin in 30%, over-expression of cathepsin D in 20-24%); and others (such as c-*myc* amplification in (20%) [3, 4]. Several prognostic markers including clinical stage, histologic grade, estrogen receptor (ER)/progesterone receptor (PR) status, human epidermal growth factor receptor-2 (Her-2), and the Ki67 proliferation index have already been identified and validated [5-12]. Apoptosis (programmed cell death) has been proposed to play a role not only in cancer onset and progression but also in sustaining decreased tumor cell sensitivity to chemotherapy which represents one of the main prognostic indicators in these cancers [13-15].

Recently, novel proteins which suppress apoptosis through caspase-dependent and caspase-independent mechanisms have been characterized, collectively defined as inhibitors of apoptosis (IAPs) [16]. Until now, 8 human IAPs have been recognized: Survivin, Livin, IAP1, c-IAP2, NAIP, XIAP, c-BRUCE, and ILP-2 [17, 18]. Survivin (encoded by baculoviral inhibitor of apoptosis repeat-containing 5 [*BIRC5*]) is one of the IAPs [19]. It is being involved in inhibition of apoptosis and mitosis regulation in malignant cells. It is over-expressed in a wide range of human tumors such as prostatic, pancreatic, lung, ovarian, and breast cancers [20, 21]. It is also an important molecular prognostic marker in many cancers and a target of cancer therapies [22-24].

In breast cancer, Survivin and its splice variants are found to be associated with an aggressive tumor behaviour [25]. *livin* is identified as an anti-apoptotic gene, connected with the death receptor signaling complexes, where it suppresses the activation of caspases. The latter are responsible for apoptosis and protect cells from different pro-apoptotic stimuli [18]. Cell proliferation, invasion, and Livin are associated with the stimulation of motility, inhibition of apoptosis in human cancer cells [26-29]. In several human cancers, Livin expression is augmented and correlated with cancer progression [16, 30-34]. In the present study, we assessed the expression of Survivin and Livin proteins in primary invasive breast cancer and their lymph node metastases, correlating Survivin and Livin expression patterns with several clinicopathological variables.

Materials & Methods

I Materials

The material includes archival samples of 78 Libyan women with invasive breast cancer diagnosed during 2010-2014 at the Department of Pathology, National Cancer Institute of Libya. All analyses were made of representative paraffin blocks available at the department archives. All relevant clinical and histopathological data of the patients were collected from the patients' records and summarized in (Table 1). All patients have been prospectively followed up until death or when last seen alive on their clinical visit, with a mean follow-up time of 26 months (ranges 2-70).

Table 1: The key clinicopathological characteristics of the patients.

Characteristic	Number of patients (%)
Age (years)	
<49 years	46(59)
≥49 years	32(41)
Site of tumor	
Rt.	43(55)
Lt.	35(45)
Histological type	
Invasive ductal carcinoma (IDC)	69(89)
Mucinous carcinoma	3 (4)
Invasive lobular carcinoma (ILC)	3(4)
Papillary carcinoma	1(1)
Secretory carcinoma	1(1)
Cribriform carcinoma	1(1)
Histological Grades	
G1	13(17)
G2	48(61)
G3	17(22)

Stage	
I	6(8)
IIA	23(29)
IIB	13(17)
III	1(1)
IIIA	18(23)
IIIB	1(1)
IV	16(21)
Primary tumor:	
T1	6(8)
T2	44(56)
T3	28(36)
Lymph node involvement	
No	35(45)
Yes	43(55)
Distance metastasis	
No	62(79)
Yes	16(21)
Chemotherapy	
No	7(9)
Yes	71(91)
Radiotherapy	
No	26(33)
Yes	52(67)
Hormone replacement therapy	
No	29(37)
Yes	49(63)
Recurrence	
No	70(90)
Yes	6(8)
Estrogen	
No	32(41)
Yes	45(58)
Progesterone	
No	31(40)
Yes	46(59)
Her-2	
No	59(76)
Yes	18(23)
Multifocality	
No	70(90)
Yes	8(10)
Free surgical margin	
No	16(21)
Yes	20(26)

Vascular invasion	
No	58(74)
Yes	20(26)
Positive family history	
No	74(95)
yes	4(5)

