IMMUNOHISTOCHEMICAL CYCLOOXYGENASE-2 (COX-2) AND CD31 EXPRESSION IN BREAST CARCINOMA WITH CORRELATION TO CLINICO-PATHOLOGICAL PARAMETERS

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ABSTRACT

Background: Breast cancer is the most common cancer in Egyptian women. COX-2 seems to be involved in malignant transformation and tumor progression by affecting cell proliferation, mitosis, cell adhesion, apoptosis, immune surveillance, and angiogenesis. Angiogenesis is an important key step in tumor progression. Microvascular density (MVD), a surrogate marker of angiogenesis can be assessed by CD31 staining. **Aims** This study aims to: 1. Evaluate COX-2 and CD31 expressions in breast cancer. 2. Determine the correlation between COX-2 and CD31 with the clinico-pathological parameters in ductal breast carcinoma. **Materials and Methods:** This study included 74 specimens of breast lesions. Patient's age, tumor size and local aggressive changes, history of recurrence and/or presence of distant metastasis were obtained. Hematoxylin and Eosin (H&E) stained sections were evaluated for histopathological tumor type, tumor grade, presence or absence of normal, hyperplastic, *in situ* component, lymphocytic infiltration, lymphovascular invasion, and axillary lymph node status. COX-2 and CD31 immunostaining was done to detect their expression using the avidin-biotin peroxidase method. **Results:** COX-2 increased with increasing grade of ductal carcinoma *in situ* (DCIS) and invasive ductal carcinomas (IDC) ($P< 0.05$ and $P< 0.002$ respectively). COX-2 expression increased progressively along the continuum of neoplastic changes from normal breast epithelium to IDC ($P< 0.01$). There was significant correlation between COX-2 and tumor size ($P< 0.05$), tumor grade ($P< 0.002$), lymphovascular invasion ($P< 0.03$) and lymph node metastasis ($P< 0.02$). CD31 staining was observed along the cell membrane of endothelial cells of microvessels in all breast specimens. The median CD31 MVD count was 10 for normal breast, increased insignificantly to 17 in hyperplastic lesions, and reached 19 for DCIS, and 66.5 in IDC ($P < 0.000$). There was significant increase in MVD between different grades of IDC ($P < 0.01$) but not in DCIS. Positive correlation was present between COX-2 & CD31 in DCIS and in IDC ($P <0.000$ for each). **Conclusion:** COX-2 was increased with poor prognostic parameters; tumor size, tumor grade, lymphovascular invasion and lymph node metastasis. CD31 increases with increasing grade of IDC. These findings might imply for new therapeutic strategies in order to prevent progression of DCIS to IDC and to improve cancer therapy.

Keywords: Breast cancer; Microvascular density, angiogenesis, DCIS, IDC, COX-2 and CD31.

Abbreviation:
Cyclooxygenase-2 (COX-2), Microvascular density (MVD), Hematoxylin and Eosin (H&E), ductal carcinoma *in situ* (DCIS), invasive ductal carcinomas (IDC), National Cancer Institute (NCI), invasive breast carcinoma (IBC), World Health Organization (WHO), immunoreactive score (IRS), quantity score (QS), intensity score (IS), non-specific type (NST), atypical ductal hyperplasia (ADH).

INTRODUCTION

Breast cancer is the most common type of cancer and the most common cause of cancer-related mortality among women worldwide (Hortotagyi et al, 2005). According to the Egyptian National Cancer Institute (NCI), breast cancer represents 18.9% of total cancer cases; 35.1% in women and 2.2% in men (Elatar, 2002). The age-adjusted rate was 49.6 per 100 000 population (Seedhom and Kamal 2011).

Mammary carcinogenesis is a multistep process with transformation of normal ductal epithelial cells→ benign proliferative breast disease→ DCIS→ IDC (Hussein and Hassan 2006). Women with benign breast disease could be prevented from developing invasive breast carcinoma (IBC) if we can exactly identify

patients with which subtype of benign lesions will subsequently develop IBC and treat them.

COX-2 is a prostaglandin synthetase enzyme that converts arachidonic acid into pro-inflammatory prostaglandins, induced in inflammation and cancer (Guastalla et al, 2004). It is not expressed constitutively like COX-1, and is not normally present or is present at very low amounts, but COX-2 is rapidly induced by growth factors, cytokines, tumors promoters, hypoxia, ionizing radiation and carcinogens (Eltarhouny et al, 2008).

COX-2 seems to be involved in the processes of malignant transformation and tumor progression by affecting cell proliferation, mitosis, cell adhesion, apoptosis, immune surveillance, and angiogenesis. An elevated COX-2 level has been shown to correlate with a worse prognosis for patients with some types of tumors including breast cancer (Cho et al, 2006). However, there have been only a few studies dealing with the association between COX-2 expression and tumor progression in breast cancer.

Angiogenesis is a prerequisite for tumor growth and metastasis. Neovascularization provides not only the route for nutrient supply to the tumor but also the conduit for tumor cells to be shed into the circulation. New proliferating capillaries have leaky basement membranes, making them more accessible to tumor cells than mature vessels. It has been demonstrated that increasing density of newly formed microvessels in growing tumors correlated closely with increasing number of tumor cells shed into the bloodstream (Frontczak-Baniewicz et al, 2007).

It has been established that endothelial cells of tumor-associated neovasculature proliferate 20–2000 times more rapidly than endothelial cells of normal tissues (Abulafia & Sherer, 1999). Intratumoral MVD determined by staining endothelial antigens on histological sections may be used as a quantitative measure of angiogenesis. Small blood vessels as well as capillaries can be detected on immunohistochemistry with a range of specific antigens. Many studies published to date have used factor VIII-related antigen (von Willebrand factor), while others have used markers such as CD31 (PECAM-1) and CD34 (Olszanecki et al.,, 2006).

The purpose of this study was to evaluate COX-2 and CD31 expression in the successive steps of breast carcinogenesis and to determine its correlation with the clinic-pathological parameters in breast cancer.

