CLINICOPATHOLOGICAL SIGNIFICANCE AND CORRELATION OF SURVIVIN, HER2 AND BCL2 EXPRESSION IN BREAST CARCINOMA

Abeer M. Abdelbary[1], Walid S. Elsayed[1], Abdelmonem A. Hegazy[2]
[1]Pathology Department, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt
[2]Anatomy and Embryology Department, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt

ABSTRACT

Background: Breast cancer is the most common cancer among females worldwide, accounting more than one third of the total percentage of all cancers in Egypt. **Objective:** This study aimed to correlate the immunohistochemical expression of survivin, HER2 and BCL2 with the clinicopathological variables in breast carcinoma to clarify the effect of marker expression on tumor behavior and prognosis. **Methods:** Immunohistochemical expressions of the three biomarkers were evaluated in 65 cases of breast carcinoma and the harvested data were correlated with clinicopathological parameters. **Results**: The specimens were 55 infiltrating duct carcinoma (IDC) and 10 cases of ductal carcinoma in situ (DCIS). Survivin was expressed in 3 cases (30%) of DCIS and in 40 cases (72.7%) of IDC. There was a positive significant association between survivin expression and tumor size**.** Also, there was a significant association between survivin expression and histological grade, tumor stage and LN metastases. Significant relationship between survivin expression and both estrogen receptor negative and progesterone receptor negative hormonal status was noticed. HER2 was expressed in 5 cases (50%) of DCIS and in 20 cases (36.4%) of IDC. There was a positive significant association between HER2expression and tumor size, tumor stage and LN metastases. The expression of HER-2 had a significant negative correlation with a status of estrogen (ER) and progesterone receptors (PR). BCL2 was expressed in 7 cases 70% of DCIS and in 42 cases (76.3%) of IDC. There was increased BCL2 expression in small sized-tumors. Also there is a highly significant association between BCL2expression and grade of tumors. There was no significant association betweenBCl2 expression and stage of tumors and LN metastases. The expression of BCL2 had a significant positive correlation with a status of ER & PR**. Conclusion:** Both survivin and HER2 are prognostic biomarkers, but BCL2 expression is associated with low grade tumor, small size and positive ER and PR.

Key words: Immunohistochemical; Survivin; HER-2, BCL2; Carcinoma; Breast

INTRODUCTION

Carcinoma of the breast is considered the most frequent cancer among females internationally, accounting for 23% (1.38 million) of the total novel cases of malignancies and 14% (458,400) cancer related mortality**. [1]** In Egypt, carcinoma of the breast is estimated to be the most frequent cancer among females accounting for 37.7% of all cancers with 12,621 new cases in 2008. It is also the principal reason of cancer related death accounting for 29.1% of their total with 6546 deaths**. [2]**

Apoptosis is a planned cell death that responsible for the removal of the damaged and unnecessary cells**. [3]** Disturbance of apoptosis pathways is responsible for numeral diseases such as malignancy, autoimmune and immunodeficiency diseases, and neurodegenerative disorders. Apoptosis cascade can be inhibited by a group of proteins called inhibitor of apoptosis proteins (IAPs) through binding directly to caspases. Survivin is considered one of such family of inhibitors of apoptosis protein (IAP). It is involved in regulation of two important cellular processes including inhibition of cell apoptosis by direct and indirect way via interfering with the action of caspases 3, 7 and 9 also promoting cell proliferations**. [4]** Survivin is as well implicated in tumor development via various mechanisms including inhibition of Bax- and Fas-induced apoptosis, regulation of cytokinesis and cell cycle sequences as it controls G2/M phase of the cell cycle by combining with mitotic spindle, and contribution in a multiplicity of signaling pathways as the p53, Wnt, hypoxia,

transforming growth factor (TGF), and notch signaling pathways**. [5]**

Survivin is expressed in embryonic and fetal cells, however is unnoticeable in normal mature tissues. Exaggerated expression of survivin has been noticed in almost all human cancers as carcinoma of bladder, lung, breast, stomach, oesophagus, liver, ovary and blood**.** Its over expression is associated with poor clinical prognosis or resistance to the chemotherapy in certain tumors**. [6]**

HER2/neu oncogene is one of tyrosine kinase family like epidermal growth factor receptor (EGFR). HER2 activation starts signal cascades counting the MAPK (mitogen activated protein kinase) and 3- kinase pathways that are necessary for cellular proliferation and differentiation. **[7]** The HER2/neu oncogene encodes a 185 kDa transmembrane protein and is present at low levels in a range of normal epithelium, as ductal epithelium of the breast, and is over expressed in 20-30% of invasive carcinoma of the breast. Several studies have shown an association of HER2 over expression in human breast carcinomas with poor clinical prognosis and therapeutic response. **[8]**

BCL2 protein, encoded by the BCL-2 gene, has anti-apoptotic function and inhibits cellular death, leading to prolonged cell survival. BCL2 is over expressed in a lot of malignancies and participates in tumor beginning, progression and resistance to treatment**. [9]** It is commonly expressed in normal breast epithelial cells also in breast malignant ones, and is recognized to be up regulated by estrogen. **[10]** BCL2 immunoreactivity in breast malignancies is frequently associated with approving prognostic factors as small tumor size, low-grade, low proliferation, ER positive and p53-negative status**. [11]** The definitive result of breast carcinoma depends on the initial stage of the cancer at diagnosis. The major prognostic factors associated with carcinoma of the breast are lymph node involvement, size of mass, histological grade, and hormone receptor status. But, tumors of the same stage can perform in a diverse way and the prognosis can be different**. [12]**

This study aimed to correlate the immunohistochemical expression of survivin and HER2 and BCL2 with the clinicopathological parameters in breast carcinoma to find the influence of biomarker expressions on tumor performance and to detect the correlations between these biomarkers.