II Methods

i Livin and Survivin Immunohistochemistry (IHC) Staining

IHC staining was performed using an automated system (BenchMark XT; Ventana Medical System, Inc., Tucson, AZ, USA). This fully automated processing system for code-labeled slides includes baking of the deparaffinization, antigen retrieval in the cell slides, solvent-free conditioning buffer CC1 (Mild: 36 minutes conditioning and standard: 60 minutes conditioning), incubation with rabbit polyclonal anti-Livin and anti-Survivin antibody, 2.0 ml ready-to-use from Spring Bioscience at a dilution of 1:100 for 30 minutes, at 37°C (Survivin, Catalog No: abx 11576, Livin, Catalog abx 48503, USA), as well as application of ultra-view^{TM} universal DAB inhibitor, ultra-view universal DAB chromogen, ultra-view universal DAB H_2O_2, ultra-view universal DAB copper and ultra-view universal HRP multimer. Counterstaining with hematoxylin II (C00758) was performed for 4 minutes, followed by post-counterstaining with bluing reagent (B11129) for 4 minutes. After staining, the sections were dehydrated in ethanol, cleared in xylene and covered with Mountex and coverslips.

ii Evaluation of Livin and Survivin Staining

IHC staining of both markers was evaluated using a regular light microscope at the magnification of x40, blinded by the clinical information and other tumor characteristics. Nuclear and cytoplasmic staining were evaluated separately. Three different grading (A, B, C) systems were applied to assess the patterns of Livin and Survivin expression in tumor cells. In system A, the staining was graded into four categories: 0, no expression (no detectable staining); 1, weak staining; 2, moderate staining; and 3, strong staining intensity. In system B, staining was graded in two categories: 1, no/weak expression; and 2, moderate/strong expression. Finally, in system C, Livin and Survivin expression were categorized simply as negative or positive. In calculating the staining indexes, cytoplasmic and nuclear index, the intensity of staining and the fraction of positively stained cells were taken into account using the following formula: $I = 0 \times f0 + 1 \times f1 + 2 \times f2 + 3 \times f3$ Where 'I' is the staining index and f0-f3 are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, the index could vary between 0 and 3 [35, 36].

iii Statistical Analysis

SPSS for Windows SPSS 19.0.1 (IBM, NY, USA) was used for statistical analysis. Frequency tables were analysed using the Chi-square test, with Fisher's exact test (where appropriate), or likelihood ratio (LR) statistics to assess the significance between categorical variables. Differences in the means of continuous variables were analysed using ANOVA (analysis of variance) or nonparametric tests (Mann-Whitney, Kruskal-Wallis) tests. Kaplan-Meier analysis was used in evaluating patient survival, with a log-rank test in comparison between the strata. Reported p-values are from two-sided tests, and in all analyses $p<0.05$ was regarded as statistically significant.

Results

I Expression Patterns of Survivin and Livin

The expression pattern of Survivin and Livin in primary cancer and their lymph node metastases was predominantly cytoplasmic, with few cases showing any nuclear expression, as illustrated in (Figures 1A-1C).

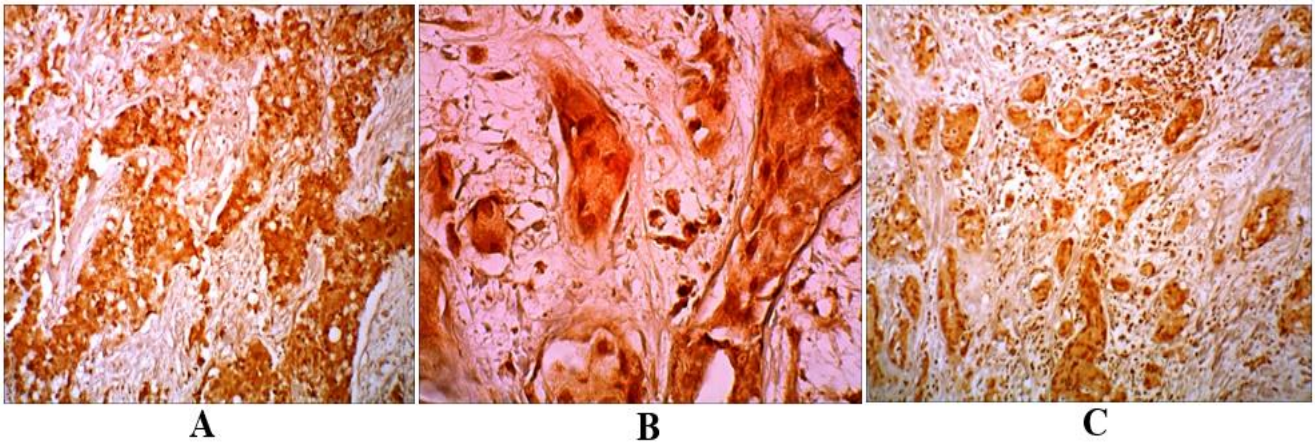

Figure 1: A) Moderate Survivin expression in breast cancer (x40 magnification). **B)** Strong Livin expression in breast cancer (x40 magnification). **C)** Moderate Livin expression in breast cancer (x40 magnification).