PATIENTS AND METHODS

A total of 74 breast specimens, 4 with normal breast tissue, 5 with typical ductal hyperplasia, 11with DCIS, and 54 with IDC, were selected from the files of the Department of Pathology, Sohag University Hospital, Egypt in the period from 2010-2011. Availability of adequate tissue material and clinical data was the only criterion for selection of patients with DCIS and/or IDC.

According to World Health Organization (WHO) criteria (Tavassoli and Deville 2003), DCIS were graded into, 2/11(18%) low grade, 3/11 (27%) intermediate grade, and 6/11 (55%) high grade. IDC were classified according to Elston and Ellis grading system (1998) into 6/54 (11%) low grade, 29/54 (54%) intermediate grade, and 19/54 (35%) high grade. All patients with carcinomas were treated by modified radical mastectomy.

Immunohistochemistry

After reviewing hematoxylin and eosin (H&E) stained slides, a representative blocks was chosen for the study. Serial sections from each block were used for immunohistochemistry. Five micron tissue sections mounted on sialinized glass slides were deparaffinized and rehydrated through descending graded alcohols to water. Tissue sections were incubated in hydrogen peroxide for 10 min to block endogenous peroxidase activity. Then slides were treated with antigen retrieval solution (citrate buffer; 10 mmol sodium citrate buffer solution, pH 6.0 for COX-2 and EDTA for CD31). The buffer was allowed to boil in microwave at 750 Watt for 15 min divided into 3 cycles. Non specific protein binding was blocked with 10 min exposure to 10% normal goat serum.

Sections were then incubated with Rabbit polyclonal for COX-2, ready to use (Catalog; Cat

RB-9072-R7, LABVISION Corporation, Fremont, USA) and mouse monoclonal antibody for CD31, ready to use (Cat # MS-353-R7, LAB VISION Corporation, Fremont, USA) for one hour at room temperature. Then biotinylated goat polyvalent was applied on each section for 10 min with Streptavidin peroxidase. DAB (14-diaminobenzidine and 0.06 % H2O2) chromogen was applied to each tissue section for 10 min then washed in distilled water. Universal staining kit (Cat # TP-015-HD, LABVISION Corporation, Fremont, USA) composed of: Hydrogen peroxide block, Biotinylated goat anti-polyvalent, Streptavidine peroxidase, DAB chromogen, DAB subsrate was used. Tissue sections were counterstained with Mayer's hematoxylin, dehydrated alcohols, cleared in xylene, and cover slipped.

Positive control: Sections from colon cancer for COX-2 and from the placenta for CD31 were used as positive control. COX-2 and CD31 showed brownish cytoplasmic and nuclear and staining respectively. **Negative control:** The negative control slides were stained in parallel, but with omission of the primary antibody.

Assessment of COX-2:

According to Spizzo et al, (2003) the immunoreactive score (IRS) was determined by combining an estimate of the percentage of immunoreactive cells; quantity score (QS) with an estimate of the staining intensity; intensity score (IS). QS was calculated as follows: no staining is 0, 1–10% stained cells was scored as 1, 11–50% stained cells was scored as 2, 51–80% stained cells was scored as 3, and 81–100% stained cells was scored as 4. IS was calculated as follows: on a scale of 0–3, where 0 was no staining, 1 was weak staining, 2 was moderate staining, and 3 was strong staining. IRS was measured by multiplying QS by SI (McLendon et al, 2000). An IRS of 0-4 was considered weak, 5-8 was moderate, and 9-12 was considered strong (Hussein et al, 2002).

For intratumoral microvessel density (MVD):

Assessment, we first identified "hot spots" (areas with the highest microvessel concentration) by scanning the section at lower power magnification (X40) using Olympus microscope. Then the number of positive vessels in four hot spots high power fields; X 200 was counted. Single immunoreactive endothelial cell, or endothelial cell clusters separate from other microvessels, were counted as a vessel (Weidner, 1995). The presence of blood cells or fibrin without any detectable endothelial cells was not sufficient to define a microvessel. Vessels with muscular walls were not counted (Svagzdys et al, 2009). The mean microvessel density for CD31 was calculated as the mean value of the vessel count in four high power fields; X 200 (Dales et al, 2004).

Statistical analysis: ANOVA test (Analysis of variance) and Pearson's Correlation Coefficient tests were used with p value <0.05 was considered statistically significant.

RESULTS

Clinical and histopathological findings of ductal carcinomas:

The mean age of the patients was 53 years (range 26–77 years). The tumor size was >2cm and ≤5cm in 23/54 (43%) cases, and >5cm in 31/54 (57%) of cases. Axillary lymph nodes were positive in 36/54 (67%) patients. IDC of non-specific type (NST) was the most common histological pattern 42/54(77.8%). Lymphovascular invasion was observed in 21/54 (39%) cases. Lymphocytic infiltrate was prominent in 14/54 (26%) of cases. Desmoplasia was prominent in 35/54(65%) of cases. A summary of these data is presented in table (1). Figure (1) shows H&E in DCIS and IDC.

Table (1): Clinical and histopathological findings of ductal carcinomas

Paremeter	**No. of cases**
Histological types	
IDC NOS	42 (77.8%)
Medullary carcinoma	5 (9.3%)
Neuroendocrine carcinoma	2 (3.7%)
Micropapillary carcinoma	2 (3.7%)
Papillary carcinoma	1 (1.9%)
Tubular carcinoma	1 (1.9%)
Mucinous carcinoma	1 (1.9%)
Tumor grade of IDC (54)	
Grade I	6 (11%)
Grade II	29 (54%)
Grade III	19 (35%)
Lymphovascular invasion	
Absent	33 (61%)
Present	21 (39%)
Lymphocytic infiltrate	
Minimal	40 (74%)
Prominent	14 (26%)
Desmoplasia	
Minimal	19 (35%)
Prominent	35 (65%)

