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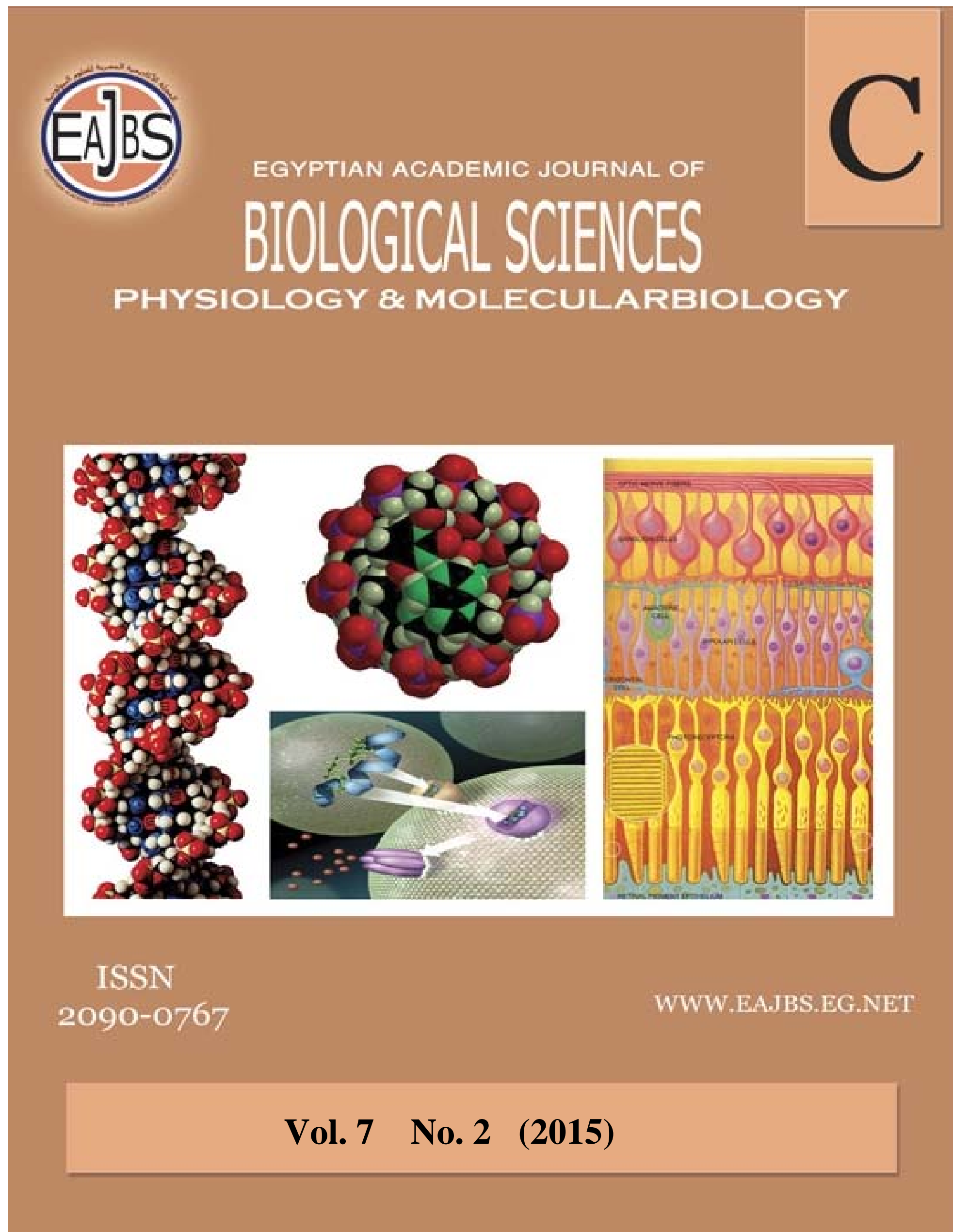