MATERIAL AND METHODS

2.1 Patients and clinical data

Retrospective study was carried out on 65 cases of breast carcinoma achieved from the Archives of the Pathology Lab. of Zagazig University, the period from January 2010 to March 2012. All cases were obtained by modified radical mastectomy. All tissue samples were formalin-fixed and paraffin-embedded. We collected the clinical, pathological, and immunohistochemical (estrogen receptor [ER], progesterone receptor [PR] information from the medical records of the patients. All of the patients did not receive any chemotherapy or radiation prior to the surgical interference. Histological typing and grading have followed the World Health Organization classification and modified Bloom-Richardson grading. **[13]** The study was carried out with full local ethical measurements. All collected blocks were cut at 4 microns and stained with hematoxylin and eosin (H&E) stain to verify the diagnosis.

2.2 Immunohistochemical staining

After cutting our blocks into 4 μm, the sections were deparaffinised with xylene, rehydrated in different alcohol grades, and located in 0.5% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase action. Antigen retrieval was done by incubation in 0.01 M citrate buffer (pH 6.0) for 5 min in a pressure cooker. The sections were exposed to the primary antibody for 60 min at room temperature. We used Streptavidin-biotin-peroxidase complex technique, for survivin (rabbit polyclonal anti survivin (1:250), (Cat.#RB-9245-R7,Thermo Fisher Scientific Inc., Fremont, CA) and HER-2 (mouse monoclonal antibody, ready to use, clone e2-

4001, catalog no. MS-730-R7-A, Lab Vision, California, USA) and (BCL2 mouse monoclonal antibody –diluted 1:80- clone 124;Dako) was used by employing diaminobenzidine (DAB) as the chromogen. Sections of colonic adenocarcinoma and breast carcinoma and samples of normal tonsil are used as positive control. Negative control was used buffer instead of primary antibodies.

Evaluation of immunohistochemical staining

Immunostaining for survivin was recorded according to staining intensity, distribution in cytoplasm and/or nucleus, and percentage of malignant cells that stained positively. Protein expression was quantified in the different samples examined using a scoring manner utilized previously. A mean percentage of positive malignant cells was detected in at least five areas at a magnification of 400 and classified to one of the following five categories: 0, <5%; 1, 5%- 20%; 2, 21%-50%; 3, 51%-75% and 4, >75%. The immunostaining intensity was scored as follows: (a) weak, 1+; (b) moderate, 2+ and (c) intense, 3+. The percentage of positive cells and the staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores of <1 were considered negative and those with scores of > 1 were considered positive**. [14]**

Tissue expressions of membranous HER2 immunoreactivity were similarly classified into four groups as follow: 0 (negative; no staining seen , or membrane staining in < 10% of the tumor cells); 1 (negative; faint ⁄ barely perceptible focal membrane staining in >10% of tumor cells); 2 (positive; weak to moderate staining of the complete cell membrane in >10% of the tumor cells); or 3 (positive; strong staining of the complete membrane in > 10% of the tumor cells) finally, for statistical analysis the HER-2 expression was grouped into negative (0, 1) and positive or overexpressed (2, 3). [**15]**

BCL2 cytoplasmic immunoreactivity was quantified by counting at least 1000 tumor cells in different random fields, using a high-power (400x) objective. Results were expressed as percentage of tumor cells staining positively for BCL2. For further statistical analysis two groups of tumors were defined: tumors containing 10% or less (BCL-2 negative) and tumors containing more than 10% positively staining tumor cells (BCL2 positive)**. [16]**

2.4 Statistics

The results from the analysis of the continuous variable are expressed as a mean ± standard deviation (SD). Analysis of categorical data was performed using the x^2 or Fisher's exact test, Spearman correlation was performed to assess the correlation between survivin, HER2 and BCL2.All statistical analyses were done using SPSS software (version 19.0; SPSS, Chicago, IL). The $P \leq 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Clinicopathological results

Out of the studied cases, 10 cases were ductal carcinoma in situ (DCIS); their ages ranged from 35-52 years. The other 55 cases were infiltrating duct carcinoma (IDC); their ages ranged from 38-71 years. Among the studied IDC, 54.5% cases were ≤ 5 and 45.5% >5. On the other hand, 63.6% of cases were of low grades (I and II), 36.4% were high grade (III). 43.7% of cases had a positive LN metastasis, 60% of cases were stage I/II and 40% were stage III/IV. 63.6% of cases were estrogen receptor positive and 40% were progesterone receptor positive (Table 1).

Immunohistochemical expression of survivin

Survivin was detected in cytoplasm and/or nucleus of malignant cells. It was expressed in 3 (30%) cases of DCIS (Fig.2) and in 40 cases (72.7%) of IDC. negative expression more common in low grade (Fig.1). Among the positive cases, 8 (20%) cases showed nuclear staining only and 32 (80%) cases showed cytoplasmic and nuclear staining (Figs. 3-6). There was no significant association between survivin expression and age of patients (p 0**.**7748). There was a positive significant association between survivin expression and tumor size (p 0.0203) as the expression increased with large tumor size, also there was

a significant association between survivin expression and histological grade , tumor stage and LN metastases (p values 0.0131, 0.0020 and 0.0055 respectively). There was a significant relationship between survivin expression and both estrogen receptor negative (p 0.0297) and progesterone receptor negative hormonal status (p 0.0002) (Table 2). As regard the subcellular localization of survivin expression, it was independent of the tumor size but 91% of large size tumor >5 showed survivin expression both cytoplasmic and nuclear. There was a statistical significant relationship between the subcellular localization of survivin and histologic grade (p 0.0040), 95.7% of high grade tumors had cytoplasmic and nuclear survivin expression. Also there was a statistical significant relationship between the subcellular localization of survivin and tumor stage (p 0.0113) as high grade showing both cytoplasmic and nuclear expression. This finding was seen with LN metastases (p 0.0005) as 100% of tumor with LN metastases showing both cytoplasmic and nuclear expression (Table 3).