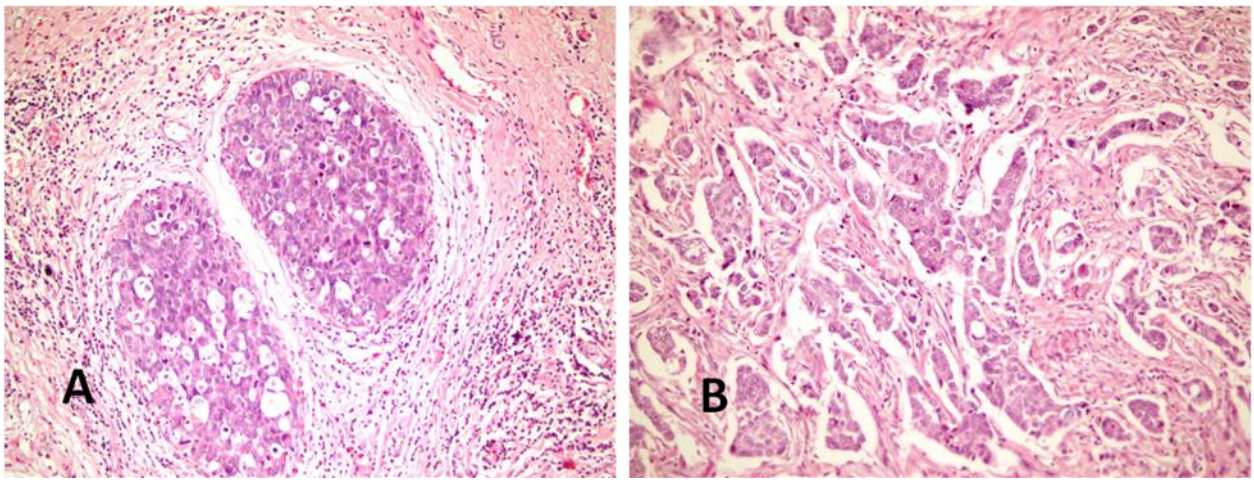

Figure (1): H&E stained sections of ***A.*** DCIS, ***B.*** IDC. Magnifications X 200 (A, B).

COX-2 expression and its relationship to clinic-pathologic features:

COX-2 immunoreactivity was brown granular cytoplasmic stain of the epithelial and myoepithelial cells. Some peritumoral mononuclear inflammatory and stromal cells also displayed cytoplasmic COX-2 staining. COX-2 expression was weak in 3/4 (75%) and moderate in 1/4 (25%) cases of normal breast tissue, whereas it was weak in 2/5 (40%), and moderate in 3/5 (60%) of cases of typical ductal hyperplasia of the breast. COX-2 was weak in 3/11(27.3%), moderate in 6/11(54.5%), and strong in 2/11(18.2%) of cases of DCIS. There was an increase in COX-2 expression with increasing grade of DCIS ($P< 0.05$; Table 2). COX-2 expression was weak in 11/54 (20.4%), moderate in 19/54 (35.2%), and strong in 24/54 (44.4%)

cases of IDC. There was an increase in COX-2 expression with increasing grade of IDC (P< 0.002; Table 3). Figure (2) shows COX-2 expression in DCIS and IDC.

Weak expression of COX-2 was often observed in normal-appearing lobular acini and ductal epithelium adjacent to IDC. When DCIS and IDC coexisted, COX-2 immunostaining in DCIS was usually less or equal to the staining of the corresponding IDC. COX-2 expression appeared to increase progressively along the continuum of neoplastic changes from normal breast epithelium to IDC (P< 0.01; Table 4).

There was statistically significant correlation between COX-2 expression and tumor size (P< 0.05), tumor grade (P< 0.002), lymphovascular invasion (P< 0.03), and lymph node metastasis (P< 0.02). However, no significant correlation between COX-2 and the age, lymphocytic infiltration, or desmoplasia was found (Table 5).

Table (2): COX-2 expression in DCIS

Tumor grade	COX-2 expression (IHCS)							IHCS (X±SD)
	weak			moderate		Strong		
	0	2	4	6	8	9	12	
Grade I (2)	0	1	0	1	0	0	0	4±2.8
Grade II (3)	0	1	1	1	0	0	0	4±2
Grade III (6)	0	0	0	2	2	0	2	8.7±2.73
P value	< 0.05 *							

ANOVA test is used, *, significant

Table (3): COX-2 expression in IDC

Tumor grade	**COX-2 expression (IHCS)**							**IHCS (X±SD)**
	Mild			**Moderate**		**strong**		
	0	2	4	6	8	9	12	
Grade I (6)	1	1	2	1	1	0	0	4±2.8
Grade II (29)	1	2	2	5	8	6	5	7.6±3.1
Grade III (19)	0	0	2	2	2	6	7	9.2±2.7
P value	< 0.002**							

ANOVA test is used, **; highly significant

Table (4): COX-2 expression in different breast lesions:

Histological stage	COX-2 expression (IHCS)							IHCS (X±SD)
	weak			moderate		Strong		
	0	2	4	6	8	9	12	
Normal breast (4)	0	3	0	1	0	0	0	3±2
Hyperplasia (5)	1	2	0	0	2	0	0	4±3.7
DCIS (11)	0	2	1	4	2	0	2	6.5±3.4
IDC (54)	2	3	6	8	11	12	12	7.74±3.3
P value	< 0.01*							

ANOVA test is used, *, significant

Table (5): COX-2 expression in IDC in relation to clinicopathological parameters:

Clinicopathological Parameter	NO	COX-2 expression			P value
		Low IHCS≤4 (11cases)	Moderate 6≤IHCS≥8 (19cases)	High IHCS>8 (24cases)	
Age					0.1 (NS)
<50	19	7	5	7	
>50	35	4	14	17	
Tumor size					0.05*
2-5	23	7	9	7	
>5	31	4	10	17	
Tumor grade					0.002**
Grade I	6	4	2	0	
Grade II	29	5	13	11	
Grade III	19	2	5	13	
Lymphovascular invasion					0.03*
Absent	33	9	13	11	
Present	21	2	6	13	
Lymphocytic infiltration					0.9 (NS)
Minimal	40	8	14	18	
Prominent	14	3	5	6	
Desmoplasia					0.6 (NS)
Absent	19	4	5	10	
Present	35	7	14	14	
Lymph node status					0.02*
Negative	18	7	6	5	
Positive	36	4	13	19	
Local aggressive manifestations					0.2 (NS)
Absent	47	10	18	19	
Present	7	1	1	5	