II Correlation of Survivin and Livin Expression with Clinicopathological Characteristics

The distribution of Survivin and Livin in the primary tumor as related to clinicopathological characteristics is presented in (Tables 2 & 3). Survivin expression in primary breast cancer showed a significant correlation with the site of the tumor (p=0.021) in that the right-side tumors had a higher expression than left side tumors. Survivin expression in the primary tumors was significantly (p=0.036) higher in patients without disease recurrence during the follow-up period. Survivin expression was also significantly (p=0.001) associated with unifocal tumors more than multifocal tumors. Moreover, Her-2 negative tumors expressed Survivin more than Her-2 positive tumors (P=0.047). There was no statistically significant association of Survivin expression with the histological grade, histological type, lymph node status, tumor stage, TNM classification, estrogen and progesterone receptors, distant metastases, chemotherapy, radiotherapy, hormone replacement therapy, vascular invasion, free surgical margins or positive family history of breast cancer (Table 2).

Table 2: Correlation between Survivin expression and clinicopathological features of the primary tumors.

Features	**Number of cases (%)**	**Survivin-expression in the primary tumors**				
		Negative (0)	Weak (+1)	Moderate (+2)	Strong (+3)	***P-value***
Age (years)						***0.650***
< 49 years	46(59%)	13(28%)	24(52%)	8(18%)	1(2%)	
≥ 49 years	32(41%)	12(37.5%)	16(50%)	4(12.5%)	0(0%)	
Site of tumor						**0.021**
Right	43(55%)	9(20.9%)	25(58.1%)	9(20.9%)	0(0%)	
Left	35(45%)	16(45.7%)	10(28.6%)	9(25.7%)	0(0%)	
Histological Type						***0.611***
Invasive ductal carcinoma	69(89%)	21(30.4%)	31(44.9%)	0(0%)	0(0%)	
Mucinous carcinoma	3(4%)	2(66.7%)	1(33.3)	0(0%)	0(0%)	
Invasive lobular carcinoma	3(4%)	1(33.3%)	2(66.7%)	0(0%)	0(0%)	
Papillary carcinoma	1(1%)	1(100%)	0(0%)	0(0%)	0(0%)	
Secretory carcinoma	1(1%)	0(0%)	1(100%)	0(0%)	0(0%)	
Cribriform carcinoma	1(1%)	0(0%)	0(0%)	1(100%)	0(0%)	
Histological Grades						**0.431**
G1	13(17%)	7(53.8%)	4(30.8%)	2(15.4%)	0(0%)	
G2	48(61%)	12(25%)	24(50%)	12(25%)	0(0%)	
G3	17(22%)	6(35.3%)	7(41.2%)	4(23.5%)	0(0%)	
Stage						**0.892**
I	6(8%)	3(50%)	1(16.7%)	2(33.3%)	0(0%)	
IIA	23(29%)	7(30.4%)	10(43.5%)	6(26.1%)	0(0%)	
IIB	13(17%)	3(23.1%)	7(53.8%)	3(23.1%)	0(0%)	
III	1(1%)	0(0%)	1(100%)	0(0%)	0(0%)	
IIIA	18(23%)	5(27.8%)	10(55.6%)	3(16.7%)	0(0%)	
IIIB	1(1%)	1(100%)	0(0%)	0(0%)	0(0%)	
IV	16(21%)	6(37.5%)	6(37.5%)	4(25%)	0(0%)	
Primary Tumor						**0.742**
T1	6(8%)	3(50%)	1(16.7%)	2(33.3%)		
T2	44(56%)	12(27%)	23(52.5%)	9(20.5%)		
T3	28(36%)	9(32%)	11(39%	8(33%)		
Lymph node involvement						**0.589**
No	35(45%)	11(31.4%)	14(40%)	10(28.6%)	0(0%)	
yes	43(55%)	14(32.6%)	21(48.8%)	8(18.6%)	0(0%)	
Distance Metastases						**0.771**
No	62(79%)	19(30.6%)	29(46.8%)	14(22.6%)	0(0%)	
yes	16(21%)	6(37.5%)	6(37.5%)	4(25%)		
Chemotherapy						**0.200**
No	7(9%)	4(57.1%)	1(14.3%)	2(28.6%)	0(0%)	
Yes	71(91%)	21(29.6%)	34(47.9%)	16(22.5%)	0(0%)	
Radiotherapy						**0.584**
No	26(33%)	9(34.6%)	13(50%)	4(15.4%)	0(0%)	
yes	52(67%)	16(30.8%)	22(42.3%)	14(26.9%)	0(0%)	

