Expression of Vascular Endothelial Growth Factor and Ki-67 in Ductal Breast Carcinoma in Central Sudan

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Abstract

Breast cancer is the second most common cancer overall, ranks the 5th as a cause of death in the world. The prevalence of breast cancer is rising every year and it is the most common and leading cause of death in Sudan. The objective of this study is to assess the significance of vascular endothelial growth factor (VEGF) protein over expression and cell proliferation marker (Ki-67) with the grade of female ductal carcinoma. The study was carried out in the Histopathology and Cytology Laboratory of Faculty of Medicine, University of Gezira in 2014. A retrospective study was employed on paraffin-embedded blocks from 49 female patients with breast cancer. One ductal carcinoma in situ grade II and one ductal carcinoma in situ grade III, 20 patients were invasive ductal carcinoma with NG II and 27 were invasive ductal carcinoma NG III. The study was designed to apply immunohistochemistry using super sensitive one step polymer – HRP IHC/DAB for detection of both vascular endothelial growth factor and Ki-67. All forty nine cases showed the expression of VEGF. From the 49 cases of the study 47 cases (95.7%) showed high density stain for Ki-67 and two moderate. The overall expression of VEGF and Ki-67 was seen in all patients showing that there is no difference due to age. This study demonstrated that the co-expression of VEGF and Ki-67 indicates the involvement of VEGF in the tumorigenesis and invasion and this supports the evidence of its role of angiogenesis and cell survival. Furthermore, angiogenesis may enhance to metastasis of mammary carcinoma to other organs making the prognosis poor and treatment difficult. The study recommended that further studies to be done on the effect of anti-angiogenic drugs on blocking the action of vascular endothelial growth factor to localize the cancer in the breast preventing growth, progression and spread of cancer making the treatment more successful.

Key words: Breast cancer diagnosis; VEGF; Ki-67; Sudan

1. Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. Breast cancer is cancer that forms in the cells of the breasts which can occur in both men and

women, but it's far more common in women. Breast cancer incidence is much higher in the Western world, whether in Europe or North America, than in third world countries. North American women have the highest incidence of breast cancer in the world (Jemal, *et al.*,2012).

Breast cancer continues to remain the most lethal malignancy in women across the world. It was estimated that 12.7 million new cancer cases and 7.6 million cancer deaths occurred in 2008 worldwide (Ferlay, et al.,2010). Approximately, 1.4 million women were diagnosed with breast cancer worldwide with corresponding 458,000 deaths. Breast cancer is the second most common cancer overall (1.4 million cases, 10.9%), but ranks 5th as cause of death in the world (458,000, 6.1%) (Ferlay, *et al.*,2010). Breast cancer is the most commonly diagnosed cancer in women and the second leading cause of death in women in Africa, with much geographical variation in incidence and mortality within the continent (Jemal, *et al.*,2012). Studies have shown that breast cancer in African women is characterized by younger age at onset, advanced stage at diagnosis, and consequently poor prognosis (Adebamowo and Ajayi, 2000). Although the current incidence of breast cancer in Sub-Saharan African countries is low compared to that in developed countries (Jemal *et al.*, 2012), the cancer prevalence is rising every year especially in Sudan and the majority of the patients were <50 years old or premenopausal. Invasive ductal carcinoma was the most common pathology (82% of the cases), and women presented with stage III or higher tumors had already metastasized (Elgaili *et al.*, 2010).

A number of epidemiological studies revealed that the commonest cancers in women are those of the breast, cervix and ovary, with breast and cervical cancer accounting for 50% of all cancers among Sudanese women (Hamad,2006) and female breast cancer is by far the leading female cancers in Sudan, it accounts for 16.12% of all cancers (30.24% of all female cancers) in 2006 (Ahmed *et al.*, 2010).

angiogenesis is necessary for tumor growth and metastasis, anti-angiogenic agents targeting VEGF signaling pathway has been suggested as a promising approach for cancer treatment, therefore strategies for treatment of cancer are currently a focus of major scientific interest. However, information about the role of immunohistochemical tumor expression of VEGF that is assumed to be the most potent angiogenesis factor is ambiguous and their correlation with microscopic grade is still limited and controversial in female infiltrating mammary carcinomas. VEGF and Ki-67 are immunohistochemical markers which are responsible for angiogenesis and cell proliferation respectively, therefore studying the relationship between these markers may aid in understanding the role of VEGF in angiogenesis and cell proliferation with histological grades of breast cancer and it will be helpful in designing treatment strategies.