ANOVA test is used, NS; none significant, *, significant, **; highly significant

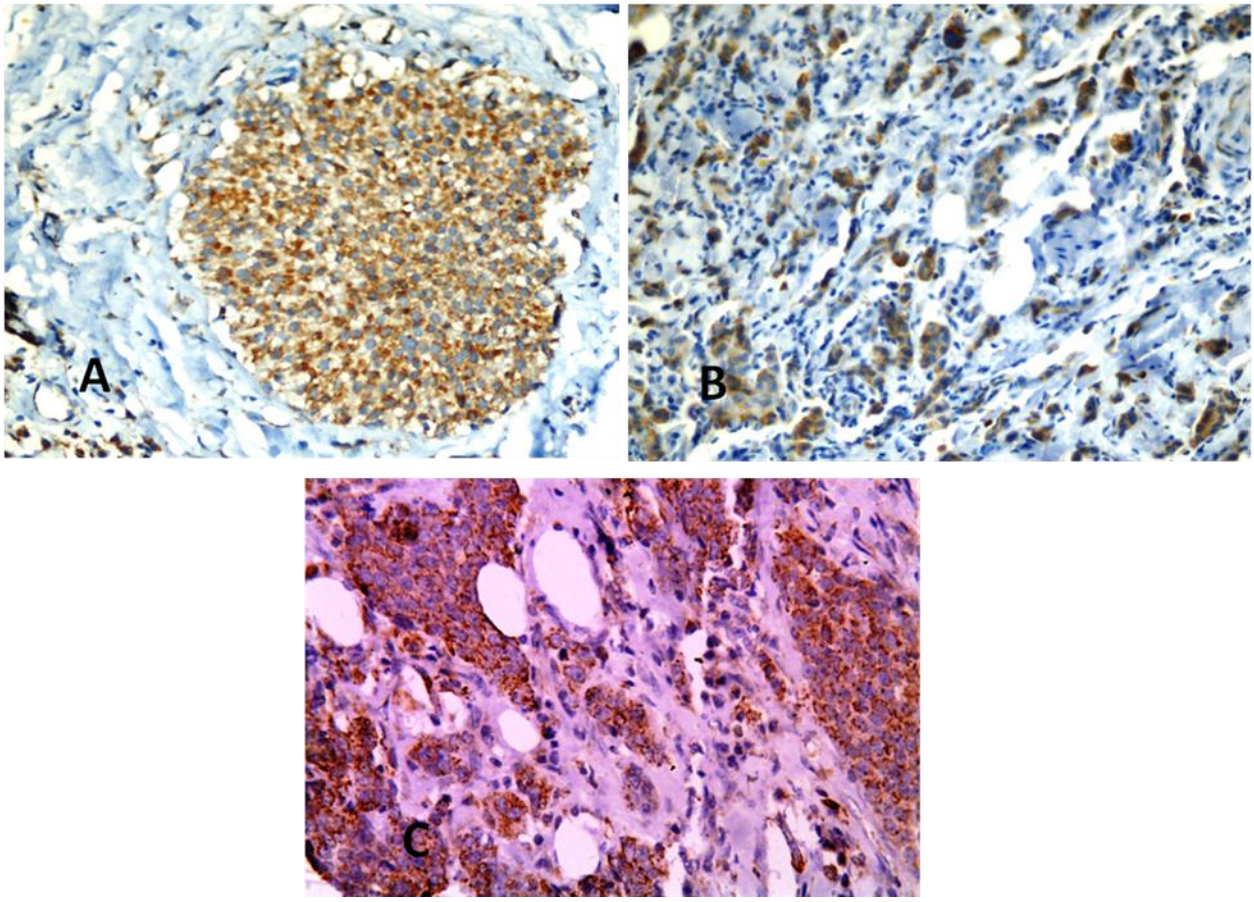

Figure (2): Moderate cytoplasmic COX-2 immunostain in ***A.*** DCIS, ***B.*** IDC grade II; Strong granular cytoplasmic COX-2 immunostain in ***C***. Magnifications X 200 (A), X400 (B, C).

CD31 expression:

CD31 staining was observed along the cell membrane of endothelial cells in all breast specimens. The median CD31 MVD count was 10 for the four cases of normal breast (range, 6-13), which increased insignificantly to 17 in the five hyperplastic lesions (range, 10- 27; P = 0.09). Then it reached 19 for DCIS (range, 10- 49; P = 0.3). But there was a highly significant increase in IDC reaching 66.5 compared with DCIS (range, 17- 165; P <0.000), Table 6.

There was insignificant increase in MVD between different grades of DCIS (P = 0.17). But there was a significant increase with increasing the grades of IDC (P < 0.01), as shown in tables (7 & 8). **Figure (3):** shows CD31 expression in IDC.

Relations between the estimated biological markers in DCIS and in IDC of the breast:
Positive correlation was present between COX-2 & CD31 in DCIS (r= 0.900, P <0.000). Positive correlation was also present between COX-2 & CD31 in IDC (r= 0.881, P <0.000)

Table (6): CD31 expression in different breast lesions

Histological type	Mean of microvascular density (MVD)	The median value of MVD for each group	P value for the difference between each two subsequent lesions
(1) Normal breast.			
1	6		
2	9	10	
3	11		
4	13		
(2)Hyperplastic lesions	10		P = 0.09 (NS)
1	11		
2	17	17	
3	23		
4	27		
5			
(3) *in situ* carcinoma	10		
1	12		
2	13		
3	15		
4	19		
5	19		
6	26	19	P = 0.3 (NS)
7	28		
8	33		
9	44		
10	49		
11			

(4) Invasive duct carcinoma			
1	17		
2	19		
3	22		
4	23		
5	27		
6	28		
7	29		
8	31		
9	33		
10	35		
11	35		
12	38		
13	40		
14	40		
15	41		
16	44		
17	44		
18	47		
19	48		
20	48		
21	52		
22	54		
23	54		
24	56		
25	61		
26	64		
27	66		P < 0.000**
28	67	66.5	
29	68		
30	72		
31	74		
32	75		
33	79		
34	83		
35	85		
36	86		
37	89		
38	91		
39	93		
40	95		
41	95		
42	97		
43	99		
44	99		
45	101		
46	103		
47	104		
48	105		
49	108		
50	108		
51	113		
52	120		
53	129		
54	165		