Immunohistochemical expression of HER2

HER2 was noticed to be expressed in the membrane of breast cells. It was expressed in 5 cases (50%) of DCIS (Fig 7) and in 20 cases (36.4%) of (IDC) (Figs. 8,9). There was no significant association between HER2 expression and age of patients (p 0.6717), and tumor grade (p 0.7986). There was a positive significant association between HER2expression and tumor size, tumor stage and LN metastases (p 0.0278, 0.0001 and 0.0157 respectively). The expression of HER2 had a significant negative correlation with a status of ER and PR (p 0.0059 and 0 .0002 respectively) (Table 2).

Immunohistochemical expression of BCL2

BCL2 was expressed in the cytoplasm of breast cells. It was expressed in 7 cases (70%) of DCIS (Fig. 10) and in 42 cases (76.3%) of IDC (Fig 11,12). There was no significant association betweenBCl2 expression and age of patients (p 0.2544). There was a significant association between BCL2 expression and tumor size (p 0.0012) as increased expression in small size tumors. Also there is a highly significant association between BCL2 expression and grade of tumors (p 0.000) as it is expressed in all cases of low grade tumors and in 50% of high grade tumors. There was no significant association between BCl2 expression and stage of tumors and LN metastases (p 0.8969 and 0.1364 respectively). The expression of BCL2 had a significant positive correlation with a status of ER and PR (p 0.0048 and 0.0382) (Table 2).

Correlation of surviving expression and HER2

There is a high significant correlation between survivin and HER2 immunohistochemical expression (Spearman correlation = .402; P = 0.002) (Table 4).

Correlation of surviving expression and BCL2

There is an inverse significant correlation between survivin and BCL2 immunohistochemical expression (Spearman correlation = - .301; P =0 .026) (Table 5).

Table 1: Clinicopathological characteristics of 55 patients with breast carcinoma

Variables	**Breast carcinoma cases No**: (%) 55: (100%)
Age (years):	
	20 (36.4%)
≤50	35 (63.6%)
>50	
Tumor size (cm):	
	30 (54.5%)
≤5	25 (45.5%)
>5	
Histological grade:	
	35 (63.6%)
Low grade (Iand II)	20 (36.4%)
High grade (III)	
Lymph node metastasis:	
	31 (56.3%)
Node –	24 (43.7%)
Node +	
Tumor stage:	
	33 (60%)
I/II	22 (40%)
III/IV	
Estrogen receptor:	
	20 (36.4%)
Negative	35 (63.6%)
Positive	
Progesterone receptor:	33 (60%)
Negative	22 (40%)
Positive	

Table2: Biomarkers expressing breast cancer tissues in relation to clinicopathological parameter

Variable	Number	Survivin		HER2		BCL2	
		(+)	(-)	(+)	(-)	(+)	(-)
Age at surgery (y)							
	20 (36.4%)						
≤50	35 (63.6%)	15 (75%)	5 (25%)	8(40%)	12(60%)	17(85%)	3(15%)
>50		25 (71.4)	10 (28.6)	12(34.3%)	23(65.7%)	25(71.4%)	10(28.6%)
P value		**0.7748**		**0.6717**		**0.2544**	
Tumor size							
	30 (54.5%)						
≤5	25 (45.5%	18 (60%)	12 (40%)	7(23.3%)	23(76.7%)	28(93.3%)	2(6.7%)
>5		22 (88%)	3 (12%)	13(52%)	12 (48%)	14(56%)	11(44%)
P value		**0.0203**		**0.0278**		**0.0012**	
Histological grade							
	29 (52.7%)						
Low grade (I and II)	26(47.3%)	17 (58.6%)	12(41.4%)	11(37.9%)	18(62.1%)	29(100%)	0(0%)
High grade (III/IV)		23 (88.5%)	3 (11.5%)	9(34.6)	17(65.4%)	13(50%)	13(50%)
P value		**0.0131**		**0.7986**		**0.000**	
Tumor stage							
	33 (60%)						
I/II	22 (40%)	19(57.6%)	14(42.4%)	5(15.2%)	28(84.8%)	25(75.5%)	8(24.5%)
III/IV		21(95.5%)	1(4.5%)	15(68.2%)	7(31.2%)	17(77.3%)	5(22.7%)
P value		**0.0020**		**0.0001**		**0.8969**	
LN metastasis							
	31 (56.3%)						
Negative	24 (43.7%)	18(58%)	13(42%)	7(22.5%)	24(77.5%)	26(83.8%)	5(16.2%)
Positive		22(91.6%)	2(8.4%)	13(54.2%)	11(45.8%)	16(66.6%)	8(33.4%)
P value		**0.0055**		**0.0157**		**0.1364**	
Estrogen receptor							
Negative	20 (36.4%)	18(90%)	2(10%)	12(60%)	8(40%)	11(55%)	9(45%)
	35 (63.6%)						
Positive		22(62.8%)	13(37.2%)	8(22.8%)	27(77.2%)	31(88.5%)	4(11.5%)
P value		**0.0297**		**0.0059**		**0.0048**	
Progesterone receptor	33 (60%)						
Negative	22 (40%)	30(90.9%)	3(9.1%)	28(84.8)	5 (15.2%)	22(66.6%)	11(33.4%)
Positive		10(45.4%)	12(54.5%)	8(36.4%)	14(63.6%)	20(90.9%)	2(9.1%)
P value		0.0002		0.0002		0.0382	