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Immunohistochemical IHC Study Expression of Vascular Endothelial Growth Factor (VEGF) and Endostatin in Breast Cancer Groups in Slahaldeen Women Patients

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ABSTRACT

Twenty Iraqi breast cancer cases were included in this study to detect the protein expression of vascular endothelial growth factor (VEGF) and endostatin (ES) in the women patients from Saladin governorate breast tissues, among breast cancer cases. Hematoxylin and Eosin (H and E) stained then immunohistochemically (IHC) designed for vascular endothelial growth factor (VEGF) and Endostatin. Regarding to histological grading, the results showed that among 20 patients 17 cases were grade II and 3 cases were grade III, while staging system 8 cases were stages (II); 12 cases were stage III. Immunohistochemical IHC study expression of Vascular endothelial growth factor VEGF in breast cancer groups shows positive expression found in 14 (70%) out of 20 cases of the Saladin breast can cercases with *P* value <0.001, Endostatin expression was observed in 15 (75%) out of 20 samples of the Saladin breast cancer patients with *P* value <0.05. Correlation between the two studied markers VEGF and ES was studied statistically and the study revealed that there was a strong significant relationship between the VEGF expression and expression of ES (*P* value <0.01), and also there was significant relationship between expression of VEGF and ES with grading (*P* value >0.001) and staging system of breast cancer patients (*P* value >0.05).

INTRODUCTION

The incidence of breast cancer increases with age, the disease is rare below the age of 25 year. Tumors of the breast are believed to arise from terminal ductal lobular unit and comprise two common morphological type: ductal and lobular neoplasia (Russo and Russo, 1999).

The vascular endothelial growth factor (VEGF) system is part of the platelet-derived growth factor gene family, and vascular endothelial growth factor A (VEGFA) is a very potent angiogenic growth factor that interacts with its specific receptors VEGFR-1 (flt-1) and VEGFR-2 (flk-1 or KDR) (Ferrara *et al*., 2003). Vascular endothelial growth factor B (VEGF-B), interacting with VEGFR-1, is another ligand in this gene family that is supposed to play a role in the maintenance of existing vessels, but this protein is less well described (Fischer *et al*., 2008; Li *et al*., 2009). VEGF-A, VEGF-B,VEGFR-1, and VEGFR-2 are expressed in a variety of normal cells, and over expression in different malignant tumors has been described (Dvorak *et al*., 1995; Ferrara *et al*., 2003; Fischer *et al*., 2008; Li *et al*., 2009).Endostatin, one of the most potent known natural inhibitors of angiogenesis (O'Reilly *et al*., 1997), is a C-terminal fragment of collagen XVIII, which is mainly localized in the basement membrane zones of the vessels (Muragaki *et al*., 1995), particularly in newly formed tumour associated blood vessels (St Croix *et al*., 2000).The aim of this study was to evaluate the immunohistochemical expression of Pro-angiogenic vascular endothelial growth factor (VEGF) and anti-angiogenicendostatin markers in cases of Iraqi breast carcinoma and the correlation between them.

MATERIAL AND METHODS

Immunohistochemistry (IHC) Technique:

Immunohistochemistry or IHC refers to the process of detecting antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos, Miller, 2014). The procedure was conceptualized and first implemented by Dr. Albert Coons in 1941(Coons *et al*.,1941). Visualising an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyze a colour-producing reaction (see immunoperoxidase staining). Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine.

Immunohistochemical Staining Procedure for Detection of VEGF and Endostatin:

All incubations were carried out in a humidified chamber to avoid drying of the tissue. Dilutions were adjusted appropriately from the results obtained. All incubation times were restricted to the protocol (Khashman, 2008).