Recurrence						**0.036**
No	70(90%)	21(30%)	34(48%)	15(21.4%)	0(0%)	
yes	6(8%)	3(50%)	0(0%)	3(50%)	0(0%)	
Estrogen						**0.357**
No	32(41%)	10(31.3%)	12(37.5%)	10(31.3%)	0(0%)	
yes	45(58%)	14(31.1%)	23(51.1%)	8(17.8%)	0(0%)	
Progesterone						**0.554**
No	31(40%)	10(32.3%)	12(38.7%)	9(29%)	0(0%)	
yes	46(59%)	14(30.4%)	23(50%)	9(19.6%)	0(0%)	
Her-2						**0.047**
No	59(76%)	17(28.8%)	31(52.5%)	11(18.6%)	0(0%)	
yes	18(23%)	7(38.9%)	4(22.2%)	7(38.9%)	0(0%)	
Multifocality						**0.001**
No	70(90%)	18(25.7%)	34(48.6%)	18(25.7%)	0(0%)	
yes	8(10%)	7(87.5%)	1(12.5%)	0(0%)	0(0%)	
Free surgical margin						**0.485**
No	16(21%)	3(18.8%)	9(56.3%)	4(25%)	0(0%)	
yes	20(26)	22(35.5%)	26(41.9%)	14(22.6%)	0(0%)	
Vascular Invasion						**0.754**
No	58(74%)	17(29.3%)	27(46.6%)	14(24.1%)	0(0%)	
yes	20(26%)	8(40%)	8(40%)	4(20%)	0(0%)	
Positive family history						**0.812**
No	74(95%)	23(31.1%)	34(45.9%)	17(23%)	0(0%)	
yes	4(5%)	2(50%)	1(25%)	1(25%)	0(0%)	

Table 3: Correlation between Livin expression and clinicopathological features of the primary tumors.

Features	**Number of cases (%)**	**Livin-expression in the primary tumors**		
		Negative (0), weak (1) vs. moderate (2+), Strong (3+)		
		Negative, weak	Moderate, strong	***P-value***
Age (years)				***0.73***
< 49 years	46 (59%)	31(68%)	15(32%)	
≥ 49 years	32(41%)	22(68.7%)	10(31.3%)	
Site of tumor				***0.645***
Right	43 (55%)	24(55.8%)	19(44.2%)	
Left	35(45%)	22(62.9%)	13(37.1%)	
Histological Type				***0.374***
Invasive ductal carcinoma	69(89%)	40(58%)	29(42%)	
Mucinous carcinoma	3(4%)	1(33.3%)	2(66.7%)	
Invasive lobular carcinoma	3(4%)	3(100%)	0(0%)	
Papillary carcinoma	1(1%)	1(100%)	0(100%)	
Secretory carcinoma	1(1%)	1(100%)	0(100%)	
Cribriform carcinoma	1(1%)	0(100%)	1(100%)	
Histological Grades				**0.852**
G1	13(17%)	8(61.5%)	5(38.5%)	
G2	48(61%)	27(56.3%)	21(43.8%)	
G3	17(22%)	11(64.7%)	6(35.3%)	
Stage				***0.47***
I	6(8%)	2(33.3%)	4(66.7%)	
IIA	23(29%)	15(65.2%)	8(34.8%)	
IIB	13(17%)	6(46.2%)	7(53.8%)	
III	1(1%)	0(0%)	1(100%)	
IIIA	18(23%)	11(61.1%)	7(38.9%)	
IIIB	1(1%)	1(100%)	0(0%)	
IV	16(21%)	11(68.8%)	5(31.3%)	
Primary Tumor				**0.584**