Table 3: Subcellular localization of survivin expression

Variables	Nuclear staining only NO % 8 (20%)	Cytoplasmic staining (only or combined with nuclear staining) 32 (80%)	P value
Tumor size			**0.0560**
	6 (33.3%)	12 (66.7%)	
≤5	2 (9%)	20 (91%)	
>5			
Histological grade			
	7 (41%)	10 (59%)	**0.0040**
Low grade (I and II)	1(4.3%)	22 (95.7%)	
High grade (III and IV)			
LN metastasis			
	8 (44.4%)	10 (55.6%)	**0.0005**
Negative	0 (0%)	22 (100%)	
Positive			
Tumor stage			
	7 (36.8)	12 (63.2%)	**0.0113**
I/II	1 (4.7%0	20 (95.3%)	
III/IV			

Table 4: The Correlation analysis between Survivin and HER-2 expression among the studied IDC cases**.**

		HER2				Total
		.00	1.00	2.00	3.00	
survivin	.00	6	6	3	0	15
	1.00	8	3	1	0	12
	2.00	6	3	5	4	18
	3.00	1	2	3	4	10
Total		21	14	12	8	55

Spearman Correlation = .402; P = .002[c]

Table 5: Correlation analysis between survivin and BCL2 expression among the studied IDC cases

		BCL2		Total
		.00	1.00	
survivin	.00	0	15	15
	1.00	3	9	12
	2.00	7	11	18
	3.00	3	7	10
Total		13	42	55

Spearman Correlation= -.301; P = .026[c]

Figures:

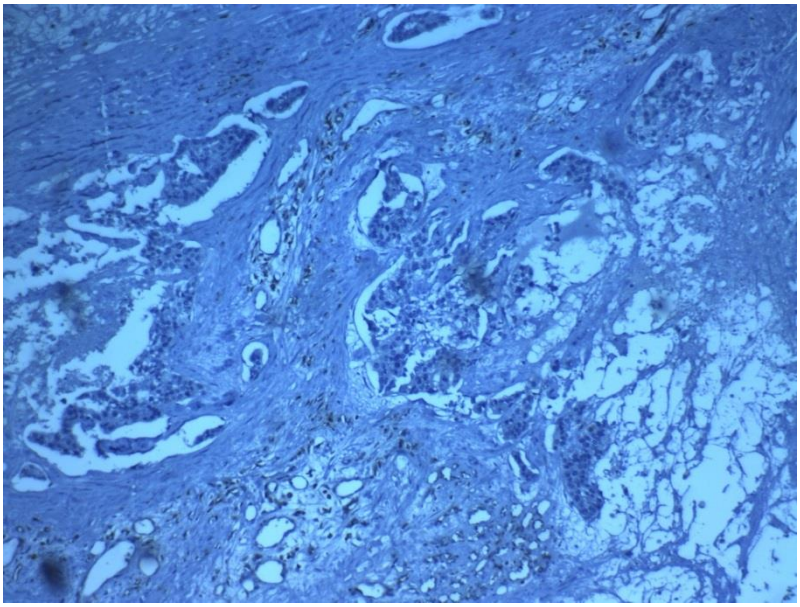

Fig (1) : Negative survivin expression in G I of IDC (X 400).

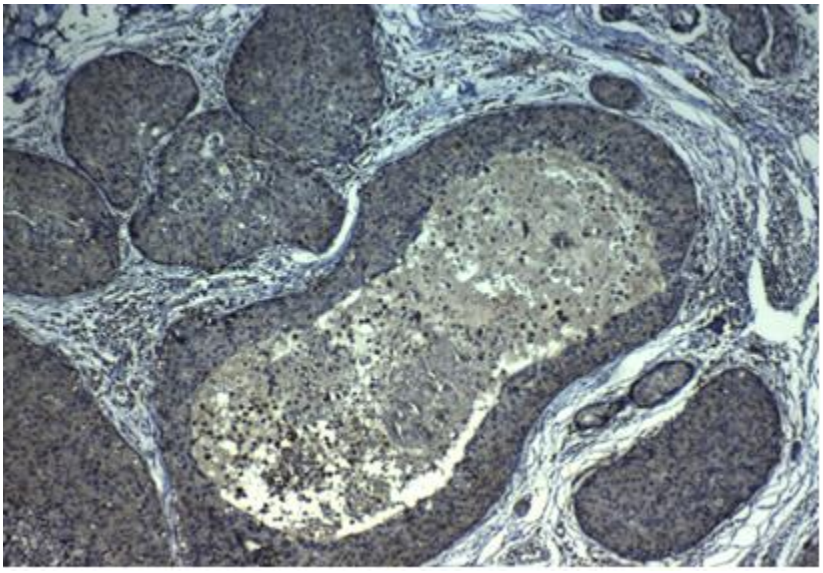

Fig (2); Strong cytoplasmic and nuclear survivin expression in DCIS comedo type (X200)

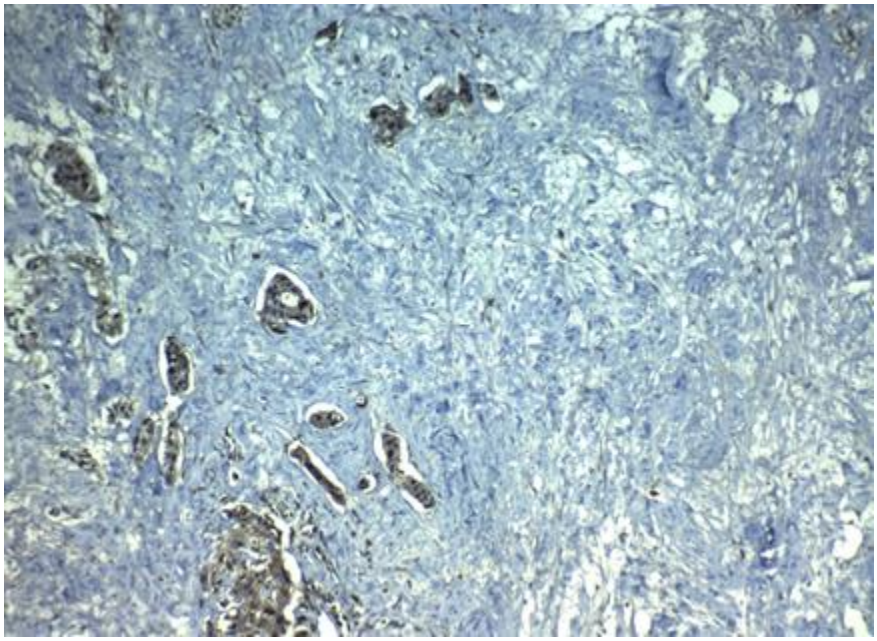

Fig (3): Weak cytoplasmic and nuclear survivin expression in GI of IDC (X200)