IHC Staining Method Done according to (Lovino *et al*., 2008):

1. Tissue must be rehydrated before commencing staining protocol.
   a. Immersed the slides in xylene (mixed isomers) 2 times for 10 minutes each.
   b. Immersed the slides in 100% alcohol 2 times for 10 minutes each.
   c. Immersed the slides in 95% alcohol for 5 minutes.
   d. Immersed the slides in 70% alcohol for 5 minutes.
   e. Immersed the slides in 50% alcohol for 5 minutes.
   f. Rinsed the slides with deionized H_2O.
   g. Rehydrated the slides with wash buffer for 10 minutes. Drain the excess wash buffer.
2. Surround the tissue with a hydrophobic barrier using a barrier pen.
3. To quench endogenous peroxidase activity, incubate the sample with 1-3 drops peroxidase blocking reagent (3% H_2O_2 in water or methanol) for 5-15 minutes.

4. Rinsed the sample, then gently wash in wash buffer for 5 minutes.
5. To reduced non-specific hydrophobic interactions between the primary antibodies and the tissue, block the section with 1-3 drops of serum blocking reagent for 15 minutes. Drain the slides and wipe away any excess blocking reagent before proceeding to the next step. Do not rinse.
6. Incubated the sample with primary antibodies in Incubation Buffer. Follow manufacturer's recommendations regarding working dilution for the primary antibody. For chromogenic IHC staining of paraffin-embedded tissue sections using R&D Systems antibodies, it is recommended to incubate overnight at 2-8 °C. This incubation regime allows for optimal specific binding of antibodies to tissue targets and reduces non-specific background staining. These variables may need to be optimized for your system.
7. Covered stained tissue with a cover slip of an appropriate size, place slides vertically on filter paper or a towel to drain excess mounting medium, and allow them to dry.
8. Visualized staining of tissue under a microscope using a bright-field illumination.

Immunohistochemical Scoring of VEGF:

The percentages of cells positive for the VEGF expression of the cancer cells were scored as follows (Saponaro *et al.*, 2013):

0 = (no immunoreactivity),1 = (< 25%), 2 = (26 -50%), 3 = (51-75%), and 4 = (>75%).

The mean percentage of positively stained cells was calculated and the staining intensity was graded as follows (Saponaro *et al.*, 2013) :

0: negative, 1:weak,2: moderate, and 3: strong.

Immunohistochemical Scoring of Endostatin:

Each section of the socket tissue samples was evaluated for the presence of intracellular brown DAB precipitate indicative of antibody binding. The staining intensity was assessed using the following evaluation (Balasubramanian *et al.*, 2007): 0 = (negative), < 3 = (minimal staining), 3 – 6 = (moderate staining), and 7 - 9 = (strong staining).

Statistical Analysis:

The values of the investigated parameters were given in terms of mean ± standard error, and person correlation using SAS computer program version 7.5. Differences in results were considered significant at probability value equal or less than 0.05 and 0.001(SAS, 2004).

RESULTS AND DISCUSSION

Immunohistochemical IHC Study Expression of Vascular Endothelial Growth Factor VEGF in Breast Cancer Groups:

The IHC study for the VEGF expression of the revealed that VEGF, positive expression was found in 14 (70%) out of 20 cases, while 6(30%) cases were not expressed or negative cases. So, there was a highly significant difference (*P* value *P*<0.001) between the expression of VEGF among the malignant cases. These results are shown in Table (1).

Table 1: Immunohistochemical of VEGF Expression in ductal carcinoma.

Score group	0	+1	+2	+3	- ve	Total positive out of (20)
Malignant	5(25%) A	5(25%) B	3(15%) C	1 (5%) C	6(30%) A	14(70%) out of (20)

***P<0.001

Difference letters meanpresence of significant difference.

Same lettersmean there is no significant difference.

Figure (1) shows the expression of VEGF in cytoplasm of ductal carcinoma stained by IHC, brown stained cytoplasm indicated positive VEGF expression and blue stained cytoplasm indicated no expression for VEGF in these cells.