The test used is Pearson correlation, NS= Not significant, *= Significant, **= Highly significant

Table (7): CD31 expression in DCIS

Tumor grade	Mean of microvascular density (MVD)	The median value of MVD for each group	P value
Low grade			0.17 (NS)
1	15	17	
2	19		
Intermediate grade			
1	10	19	
2	19		
3	26		
High grade			
1	12		
2	13		
3	28	30.5	
4	33		
5	44		
6	49		

The test used is Pearson correlation, NS= Not significant,

Table (8): CD31 expression in IDC

Tumor grade	Mean of microvascular density (MVD)	The median value of MVD for each group	P value
a-Grade I			0.01*
1	22		
2	35		
3	48	51	
4	54		
5	68		
6	83		
b- Grade II			
1	19		
2	23		
3	27		
4	28		
5	31		
6	35		
7	38		
8	40	61	
9	41		
10	44		
11	47		
12	48		
13	52		
14	56		
15	61		
16	64		

17	67		
18	72		
19	75		
20	79		
21	85		
22	89		
23	91		
24	93		
25	95		
26	99		
27	101		
28	103		
29	105		
c- Grade III			
1	17		
2	29		
3	33		
4	40		
5	44		
6	54		
7	66		
8	74		
9	86	95	
10	95		
11	97		
12	99		
13	104		
14	108		
15	108		
16	113		
17	120		
18	129		
19	165		

The test used is Pearson correlation, *= Significant

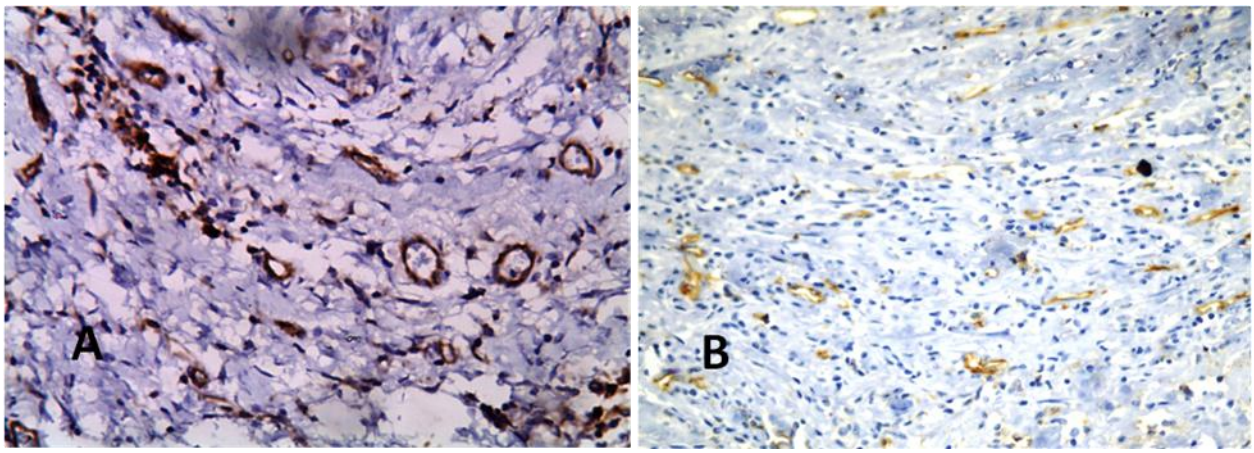

Figure (3): CD31 in IDC 108 microvessel/mm2 in ***A,*** CD31 in IDC MVD is 95 microvessel/mm2 in ***B;*** Magnifications X 200 (A) & X400 (B).

DISCUSSION

Breast cancer is a malignant tumor threatening women's health with an increasing incidence worldwide. There are still many deaths due to relapse or metastasis (Wang et al, 2009). Age is an independent prognostic factor for overall survival and disease free survival in breast cancer patients (Lin et al, 2005). In our study, patient ages ranged from 26- 77 years, with the highest age incidence between 45-59 years (mean age was 53 years) this is consistent with Nouh et al, (2004) who reported that the mean age was 47 years, in a larger study made in Upper Egypt. In developed countries, however there is a decrease in breast cancer incidence in women over 40 years due to decrease in using hormone replacement therapy (Jemal et al, 2007).

Tumor size is one of the most powerful predictors of tumor behavior in breast cancer. It constitutes the basis of major staging systems (Fryback et al, 2006). Our data showed that in 52% of the patients tumor size was more than 5 cm in diameter. This is in keeping with the National Cancer Institute (2003), which stated that the mean tumor diameter in Egyptian patients was 4.5 cm (Omar et al, 2003 & Seedhom and Kamal 2011). While, in developed countries, higher percentages of small sized tumors were present due to periodic examination and early detection using screening mammography (Nystrom et al, 2002).

The current hypothesis of tumorigenesis in humans suggests that cancer cells acquire their hallmarks of malignancy through the accumulation of gene activation and inactivation events over long periods of time. For breast cancer, this multistep process may manifest itself as a sequence of pathologically defined stages. It is widely held that breast cancer initiates as the pre-malignant stage of atypical ductal hyperplasia (ADH), progresses into the pre-invasive stage of DCIS, and culminates in IDC (Ma et al, 2003).

In our study *in situ* component was evident only in 6/54(11%) cases. This percentage is much lower than the findings of Tavassoli, (1999), who found foci of associated DCIS in up to 80% of cases of IDC. This marked difference may be explained at least in part by the late discovery of cases in our locality. Omar et al, (2003), found even smaller percentage of carcinoma *in situ* component (1.5%), in breast cancer patients in Upper Egypt.

IDC NST is the most commonly encountered form of IDC (Munirah et al, 2011). In our series most of the specimens were IDC NST (77.8%). This ratio looks near to that found by Li et al, (2003), Denkert et al, (2004), and El-Gendi & Abdel-Hadi (2009) who found that the frequency of IDC NST was 72.8%, 80% and 85%, respectively.