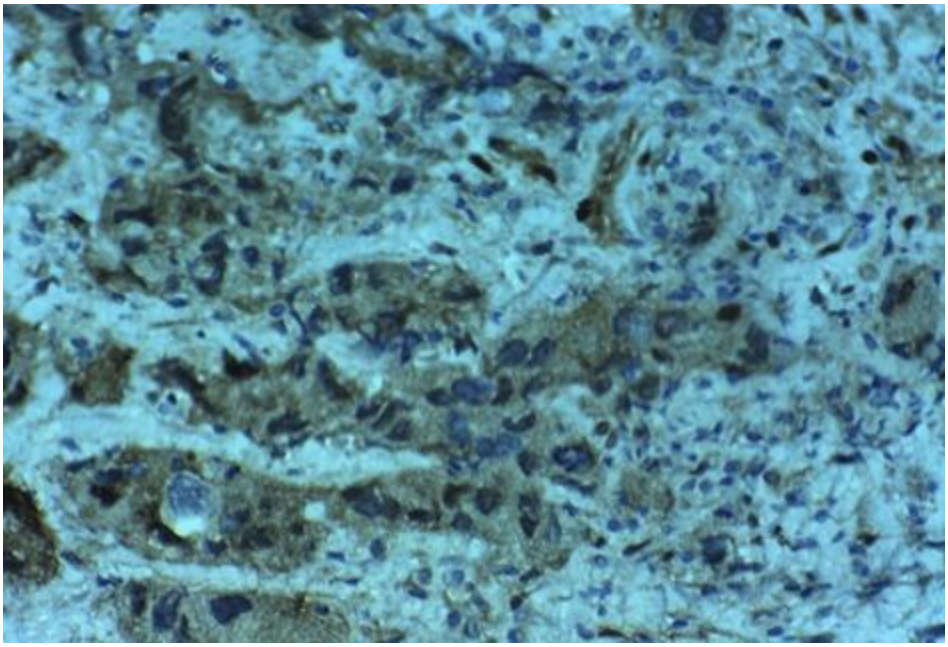

Fig (4): Moderate cytoplasmic and nuclear surviving expression in GII of IDC (X400)

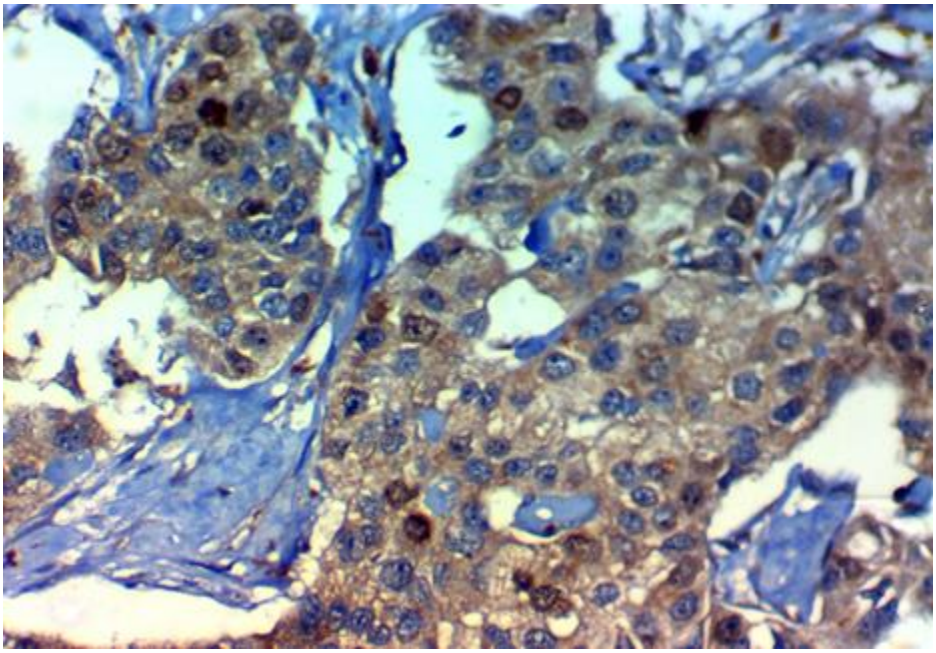

Fig (5): Moderate cytoplasmic and nuclear survivin expression in GIII of IDC (X400)