T1	6(8%)	2(33.3%)	4(66.7%)	
T2	44(56%)	24(54.5%)	20(45.5%)	
T3	28(36%)	12(42.8%)	16(75.2%)	
Lymph node involvement				***0.820***
No	35(45%)	20(57.1%)	15(42.9%)	
yes	43(55%)	26(60.5%)	17(39.5%)	
Distance Metastases				**0.410**
No	62(79%)	35(56.5%)	27(43.5%)	
yes	16(21%)	11(68.8%)	5(31.3%)	
Chemotherapy				**0.694**
No	7(9%)	5(71.4%)	2(28.6%)	
Yes	71(91%)	41(57.7%)	30(42.3%)	
Radiotherapy				***0.330***
No	26(33%)	13(50%)	13(50%)	
yes	52(67%)	33(63.5%)	19(36.5%)	
Recurrence				***0.683***
No	70(90%)	42(60%)	28(40%)	
yes	6(8%)	3(50%)	3(50%)	
Estrogen				***0.353***
No	32(41%)	17(53.1%)	15(46.9%)	
yes	45(58%)	29(64.4%)	16(35.6%)	
Progesterone				**0.488**
No	31(40%)	17(45.2%)	14(54.8%)	
yes	46(59%)	29(63.0%)	17(37%)	
Her-2				***0.414***
No	59(76%)	37(62.7%)	22(37.3%)	
yes	18(23%)	9(50%)	9(50%)	
Multifocality				**1.000**
No	70(90%)	41(58.6%)	29(41.4%)	
yes	8(10%)	5(62.5%)	3(37.5%)	
Free surgical margin				**0.570**
No	16(21%)	8(50%)	8(50%)	
yes	20(26%)	24(38.7%)	38(61.3%)	
Vascular Invasion				**0.299**
No	58(74%)	32(55.2%)	26(44.8%)	
yes	20(26%)	14(70%)	6(30%)	
Positive family history				**0.025**
No	74(95%)	46(62.2%)	28(37.8%)	
yes	4(5%)	0(0%)	4(100%)	
Hormone replacement Therapy				**0.640**
No	29(37%)	16(55.2%)	13(44.8%)	
Yes	49(63%)	30(61.2%)	19(38.8%)	

Livin expression in primary breast cancer showed a significant correlation (p=0.025) with a positive family history, in that all patients with positive family showing over-expression of Livin. On the other hand, Livin expression was not significantly associated with the site of tumor, histologic type, grade, LN status, TNM classification, tumor stage, recurrence, distant metastasis, chemotherapy, radiotherapy, estrogen- and progesterone receptor status, Her-2, hormone replacement therapy, multifocality, vascular invasion and free surgical margins (Table 3). Survivin and Livin expression were also analysed in the lymph node metastases (secondary breast cancer). A total of 44 patients had lymph node metastases, 15 patients (34%) showing cytoplasmic expression of Survivin, whereas 29 (66%) were considered negative. Altogether, 37 patients (84%) showed cytoplasmic expression of Livin, while 7 patients (16%) were negative. The primary tumors showed higher Survivin expression (82%) than did the lymph node metastases (34%), whereas a Livin expression did not differ between the primary (71%) and the secondary tumors (84%). Survival analysis using the Kaplan-Meier test showed no statistically significant impact of Survivin expression (in the primary breast cancer) one disease-specific survival (DSS) (p=0.775) (Figure 2).