2. Definition of Study and Study Area

This is a retrospective and descriptive laboratory based study for assessment the expression of vascular endothelial growth factor (VEGF) protein and cell proliferation marker (Ki-67) in ductal breast carcinoma.

The study was done in Medical laboratory, Faculty of Medicine, University of Gezira, Wad-Madani, Sudan in 2014. Wad-Madani is the capital of the Al-Gezira state in east-central Sudan which lies on the west bank of the Blue Nile, nearly 115 miles (186 km) southeast of the capital city, Khartoum.

3. The Ordinary Haematoxylin And Eosin (H&E) Stain

From the selected paraffin embedded breast tissue cases, 4μm section were cut using microtome, the sectioned tissue was taken by slides from water bath and then the

slides put in oven for 30 minutes in temperature of 65 ºC to melt the wax, then the slides were deparaffinized in three changes of Xylene for 5 minutes, then the slides were hydrated by immersing in decreasing concentration of alcohol at 100%, 95% through 70% to distilled water for 2 minutes in each stage. For staining of the nucleus, the sections treated with Mayer's Haematoxylin for 8 minutes and differentiated by rinsing in acid alcohol for seconds, bluing in running tap water for 8minutes, counterstaining in Eosin for 1 minute, and rinsed in water. The sections dehydrated in 70% alcohol through 95% and 100% alcohol, and then blotted in a filter paper, cleared in xylene and mounted in DPX, after that the smears were ready for microscopic examination.

Interpretation of the results: Nucleus; deep blue colour. Cytoplasm and background tissue; pink colour. RBCs; orange colour (Bancroft JD and Marylin, 2002)

4.   The Immunohistochemistry Method (Super Sensitive One Step Polymer- HRP IHC/DAB)

Sectioning: The tissue sections were cut 4μm thickness using microtome and taken by positively charged slides from water bath. Then the slides were put in oven for 30 minutes to melt the wax.

Depparafinizing: The wax from the tissues was removed by EZ deparaffinizing solution. Preparation of EZ deparaffinization solution by taking equal volumes of concentrated EZ deparafinization solution and absolute alcohol (25ml of EZ deparaffinization solution + 25ml of absolute alcohol). The slides were immersed at three changes of EZ solution for three minutes each, and then the slides were washed in running tap water for 2-3 minutes.

Antigen retrieval: Most formalin fixed tissue requires an antigen retrieval step before immunohistochemical staining can proceed. This is due to the formation of methylene bridges during fixation, which cross-link proteins and therefore mask antigenic sites. The two methods of antigen retrieval are heat mediated (also known as heat induced epitope retrieval, or HIER) and enzymatic. In this study HIER was used by putting slides in EZ –AR solution of acidic PH in water path.

Antigen retrieval steps: The AR solution in a coplin jar was pre –heated at water path of 95ºC - 98 ºC, the slides were put in the AR solution in water path for 40 minutes, then the slides were put at room temperature for 20 minutes for cooling. The slides were washed in running tap water for 1 minute.

Blocking Endogenous peroxidase: Before putting the peroxidase block on the slides, the slides were washed using a buffer (PBS) and the buffer was prepared by adding wash buffer solution to distilled water 1:19 (16ml of PBS + 284ml of Distilled water). The slides were washed by buffer 3-times the tissue was circulated by pap pen and PBS was added on the tissue to prevent dryness. 3% hydrogen peroxide in water was used to block endogenous peroxidase present in the tissue to prevent false positive reactions. The PBS were discarded and replaced by 100μL peroxidase block for 10 minutes.