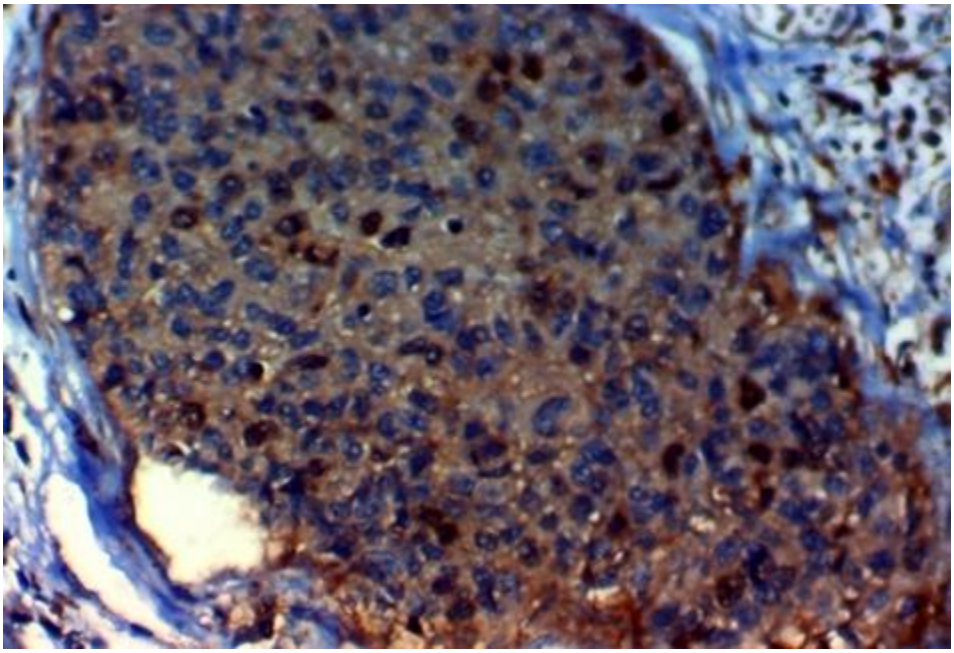

Fig (6); Strong cytoplasmic and nuclear survivin expression in GIII of IDC (X400)

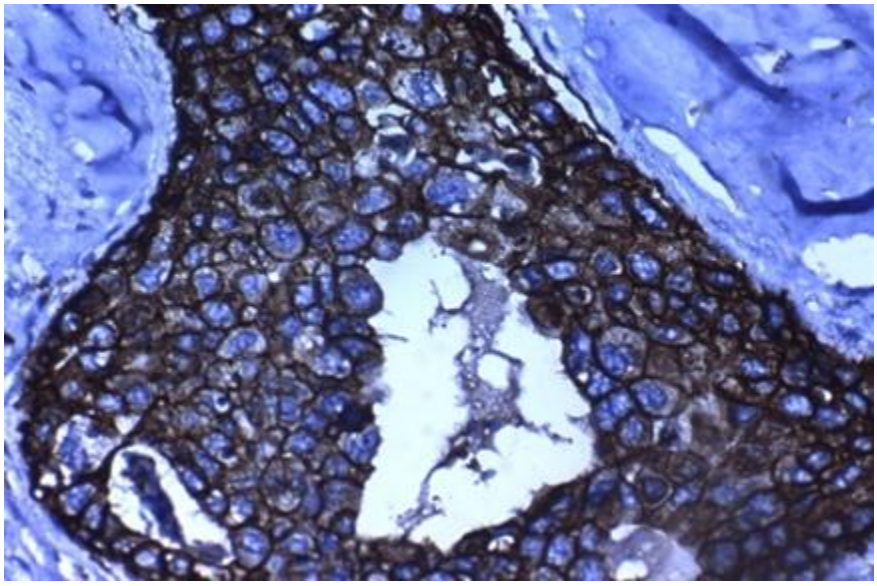

Fig (7): Moderate membranous HER2 expression in comedo carcinom (X400)

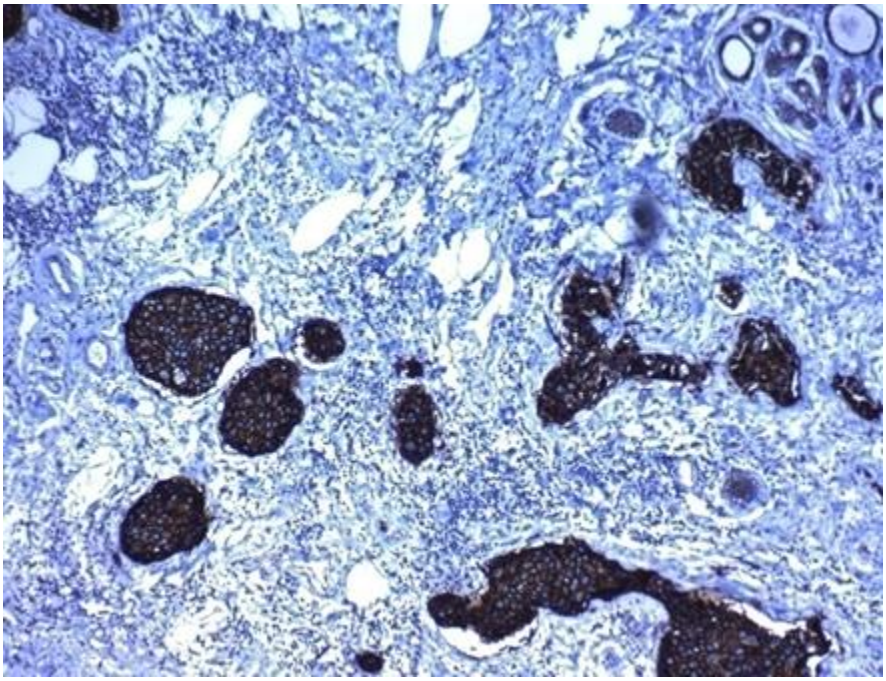

Fig (8): Moderate membranous HER 2expression in GII of IDC (X200)