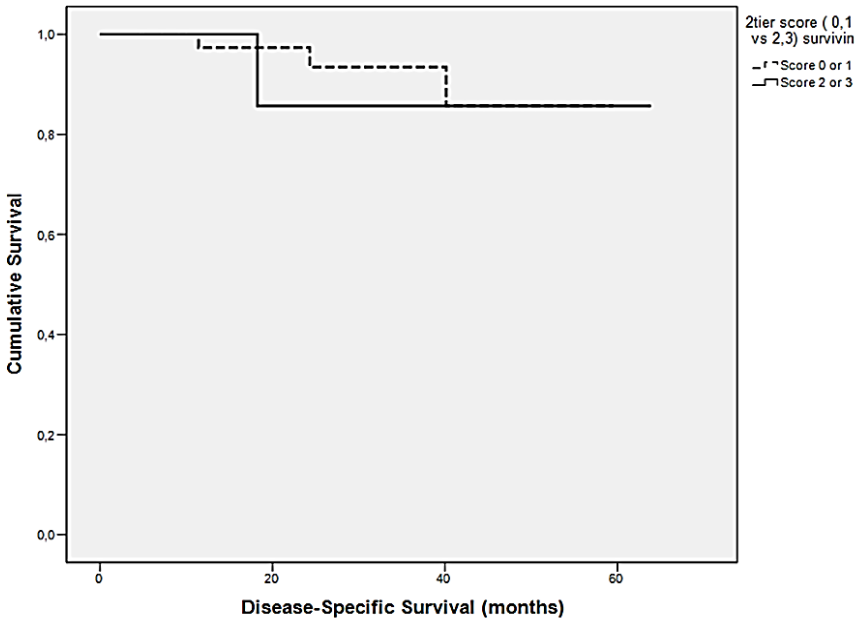

Figure 2: Survivin expression (neg-weak vs. mod-strong) as determinant of disease-specific survival (DSS) in univariate (Kaplan-Meier) analysis (p=0.775 log-rank).

Discussion

The need for informative molecular markers that provide prognostic information additional to that given by conventional pathological staging of breast cancer has been recently highlighted. The aim of our study was to investigate the expression of Survivin and Livin proteins in primary breast cancer and their lymph node metastases using immunohistochemistry. Survivin expression was detected in 82% of the primary breast cancer and in 34% of their lymph node metastases. Livin was detected in 71% of the primary tumors and in 84% of their metastases. The IHC staining of Livin and Survivin revealed that their expression was predominantly cytoplasmic with very little nuclear staining. Thus, only the cytoplasmic staining of Livin and Survivin was evaluated in the present study. The role of Survivin and Livin as prognostic markers in breast cancer is controversial. In the present series, cytoplasmic Survivin expression in breast cancer is significantly associated with several indicators of favourable prognosis including unifocal tumor, no recurrence and Her-2 negative tumors. This is in line with the study of, Shaaban *et al.* who demonstrated that cytoplasmic staining of Survivin was correlated with favourable prognostic indicators [37]. Similarly, Kennedy *et al.* confirmed that nuclear expression of Survivin is an indicator of favourable disease outlook [38].

Hormonal receptors, as well as Her-2 status, are widely accepted as prognostic and predictive indicators in breast cancer. In the present series, a significant correlation (p=0.047) was established between Survivin expression and Her-2 status, while no such correlation was found with ER (p=0.35) and PR (p=0.55) expression being in alignment with other studies [39-41]. Livin over-expression is associated with tumor progression, more aggressive behaviour, e.g., migration and resistance to radiotherapy and chemotherapy, in several types of human malignancies [42, 43]. The present results disclose a statistically significant difference in Livin expression between the patients with and without a positive family history of breast cancer. Although a positive family history of breast cancer is a well-established risk factor of incident breast cancer, it is not known whether it has an impact on mortality after breast cancer diagnosis. Some studies have reported that breast cancer cases with family history are more likely to have smaller and earlier-stage tumors [44, 45]. However, in the present series, we failed to demonstrate any statistically significant correlation between Livin expression and ER, PR, Her-2 status, or tumor grade, thus, confirming the findings reported in some previous studies [46]. As to the lymph node status in our series, there was no significant association between Livin and Survivin expression, being in agreement with the data of Soliman *et al.* [47]. Finally, we also analysed the association of Survivin and Livin expression in the (lymph node metastases). Altogether, 44 patients had lymph node metastases, of whom 15 patients (34%) showed cytoplasmic expression of Survivin, and 37 patients (84%) showed cytoplasmic expression of Livin. Accordingly, Survivin expression in the primary tumors (82%) is significantly more common than in their lymph node metastases (34%) whereas no such difference exists in Livin expression (71%) and their metastases (84%) respectively.

Conclusion

Taken together, the present results indicate that Survivin and Livin are frequently expressed in primary breast cancer and their lymph node metastases. Survivin expression in primary breast cancer is significantly associated with several indicators of favourable disease outlook, including unifocal tumor, no recurrence, and Her-2 negative tumors. However, in univariate survival analysis (Kaplan-Meier), Survivin was not a significant predictor of DSS. Livin expression in primary breast cancer is significantly associated with positive family history. These findings advocate further research focused on these two molecular markers in breast cancer.

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