Tumor grade has been a highly valuable prognostic factor for breast cancer, as poorly differentiated lesions are associated with significantly poorer clinical outcome (Page et al, 2001). Histological grade may also provide useful information with regard to response to chemotherapy and, therefore, be a predictive factor as well as a prognostic indicator (Fryback et al, 2006).

In our study; 6/54 (11%) of IBC were grade I, 29/54 (54%) were grade II, and 19/54 (35%) were grade III. Which is consistent with the findings of Omar et al, (2003), who found that pathological grading of IDC in Egyptian patients showed a low incidence of grade I tumors (5.4%), while grades II and III tumors were 66.0% and 28.6% respectively. Our findings are in variance with Cho et al, (2006) who found that in IDC; 21.2% were grade I, 43.4% were grade II and 35.4% were grade III. This could be explained by the presence of certain genetic or environmental carcinogens which may lead to the development of aggressive tumor phenotypes in our locality.

Axillary lymph node is the most important prognostic factor affecting local control, disease-free and overall survival (Kim et al, 2005 and Seedhom & Kamal, 2011). In our study axillary lymph nodes were involved in 67% (36/54). This is in agreement with Jatoi et al, (1999) who found axillary lymph node involvement in 63.3% (1.068/1.696) of studied cases, and the finding of El-Bolkainy, (2000), on Egyptian patients, who reported that the frequency of axillary lymph node metastases in breast cancer was 75%. This finding is in contrast with that of Silverstein et al, (2001),

Denkert et al, ((2004), and Cho et al, (2006); who reported axillary lymph node metastasis in 36% (680 /1891), 52% (108/ 208), and 49.5% (49/ 99) respectively. This marked difference may be explained at least in part by the late discovery of cases in our locality.

Invasion of the lymphovascular channels is a necessary gateway to the metastatic process and is an independent prognostic indicator in breast cancer (Phelan et al, 2007). In our study, lymphovascular invasion was present in 39% (21/54) of breast cancer patients. Similar incidence was found by Cho et al, (2006), Mohammed et al, (2007), and Zhang et al, (2008), who found lymphovascular invasion in 42.4% (42/99), 31.6% (56/177) and 35.7% (25/70) respectively. However, higher incidence 78% (54/69) of lymphovascular invasion was found by Ito et al, (2007). This is most likely due to the use of lymphatic endothelial markers; D2-40 and podoplanin in the latter study. These are one useful markers in accurate detection of lymphovascular invasion by tumor cells.

There is no definitive conclusion regarding the efficacy of T cell-dependent immune mechanisms or regarding the correlation between the extent and type of lymphocyte infiltration and tumor progression in most subtypes of breast carcinoma (Vgenopoulou et al, 2003). Some studies noted an adverse effect on clinical outcome (Lee et al, 1997), and others observed either no significant effect (Hussein & Hassan, 2006), or a beneficial effect (Schumacher et al, 2001). Our study showed that lymphocytic infiltration was prominent in 26% (14/54) of breast cancer which is patient consistent with Demaria et al, (2001), who found that lymphocytic infiltrate was minimal in the majority of patients and showed no relationship with the clinical response.

Desmoplastic reaction is characteristic of IDC of the breast, and the intensity of this reaction can be different from case to case. The interactions between the tumoral stroma and the neoplastic cells are very important, and the tumor stroma can act as a regulator of neoplastic behavior (Ferrini and Rossi, 2001). In our study, desmoplastic stroma was evident in 65% (35/54) of cases which is in agreement with Ferrini and Rossi (2001), who found a ratio of 74% of tumors with prominent desmoplasia.

COX-2 is recognized as a promising pharmacological target for the prevention and treatment of many human cancers, on the basis of epidemiology, expression patterns, and preclinical studies. Several epidemiological studies reported an inverse correlation between breast cancer incidence and regular use of non-steroidal anti-inflammatory drugs including aspirin. In rodent tumor models, it has been shown that treatment with COX-2 inhibitors reduces the occurrence and growth of breast carcinomas (Cho et al, 2006).

COX-2 over-expression has been described in human breast cancer (Spizzo et al, 2003 and O'Connor et al, 2004). These studies found that the role of COX-2 in breast tumor progression was limited. In the current study COX-2 was often weakly expressed in normal appearing lobular acini and ductal epithelium. Weak expression of COX-2 was seen in 75% (3/4) cases, while moderate expression was observed in one case (25%).this is in agreement with Cho et al, (2006), who found that out of 15 normal breast cases, COX-2 expression was weak in 14 and cancer moderate in one case. In contrast Leo et al, (2006), found that in normal breast epithelium, 54% exhibited moderate or strong COX-2 expression, whereas 46% were negative for COX-2. This may be due to differences in methods, antibody used, scoring system, and protein expression cut- off levels.

COX-2 protein expression was weak in 40% (2/5) and moderate in 60% (3/5) of hyperplastic lesions. Although the number of patients studied is small yet this finding is supported by Cho et al, (2006), who found that COX-2 expression was strong in 1/15 (7%), moderate in 5/15 (33%), and weak in 60% (9/15) of cases similarly. Also Visscher et al, (2008), found that 23 (10%) showed no COX-2 staining, 107 (46%) showed weak, 71 (30%) showed moderate and 34 (14%) showed strong COX-2 staining.

In the present study COX-2 was weak in 27.3% (3/11), moderate in 54.5% (6/11), and strong in 18.2% (2/11) of DCIS, and its expression correlated positively with higher tumor grade ($p < 0.05$). This observation is comparable

with that of Cho et al, (2006), who found weak COX-2 expression in 11/30 (37%), moderate expression in 14/30 (47%), and strong expression in 5/30 (17%) of DCIS. But it was obviously highly different from that of Leo et al, (2006), who found moderate or strong COX-2 staining in 55% of DCIS, whereas 45% had negative COX-2 expression. This may be due to the smaller number of patients in our study.

In this study, COX-2 expression was weak in 11/54 (20.4%) of cases, moderate in 19/54 (35.2%), and strong in 24/54 (44.4%) of cases of IDC. Slightly different ratios for COX-2 expression was reported by Cho et al, (2006) who found weak COX-2 expression in 32/ 99 (32.3%), moderate expression in 35/99 (35.4%), and strong expression in 32/99 (32.3%). Leo et al, (2006) found that COX-2 expression was weak in 41% of cases, while moderate and strong expression was found in 55% of lesions collectively. This difference is most likely due to variable proportions of different tumor grades in those studies.

