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Angiogenic activities of interleukin-8, vascular endothelial growth factor and matrix metalloproteinase-9 in breast cancer

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Abstract

Angiogenesis is a major contributor to tumor growth and metastasis within breast cancer tumor microenvironment in which different proangiogenic factors have been identified and associated with tumor progression, metastasis and poor prognosis. The aim of the current study was to evaluate the angiogenesis among breast cancer patients through *ex vivo* assessment of the angiogenic factors interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF)-A expressions in excised tumor tissues as well as matrix metalloproteinase 9 (MMP-9) serum levels as well as the prognostic value of MMP-9. Our study included 28 invasive ductal carcinoma female patients who were scheduled for modified radical mastectomy at Medical Research Institute, Alexandria University, Egypt and 10 control subjects. Both IL-8 and VEGF-A expressions were immunohistochemically detected in tumor tissues and serum MMP-9 was determined by ELISA. Although no significant correlations were found between each of IL-8, VEGF-A, MMP-9 levels, and patients' clinicopathological parameters, a significant positive correlation was found between these angiogenic factors each other suggesting their synergistic roles in proceeding angiogenesis. Higher serum MMP-9 level was detected in breast cancer patients compared to the control group, indicating that it can be used as a prognostic biomarker in breast cancer patients.

Keywords: breast cancer, angiogenesis, IL-8, VEGF-A, MMP-9

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Introduction

In Egypt, breast cancer is estimated to be the most type of cancer among females accounting for more than 34.4% of cancer cases and 29.1% of cancer related deaths.[1, 2]

Breast cancer is a complex, multifactorial disease where there is a strong interplay between genetic and environmental factors.[3] It is known that tumorigenesis is a complex process consisting of different stages: initiation, progression and metastasis.[4] Tumor microenvironment (TME) is linked to each of these stages.[5] Activation of angiogenesis is considered as a prerequisite for tumor growth and metastasis.[6] Hypoxia can trigger angiogenesis by increasing the expression of inflammatory cytokines that help in the recruitment of vascular cells and neovascularization.[7]

In breast cancer, different proangiogenic growth factors have been identified such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), matrix metalloproteinase 9 (MMP9), platelet derived growth factor (PDGF), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1β and interleukin-8 (IL-8).[8, 9]

IL-8 (CXCL-8) is a chemokine which is implicated in the initiation of leukocyte infiltration, neovascularization, and angiogenesis. IL-8 secreted by tumor cells, has an effect on endothelial cells and other cells that express IL-8 receptor leading to increased production of growth factors that are involved in metastasis.[10, 11] Specifically, a study of Ju et al., 2017,[12] showed that IL-8 activates VEGF expression in endothelial cells through autocrine signaling, thereby promoting angiogenesis. Furthermore, IL-8 enhances the production of matrix metalloproteinases like MMP-2 and MMP-9 in tumor cells, thereby promoting tumor dissemination. [13]

VEGF family plays a major role in vascular maintenance, inflammation and cancer progression.[14] The most prominent member of this family is VEGF-A.[15] Increased expression of VEGF and its receptor has been observed in different types of cancer.[16] In breast cancer, high expression of this angiogenic factor is correlated with poor prognosis and decreased patients' survival.[17] A study carried out by Ronald et al., 2009,[18] showed that anti-VEGF therapy inhibits the growth of different tumors by limiting vasculature and the infiltration of tumor cells.

MMPs are a family of proteases that play a vital role in cancer development and progression.[19] MMP9 which is an important member of this family has been associated with tumor invasion and metastasis through its ability to degrade collagens within the extracellular matrix and this facilitates the migration and detaching of tumor cells.[20] MMP-9 overexpression has been observed in different types of cancers including breast cancer and is correlated to poor prognosis.[21]

The aim of the current study was to evaluate the angiogenesis in breast cancer TME through assessment of the angiogenic factors IL-8, VEGF-A and MMP-9 as well as the prognostic value of MMP-9.

Subjects and Methods

Subjects

The current study was conducted on 28 Egyptian females with breast cancer histologically proved to be invasive ductal carcinoma (IDC) and scheduled for modified radical mastectomy. Patients were recruited from the Department of Surgery and Experimental Medicine, Medical Research Institute, Alexandria University. Smokers, diabetics, and hypertensive patients were excluded from the study. None of the patients had an immunologic mediated disease. The study protocol was reviewed and approved by the Research Ethics Committee, Medical Research Institute, Alexandria University (April 2013). An informed consent was obtained from each patient participating in the study according to the Ethical Committee of Medical Research Institute.