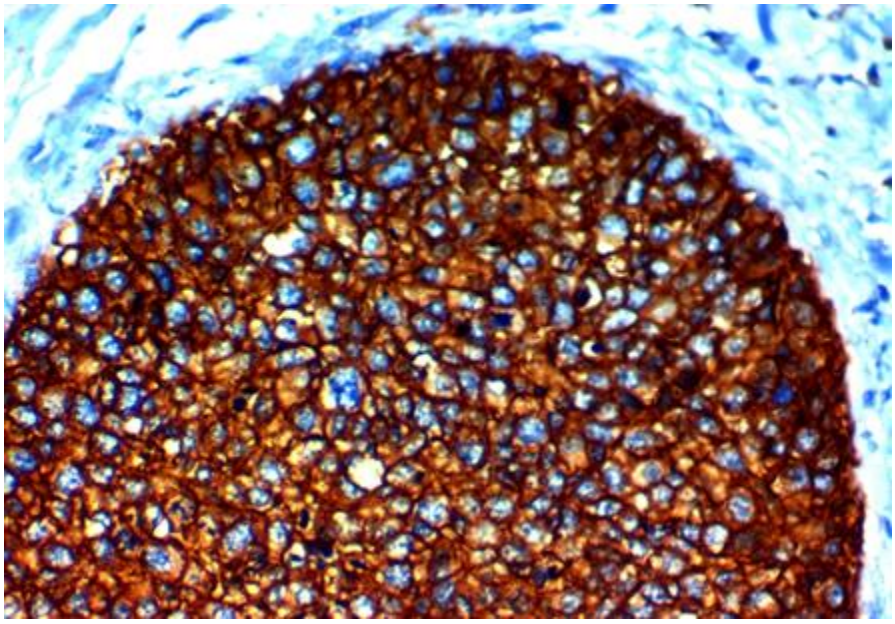

Fig (9): Strong membranous HER2 expression in GIII of IDC (X400)

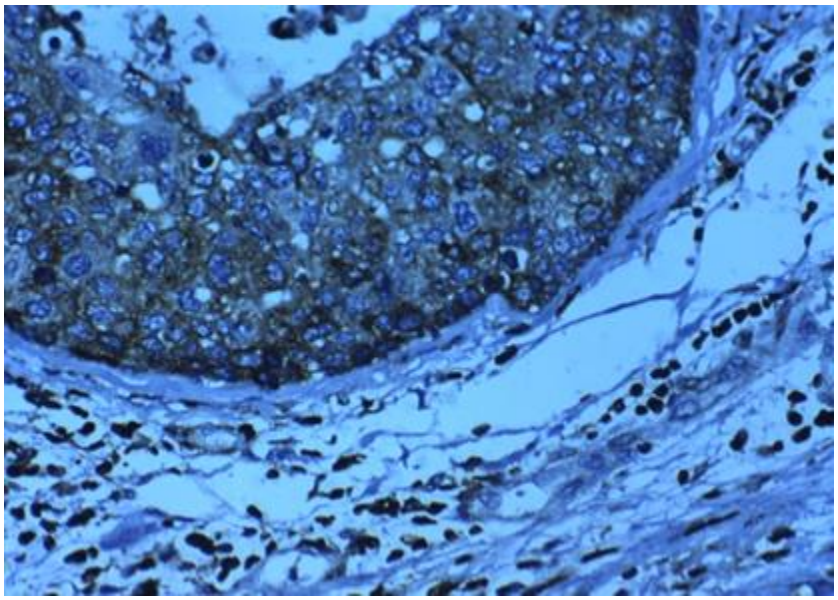

Fig (10): Weak cytoplasmic BCL2 expression in Comedo carcinoma (X200)

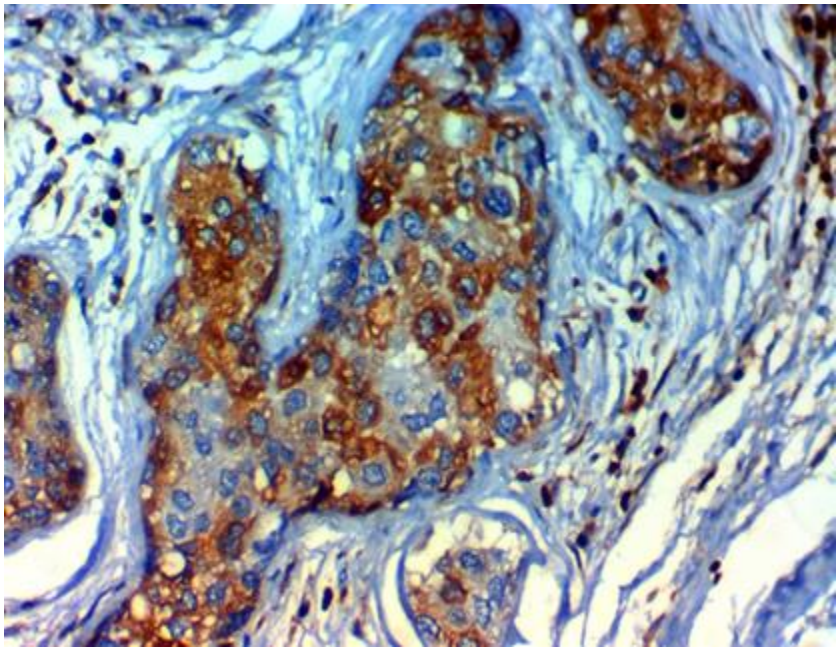

Fig (11) Moderate cytoplasmic BCL2 expression in GII of IDC (X400)