Blocking non specific background: Antibodies and other proteinaceous reagents tend to bind non specifically to membranes and tissues. To minimize this non-specific adsorption, a blocking buffer was used. Primary requirements of an ideal blocking buffer include enhancement of the signal and no cross-reactivity with the detection components. Power block reagent shows no cross reactivity, as it does not contain biotin or enzymes found in non-fat milk preparations, and it blocks more effectively than serum albumins. After washing the peroxidase block by PBS, the slides were incubated by power block for 10 minutes.

Primary antibody: The power block was not washed by PBS, primary antibodies were added on slides after discarding the power block and incubated for one hour. The primary antiboidies were anti-Ki-67 and anti-VEGF. Ki-67 used in the study was rabbit monoclonal antibody to Ki-67 purified ascites diluted in PBS, PH 7.6 containing 1% BSA and 0.09% sodium azide. Anti-VEGF used in the study was rabbit polyclonal antibody to VEGF from purified immunoglobulin fractions diluted in PBS, PH 7.6 containing 1% BSA and 0.09% sodium azide.

Interpretation of Ki-67: According to the St Gallen International Expert Consensus, the Ki-67 immunoreactivity was quantified as high (immunostaining ≥30%), intermediate (between 16 to 30%) and low (immunostaining <15%)(Goldhirsch et al., 2009; 2011). As negative controls, the primary antibody was omitted. The result was interpreted as negative, low, moderate and high.

Interpretation of VEGF: The primary antibody was omitted for negative control and the result was interpreted as negative and positive

5. Results

5.1 The age distribution of patients:

Age ranged from 31 to 85 years and mean value was 51.7 years with 12.85 Standard Deviation (table 5.1).

5.2 The distribution of in situ carcinoma according to the histological grade:

Two of the study cases were in situ carcinoma, one of them grade III and the other grade II. No case of grade I was detected (table 5.2).

5.3 The distribution of infiltrative carcinoma according to the histological grade:

Forty seven of the study cases were infiltrating ductal carcinoma, Twenty seven of them were grade III and the other twenty cases were grade II. No case of grade I was detected (table 5.3).

5.4 The relationship among grades of breast carcinoma with VEGF and Ki-67:

VEGF was over expressed in all cases, Ki-67 showed dense stain in most cases (table 5.4).

Table (5. 1) shows the age distribution of patients

Age	Frequency	Percent
30-45	16	32.7
46-60	23	46.9
61-75	8	16.3
more than 75	2	4.1
Total	49	100.0

Table (5.2) shows the distribution of in situ carcinoma according to the histological grade

Grade of BC	Frequency	Percent
DCIS I	-	-
DCIS II	1	50%
DCIS III	1	50%
Total	2	100.0

Table (5.3) shows the distribution of infiltrative carcinoma according to the histological grade

Grade of BC	Frequency	Percent
IDC I	-	-
IDC II	20	42.6
IDC III	27	57.4
Total	47	100.0

Table (5.4) shows the relationship among grades of breast carcinoma with VEGF and Ki-67

Grade of BC	VEGF		Ki-67	
	Positive	Negative	High density	Intermediate
DCIS II	1	-	1	-
DCIS III	1	-	1	-
IDC II	20	-	19	1
IDC III	27	-	26	1

6. Discussion

Breast cancer is a threatening malignancy that compromise the most common non skin malignancy among female (Sharma *et al.*, 2010) and the most common cause of cancer-related deaths in this gender after lung cancer (Kumar *et al.,* 2010). Breast Cancer is the most common and leading cause of death of females in Sudan (Elgaili *et al.*, 2010). Therefore, assessment of factors promoting tumor progression is necessary.

The heterogeneity of breast cancer has been sustained by the development of microarray-based prognostic gene signatures. This was acclaimed as a major breakthrough for the management of breast cancer patients (Stathopoulosa G.P. *et al.*, 2014).

Vascular endothelial growth factor was detected in the cytoplasm and on the membranes of the carcinoma cells.