COX-2 expression showed progressive increase along the continuum of neoplastic changes from normal breast epithelium to IDC ($P< 0.01$). This is supported by the findings of Ciris et al, (2011). These results suggest that COX-2 expression may be involved in the progression of breast cancer, and may provide a clinically useful biomarker for estimating tumor aggressiveness. COX-2 may be a useful target for chemoprevention or increased therapeutic effectiveness in breast cancer.

We found positive correlation between COX-2 expression and tumor grade of IDC ($P< 0.002$). This was elucidated previously by several studies e.g. Ristimaki et al, (2002), Singh and Lucci (2002), Schmitz et al, (2006), Ciris et al, (2011), and Kim et al, (2012). These findings indicate that up-regulation of COX-2 expression is common in advanced breast carcinomas. on the contrary other studies revealed no significant correlation between COX-2 expression and tumor grade e.g. Nam et al, (2005), Cho et al, (2006), Leo et al, (2006), Dillon et al, (2008), and Zhang et al, (2008).

In this study, no correlation was present between COX-2 expression and age of the patients ($p <0.1$). This is consistent with the findings of Nam et al, (2005), Cho et al, (2006), and Zhang et al, (2008).

Tumor size is one of the strongest predictive factors for local recurrence, and tumors greater than 2 cm leads to decreased disease free survival (Lee et al, 2011). We found positive correlation between COX-2 expression in IDC and tumor size ($P <0.05$). This is consistent with the finding of Cho et al, (2006). While other studies revealed no significant correlations between COX-2 expression in IDC and tumor size e. g. Nam et al, (2005), Cho et al, (2006), Leo et al, (2006), Dillon et al, (2008), and Zhang et al, (2008).

We found positive correlation between COX-2 expression in IDC and lymphovascular invasion ($P <0.03$). This is consistent with the findings of Zhang et al, (2008). These data suggest that elevated COX-2 expression in breast carcinoma may reflect a more aggressive biological behavior. on the contrary other studies of Cho et al, (2006), and Leo et al, (2006), found no significant correlation between COX-2 expression and lymphovascular invasion.

Axillary lymph node metastasis is an important prognostic factor, and metastasis occurs through the lymphatic route (Lee et al, 2011). Current study showed positive correlation between COX-2 protein expression and lymph node positivity ($P< 0.02$)and this is consistent with Nam et al, (2005), Zhang et al, (2008), and Dillon et al, (2008). on the contrary Cho et al, (2006), and Leo et al, (2006) found no significant correlation between COX-2 protein expression and lymph node status.

Our results showed that COX-2 overexpression is a significant unfavorable prognostic factor in breast cancer, and provide selective criteria for anti-COX-2 combinations in IBC therapy. But we found no significant correlation between desmoplasia ($p< 0.6$), lymphocytic infiltration ($P< 0.9$), and local aggressive manifestations ($P< 0.2$). To the best of our knowledge, no previous studies commented on these relations.

Most breast cancer related deaths are not due to cancer at the primary site, but rather due to distant metastasis. Metastasis entails numerous

biological functions that collectively enable cancerous cells from a primary site to disseminate and overtake distant organs. Breast cancer metastasis can be undetectable and remain latent for many years following primary tumor removal only to emerge as incurable lesion that are triggered by unknown causes in a variety of organs including bone, lung, lymph nodes, liver and brain (Eltarhouny et al, 2008).

Due to its paramount clinical significance, much effort was devoted to exploring the molecular mechanism of metastasis. There is a public demand to identify hematogenic metastasis as early as possible in order to help those patients and improve their survival rate (Eltarhouny et al, 2008).

An important key step in tumor progression is the formation of new blood vessels from a pre-existing vascular network, known as angiogenesis (Richter-Ehrenstein et al, 2007). It has been demonstrated that an important event in the process of angiogenesis is the recruitment of endothelial progenitor cells to sites of the new vessel formation with subsequent differentiation into mature endothelial cells. In the mammary gland, the formation of vascular stroma was found to precede invasion (Vleugel et al, 2004), while higher levels of angiogenic marker molecules seem to be associated with poor prognosis (Martin et al, 2005).

It has been postulated that tumor cells do not invade into normal breast stoma but rather into a richly vascular stroma that they have induced. This process of neovascularization is driven by growth factors released into the stroma by tumor cells and immune cells. These findings might imply for new therapeutic strategies using anti-angiogenic factors in order to prevent the progression of DCIS to IDC (Pavlakis et al, 2008).

The process of developing a high-density vascular network that connects the tumor and host circulation, termed the 'angiogenic switch,' is a crucial step for the progression of a tumor from a benign to malignant state (Folkman et al, 1989). It has been suggested that the initiation of angiogenesis occurs simultaneous to invasion (Bergers & Benjamin, 2003). Our data supported the view significant increase in angiogenesis occurs in the stage of transition between DCIS to IDC ($P < 0.000$) on the other hand the increase in angiogenesis occurring on transition from normal to hyperplasia, and from hyperplasia to carcinoma *in situ* did not achieve significance.

Bluff et al, (2009) found that angiogenic switch occurs at the onset of hyperplasia in the mammary milk duct before any morphological evidence of atypia, although the greatest increase in angiogenesis occurs between *in situ* and invasive disease. Other studies with a larger number of benign and preinvasive breast lesions are needed to confirm or exclude these findings. These results also might point to new therapeutic strategies using anti-angiogenic factors in order to prevent the progression of DCIS to IDC. Previous studies have shown that a high MVD significantly predicts poor survival of breast cancer patients (both relapse-free survival and overall survival) (Uzzan et al, 2004).

Although not as strong as tumor size and axillary lymph node status, tumor grade is associated with poor prognosis, and higher grade tumors show aggressive behaviors (Lee et al, 2011). In our results, tumors with the highest MVD were histologically grade III, and there was a significant increase in MVD with increasing the grades of IDC ($P < 0.01$). This can be explained by the speculation that aggressive tumors are more capable of angiogenesis.