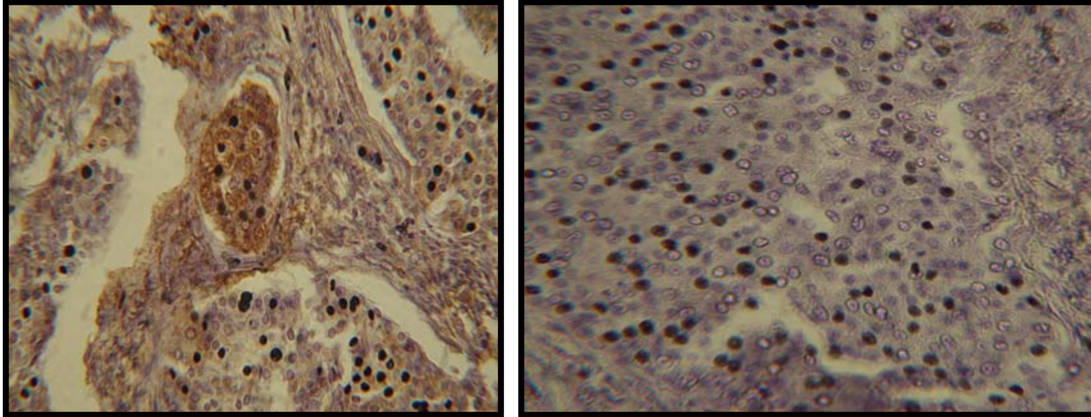

Fig. (1 A, B): Immunohistochemicalstaning in breast cancer sections. Immunostaining by peroxidase /DAB (brown) counterstained with heamatoxyline (blue), (A) positive VEGF expression(400X), (B) negative (no expression) for VEGF (400X).

Like all other solid malignant tumors, breast carcinomas need a vascular system for nutrition, oxygen supply, and to set metastases (Folkman, 1996). Angiogenic factors are necessary for the development of the tumor vasculature. VEGF has been suggested to be a promoter of tumor angiogenesis in breast carcinoma (Gasparini and Harris, 1995). It has been confirmed by other studies which have demonstrated that the increased level of VEGF is correlated with angiogenesis and cancer development (Kranz *et al.*, 1999) showing the leading role of VEGF in tumor angiogenesis and progression in many different cancers (Delli Carpini *et al.*, 2010; Tayama *et al.*, 2011). Excessive proliferation of tumor vessels caused by over-expressed pro-angiogenic factors and down-regulated angiogenesis inhibitors is the histopathological basis for the growth and metastasis of malignant tumors(Folkman, 2006). Some studies note that key angiogenic molecules, such as VEGF and VEGFR, could exhibit direct inhibition of tumor cell survival signals and result in apoptosis(Ling *et al.*, 2007).

Our immunoreactivity of VEGF was almost exclusively restricted to the tumor cells, which agrees with the results of Yoshiji *et al.*, (1996) who showed that most of the positive VEGF staining was associated with the tumor cells. Those results also are in agreements with Brown *et al.*, (1993). On the other hand, our results demonstrate that these significant association between VEGF level and breast cancer agrees with study of Lovino *et al.*, (2008), who clearly indicated that these over expression of VEGF of breast cancer patients. This result was similar to the study presented by Al Khafaji *et al.*, in 2010 as they showed that 70% of breast cancer patients were positive for VEGF by immunohistochemistry.

Immunohistochemical IHC Study Expression of Endostatin in Breast Cancer Groups:

The positive expressions of the endostatin were observed in 15 out of 20 samples (75%) a fact that +3 represents an over expression of endostatin. As in Table (2) that shows statistical analysis of the endostatin expression revealed significant difference (*P* value $P<0.05$) in the immunoexpressionamong the malignant cases. Figure (2) shows the expression of endostatin in ductal carcinoma stained by IHC, brown stained cytoplasm indicated positive ES expression and blue stained cytoplasm indicated no expression for ES in these cells.

Table 2: Immunohistochemical of Endostatin Expressionin ductal carcinoma.