All the cases which were selected in this study were positive for VEGF with over expression. There was no nuclear staining and in the majority of cases staining intensity was similar in all tumor cells. As negative controls, the primary antibody was omitted. Correlations between VEGF expression and the grades of the Breast cancer was not possible by Pearson method due to 100% positivity of the cases, but the result indicates that there is strong VEGF positivity which promotes angiogenesis. Our findings agree with (Ghasemi M *et al.* 2011), who found over expression of VEGF in 72.54% of the breast cancers. It also agrees with studies done by Al- Harris *et al* in 2008 that in 62.7% of malignant cells there is over expression of VEGF. This indicates high angiogenic activity in mammary ductal carcinoma in our study. This is true for in situ and infiltrative cancer and also true for intermediate and high grade tumors.

Angiogenesis is essential for tumor growth and invasiveness. VEGF, also known as a vascular permeability factor (VPF) is recognized to be the most potent stimulant and the key regulator for angiogenesis (Adams J, *et al*., 2000) and it stimulates extravasation of plasma proteins, such as fibrin, which when deposited in the extracellular matrix, may serve as a foundation for the formation of tumor stroma and new capillary network (Dvorak, H. F. *et al*,.1987). The VEGF are not only expressed in tumor cells but also in occasional stromal cells adjacent to necrotic zones in adenocarcinomas of the gastrointestinal tract (Hlatky. L. *et al*,.1994).

The relations of vascular endothelial growth factor (VEGF) with other clinicopathologic features of breast cancer remain uncertain (Ghasemi M *et al*., (2011). There is some molecular evidence of vascular permeability factor receptor mRNA strong expression in endothelial cells of small vessels adjacent to malignant tumor cells in DCIS, infiltrating ductal carcinoma, and metastatic ductal

carcinoma. In contrast, no definite labeling for receptor mRNA was detected in infiltrating lobular carcinoma or nonmalignant breast tissue (Brown, L. F.*et al.*,1993).

There have been nine independent studies published on the evaluation of the prognostic significance of VEGF in operable human breast cancer using different methods, eight out of the nine studies confirmed the prognostic relevance of VEGF (Gasparini.G *et al*,. 2001) .

Ki- 67 antigen was originally identified by Gerdes *et al* in early 1980s (Yerushalmi R, *et al*.2010). The advent of new genetic tests has emphasized the role of proliferative genes, including Ki67, as prognostic and predictive markers (Assersohn L, *et al.*, 2003). American society of clinical oncology does not include Ki- 67 assessment as a part of their existing guidelines as routine biological marker that can be used in treatment of breast carcinoma. However, its role as a prognostic marker for breast carcinoma is undeniable as it serves as a predictive tool in identifying patients who can benefit from chemotherapy or hormonal treatment (Yerushalmi R, *et al.*2010).

Breast cancer aggressiveness appears to be directly related to the percentage of Ki-67 positive cancer cells (Tavassoli FN,1992; Papantoniou *et al.*, 2004). Ki-67 is considered to have prognostic value as a proliferative marker. It is also considered to be modulator and has been shown to be an appropriate end point for preoperative studies involving hormonal therapies (Dowsett M, , *et al.*, 2007; Stathopoulosa G.P *et al.*, 2014).

In our study we found that forty seven densely positive and two intermediate positive cases. Our findings agree with Al- Harris *et al* in 2008 that in malignant cells there is overexpression of Ki-67. This indicates high proliferative index in mammary ductal carcinoma in our study. This is true for in situ and infiltrative cancer and also true for intermediate and high grade tumors.

7. Conclusion

Both VEGF and Ki-67 markers are highly expressed in mammary ductal carcinoma in Sudanese patients.

Breast cancer in Sudanese patients is rich in vascular formation and has high proliferative index, therefore, expected to be aggressive and metastasizes readily.

Ethical Approval

Ethical clearance was obtained from ethical committee of the ministry of Health, Gezira Estate. The samples were collected from the bank of the Laboratory and all cases were reviewed for histological type, histological grade. Grading was done according to Nottingham Grading System.

Competing Interests

Authors have declared that no competing interests exist.

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