Most of the normal, hyperplastic lesions and *in situ* carcinoma in the current study were not different from the invasive tumors and a further evaluation in patients with only these lesions in alarger study is warranted.

Relation between COX-2 and CD31:

In this study we found a highly significant positive correlation between the expression of COX-2 and CD31, in DCIS ($P<0.000$), and in IDC ($p<0.000$). Angiogenesis is a complex process where several proteins and enzymatic pathways converge. COX-2 contributes to the regulation of angiogenesis by various genes, including platelet derived growth factor, VEGF, fibroblast growth factor-a, and TGF-b (Marrogi et al, 2000). Using selective inhibitors of COX-2, Tsujii et al, (1998) were able to block the expression of several angiogenic factors including

VEGF. Furthermore, Celecoxib (NS398), a specific COX-2 inhibitor, has been shown to inhibit the growth of 97% of colon cancer cells by reducing the number of hotspots in the tumor-stromal bed (Kawamori et al, 1998). similarly Marrogi et al, (2000) have shown that COX-2, NOS2, and VEGF levels correlated with the degree of MVD individually, and their levels appear to correlate with each other. Thus, it is likely that angiogenesis-based treatment protocols might target individual proteins in order to modumate yields in breast cancer therapy.

Conclusions:

COX-2 was increased with poor prognostic parameters; tumor size, tumor grade, lymphovascular invasion, lymph node metastasis and lymphocytic infiltration. CD31 increases with increasing grade of IDC. These findings might point for new therapeutic strategies in order to prevent progression of DCIS to IDC and to improve cancer therapy.

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التعبير المناعي الهستوكيميائي لتعبيري السيكلو أوكسيجينيز-٢ وال سي دي ٣١ وعلاقتيهما بالمؤشرات الاكلينيكية الباثولوجية في سرطان الثدي

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أقسام الباثولوجيا [١-٣]، والجراحة العامة [٤] وجنوب الوادي [٢] بطب سوهاج [١،٤] وأسيوط [٣]،

الخلاصة: يعد سرطان الثدي هو السرطان الأكثر شيوعا لدى السيدات المصريات. ويبدو أن السيكلو-أوكسيجينيز-٢ من المشاركين في التحول السرطاني وتطور الورم عن طريق التأثير على تكاثر الخلايا، الانقسام، التصاق الخلية، موت الخلايا المبرمج، والمراقبة المناعية، وتكون الأوعية الدموية. ويعتبر تكوين الأوعية الدموية خطوة مهمة ورئيسية في تطور الورم. ويمكن تقييم كثافة الاوعية الدموية الدقيقة عن طريق تقييم تعبير ال سي دي٣١. وتهدف هذه الدراسة الى:١. تقييم تعبيري السيكلو-أوكسيجينيز-٢ وال سي دي ٣١ في الخطوات المتتالية من تسرطن الثدي. ٢ .تقييم علاقة تعبيري السيكلو-أوكسيجينيز-٢ وال سي دي ٣١ بالقيم النذيرية الاكلينيكى الباثولوجية المحتملة في سرطان القنوات في الثدي.

المواد والطرق المستخدمة: وقد شملت هذه الدراسة على ٧٤ عينة من سرطان الثدي، وقد تم الحصول على عمر المريض وحجم الورم والتغيرات الموضعية العدوانية، وتاريخ وجود التكرار أوالانبثاث للورم بعيدا. وجرى تقييم القطاعات المصبوغة بالهيماتوكسيلين والايوسين لنوع الورم، ودرجة الورم، ووجود أو عدم وجود اجزاء عادية أو مفرطة التصنع، ووجود الورم في الموقع، والارتشاح الليمفاوي، وغزو الاوعية الدموية الليمفاوية، ووضع الغدد الليمفاوية في الإبطين. و تم عمل الصبغة المناعية للكشف عن تعبيري السيكلو-أوكسيجينيز-٢ وال سي دي٣١ بطريقة البيروكسيداز أفيدين - بيوتين.

النتائج: وقد ازداد تعبير السيكلو-أوكسيجينيز-٢ مع زيادة درجة سرطان القنوات في الموقع وسرطان الثدي الغازي. وقد كانت هناك زيادة في تعبير السيكلو-أوكسيجينيز-٢ تدريجيا على طول سلسلة متصلة من التغيرات الورمية من ظهارة الثدي الطبيعي الى سرطان الثدي الغازي. وقد وجدنا علاقة احصائية بين تعبير السيكلو-أوكسيجينيز-٢ وحجم الورم ودرجة الورم وغزو الاوعية الدموية الليمفاوية. وقد وجدنا علاقة احصائية بين تعبير السيكلو-أوكسيجينيز-٢ وايجابية الغدد الليمفاوية للورم. وقد لوحظ وجود صبغة ال سي دي٣١ على طول غشاء الخلايا البطانية للأوعية الدموية الدقيقة في جميع عينات الثدي. وقد بلغت كثافة الاوعية الدموية الدقيقة ١٠ للثدي الطبيعى، مع زيادة غير معنوية إلى ١٧ في الآفات المفرطة التصنع، ووصلت الى ١٩ في سرطان القنوات في الموقع و٦٦.٥ في سرطان الثدي الغازي وهي زيادة احصائية عالية الدلالة. وقد وجدنا زيادة احصائية لتعبير ال سي دي٣١ ذات دلالة مع زيادة درجة الورم في سرطان الثدي الغازي وليس في سرطان القنوات في الموقع.

الاستنتاجات: كانت هناك زيادة في تعبير السيكلو-أوكسيجينيز-٢ مع زيادة الدلالات السلبية في تشخيص سرطان الثدي في كونه يرتبط إيجابيا مع حجم الورم ودرجة الورم وغزو الاوعية الدموية الليمفاوية وايجابية الغدد الليمفاوية للورم. ويزداد تعبير ال سي دي٣١ مع زيادة درجة الورم في سرطان الثدي الغازي. و قد تعني هذه النتائج وضع اساس لاستراتيجيات علاجية جديدة لمنع تطور سرطان القنوات في الموقع إلى سرطان الثدي الغازي وتحسين علاج سرطان الثدي.