Scoren Group	+1	+2	+3	-VE	Total positive out of (20)
Malignant	7(35%)A	4(20%)D	4(20%) D	5(25%) C	15(75%) out of (20)

**P<0.05
Difference letters meanpresence of significant difference.
Same lettersmean there is no significant difference.

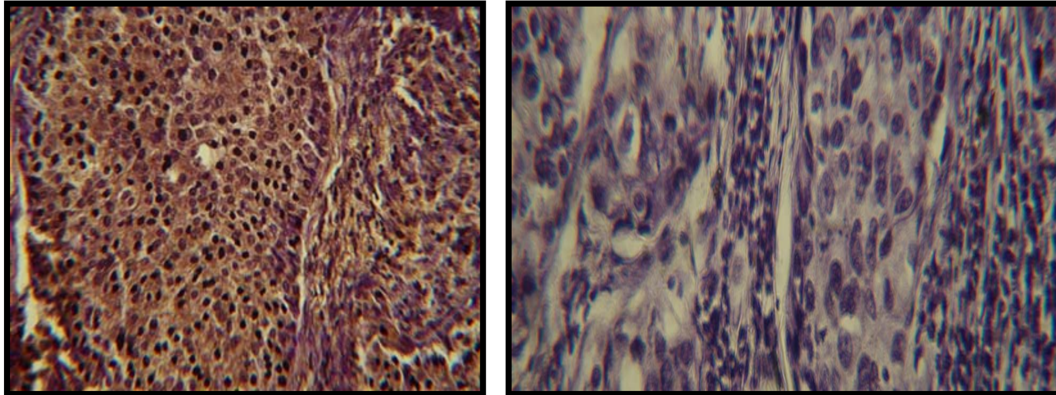

Fig. (2 A,B): Immunohistochemicalstaning in breast cancer sections. Immunostaining by peroxidase /DAB (brown) counterstained with heamatoxyline (blue), (A) positive ES expression (400X), (B) negative for ES (400X).

Endostatin is thought to be important in the preventing tumour progression mainly by inhibition of angiogenesis (Wickstrom *et al*., 2005). This has resulted in its evaluation in clinical trials in advanced cancer (Abdollahi *et al*., 2005). Our study shows highly expressed of endostatin in patients with breast cancer, this finding was in agreement with Zhao *et al*., (2004), who showed that endostatin level in patients with breast cancer is significantly higher than that in benign breast disease patients.Although the endostatin is an inhibitor of the angiogenesis, but that in the advanced stage of cancer contributes in the angiogenesis(John and Bradford, 2006).

Statistical Correlations of VEGF and ES Expression Markers:

The mode of correlations between the VEGF and endostatin markers in the benign samplestudied statically according to the Pearson correlation as shown in Table (3), there was a significant correlation between endostatin and VEGF (*P* 0.001).

Table 3: The correlations between the three IHC markers in the malignant BC cases.

Marker		VEGF	Endostatin
VEGF	Pearson Correlation Sig. (2-tailed) No.	_ _ 20	0.348 *** 0.005 20
Endostatin	Pearson Correlation Sig.(2-tailed) No.	0.348 *** 0.005 20	_ _ 20

***P<0.001
**Pearson's correlation between two variables is defined as the covariance of the two variables divided by the product of their standard deviations.

Endostatin (ES) is a potent inhibitor of angiogenesis and exhibits antiangiogenic activity by inhibiting the proliferation and migration of endothelial cells (ECs) in addition to inducing endothelial cell (EC) apoptosis at this time the level of VEGF increase.

In the present study, there was significant association between VEGF monoclonal antibody and Endostatin expression. These results are in accordance with other studies that elevated and showed a significant correlation between these markers in cancer patients (Feldman *et al*., 2000; Zhao *et al*., 2004).

Several proangiogenic growth factors and endogenous inhibitors of angiogenesis have been identified in breast cancer, among which the most important angiogenic regulatorsare considered to be VEGF and ES (Lantzsch *et al*., 2002).

CONCLUSION

Development of mor eresearch and use *in situ* hybridization(ISH) of detections and molecular studies using PCR technique for detection the cluster of genes responsible of breast cancer and comparison between them.

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