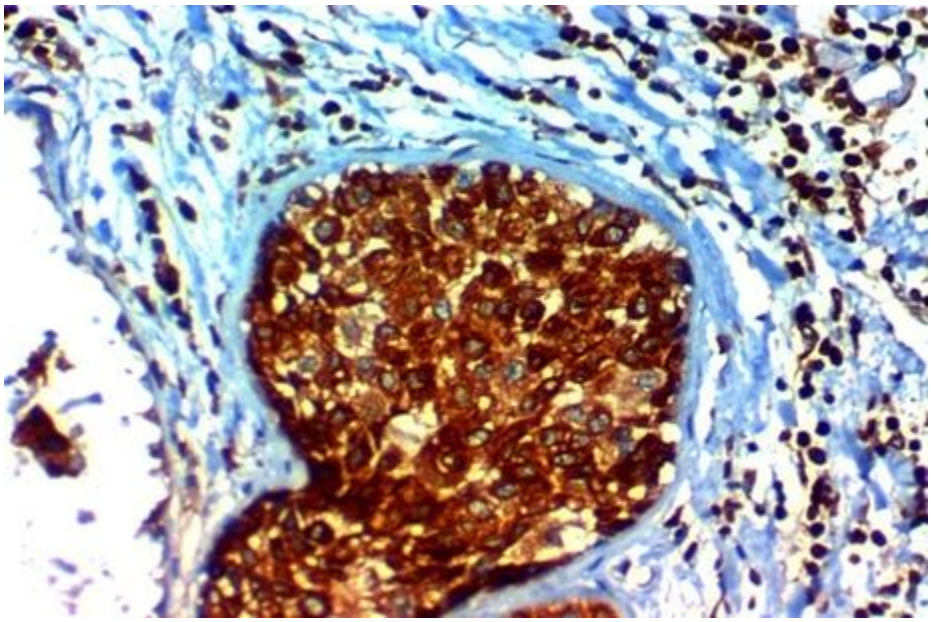

Fig (12): Strong cytoplasmic BCL2 expression in G II of IDC (X400)

DISCUSSION

In our study, we examined Survivin, HER2 and BCL2 expression in a group of Egyptian breast cancer patients. The study included 55 cases of IDC, 10 cases of DCIS. In our study survivin were expressed in 3 (30%) cases of DCIS and in 40 cases (72.7%) of IDC; this near to the results of Chiou et al [**17]** who found survivin expression in 70.7% in cases of IDC, study of Athanassiadou et al **[18]** who found survivin expression in 60% of IDC and the study made by Chu et al **[19]** who found survivin

expression in 81% of IDC.in our study we found no significant association between survivin expression and age of patients. These results are in agree with that made by Singh et al **[20]** who found no significant association between survivin expression and age of the patients. There was a positive significant association between survivin expression and tumor size as the expression increased with large tumor size, also there was a significant association between survivin expression and histological grade , tumor stage and LN metastases. there was a significant relationship between survivin expression and both estrogen receptor negative and progesterone receptor negative hormonal status**;** these results in agree with those made by Singh et al **[20]** In contrast to studies made by Tanaka et al **[14]** and Kennedy et al **[21]** who founds no significant association between survivin expression and tumor stage, size, lymph node metastasis and hormonal receptors (ER and PR), this variance may be due to different procedure and different method of analysis of the marker expression. As regard the Subcellular localization of surviving expression, was independent of the tumor size but 91% of large size tumor **>5** showing survivin expression both cytoplasmic and nuclear. There was a statistical significant relationship between the subcellular localization of survivin and histologic Grade**,** (95.7%) of high grade tumors have cytoplasmic and nuclear survivin expression. Also there was a statistical significant relationship between the subcellular localization of survivin and tumor stage as high grade showing both cytoplasmic and nuclear expression also this finding seen with LN metastases p value (.0005**)** as 100% of tumor with LN metastases showing both cytoplasmic and nuclear expression. Our results in agree with that of Okada et al **[22].**

In the present study we found HER2 was expressed in 5 cases (50%) of DCIS and in 20 cases (36.4%) of IDC; this in agree with the study made by Kumar et al **[23]** who found HER2 expression in 46.3% of cases of IDC**.** There was no significant association between HER2 expression and age of patients and tumor grade. There was a positive significant association between HER2expression and tumor size, tumor stage and LN metastases. These findings are near to the results of Sharifah et al **[24].** The expression of HER2 had a significant negative correlation with a status of ER and PR respectively, which is consistent with many previous studies. **[25,26]**

In the present study we found Bcl2 was expressed in 7 cases 70% of (DCIS) and in42 cases (76.3%) of (IDC), this is in agree with the study made by Hellemans et al **[27]** who found Bcl2 expression in 75% of cases of IDC, also near to the results of Alireza et al **[28]** who found BCL2 expression in 65.7% of IDC.

We found no significant association between BCL2 expression and age of patients, we noticed a significant association between BCl2 expression and tumor size as increased expression in small size tumors, but in the study made by Hellemans et al **[27]** found no significant association between BCl2 expression and tumor size. In our research we found, a highly significant association between BCL2 expression and grade of tumors p (.000) as it is expressed in all cases of low grade tumors and in 50% of high grade tumors but in the study made by Coppola et al **[29]** concluded that absent or decreased BCl2 expression in low grade tumors but increased expression in high grade tumors. Also we found there was no significant association between BCL2 expression and stage of tumors and LN metastases. This in agree with the study made by Hellemans et al **[27]** who found no significant association betweenBCl2 expression and stage of tumors and LN metastases. We also found The expression of BCL2 had a significant positive correlation with a status of ER & PR**.** This is in agree with Hellemans et al **[27]** who found strong positive relationship between bcl-2 immunoreactivity and estrogen and progesterone receptor status also this results parallel to previous studies **[10,30]**. We found high significant correlation between survivin and HER2 immunohistochemical

expression as expression of both biomarkers increased with large tumor size, tumor stage and LN metastases and negative correlation with a of ER and PR status.

Also we found an inverse significant correlation between survivin and BCl2 immunohistochemical expression (Spearman Correlation = - 301; P value= .026).

CONCLUSIONS

Both survivin and HER2 are prognostic biomarkers. Their expression increase with large tumor size, tumor stage and LN metastases and negative correlation with ER and PR status, but BCL2 expression is associated with low grade tumor, small size and positive ER and PR.

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