All patients were subjected to full history taking, thorough clinical examination with special reference to the stage of the disease as well as lymph node involvement. Fine needle

aspiration cytology (FNAC) was performed. Data of radiological studies (plain chest X-ray, mammography for both breasts, and abdominal ultrasonography for liver and other abdominal organs) were obtained from patients' records.

Methods

Tumor Tissue Samples' Preparation

A tumor sample was obtained from surgically excised breast of each patient, fixed in 10% phosphate-buffered formalin pH 7.4 for 24 hours and processed for preparation of paraffin wax blocks- and microscopic slides. One slide was stained with Haematoxylin and Eosin stain (H&E) for histological evaluation, and the other slides were immunohistochemically evaluated for the level of IL-8 and VEGF-A as markers of angiogenesis.[21-23]

Detection of IL-8 and VEGF-A

Tissue blocks were cut into 4-5 µm thick sections using rotatory microtome, then collected on clean glass slides, deparaffinized and rehydrated. Slides were incubated in 3% hydrogen peroxide (Thermo Fisher Scientific, UK) for 10-15 minutes to block endogenous peroxidase. The slides were then washed 2 times in phosphate buffer saline (PBS) and placed in a jar filled with retrieval sodium citrate buffer 0.01 M, pH: 6.0, then heated in a microwave oven at 100^{o}C for 3 minutes with interval of 1 minute. Slides were then incubated with normal goat serum diluted 1:5 in phosphate buffered saline (PBS, Thermo Fisher Scientific, UK) for 30 minutes to reduce non-specific binding of biotinylated secondary antibody. The sections were covered with 2 drops of the diluted rat monoclonal IgG1 (Clone: NAP11, eBioscience, UK) (10µg/ml) for IL-8 detection and diluted rabbit polyclonal antibody for VEGFA detection (Prosci-INCORPORATED, USA) (5µg/ml), then the sections were covered and incubated overnight at 4^{o}C in a humid chamber. The slides were then washed 4 times with PBS. Biotinylated goat anti-polyvalent was applied and the slides were incubated for 10 minutes at room temperature. Streptavidin peroxidase was applied, and the slides were then incubated for 10 minutes at room temperature. One drop of 3,3' Diaminobenzidine (DAB) plus Chromogen (Sky Tek Laboratories, USA) was added to 1 ml of DAB plus substrate, and the slides were then incubated for 5-15 minutes. Finally, the slides were counterstained in Mayer's Haematoxylin and Eosin and coversliped using a permanent mounting media. Visual assessment of immunohistochemistry (IHC) staining was performed with a light microscope using the semi-quantitative scale described by Bromley and Colleague.[24] The DAB chromogen yielded brown color reaction at the site of target antigen. According to the intensity of color staining, the tumor samples were graded as negative, weak (+), moderate (++), and strong (+++).

Detection of MMP-9 in serum

It was achieved by human MMP-9 ELISA kit (Human MMP-9 Platinum ELISA Kit eBioscience, UK) according to the manufacturer's instructions. A standard curve was created using seven MMP-9 standard dilutions provided in the kit and MMP-9 samples concentration were determined. The detection threshold of MMP-9 was 0.05 ng/Ml and the intra-assay coefficient of variation was 5-10 %. All tests were run in duplicates according to the manufacturer's instructions.[25]

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov was used to verify the normality of distribution of variables. Quantitative data were described using range as well as mean ± standard deviation (mean±SD). Comparison between two independent population were done using independent t-test. Spearman coefficient was used to correlate between quantitative variables. Significance of the obtained results was judged at the 5% level.[26]

Results

The mean age of our patients was 53.46 ± 10.44 years. The histological type of our patients' breast cancer was of IDC. According to the modified Bloom and Richardson (1992) classification, grade II was found in all these

patients. Tumor size, lymph node involvement and metastasis are demonstrated in Figure 1. Pathological staging and hormonal status are demonstrated in Figures 2, 3 A and B.

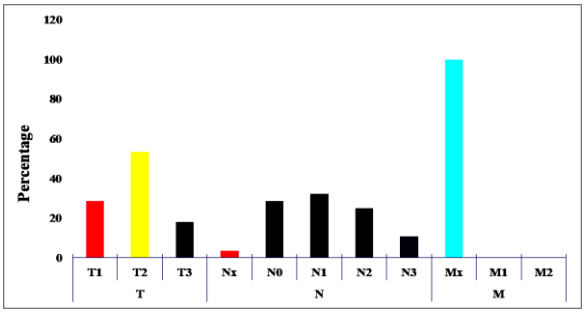

Figure 1. Distribution of studied patients according to tumor size (T), lymph node involvement (N), and metastasis (M).

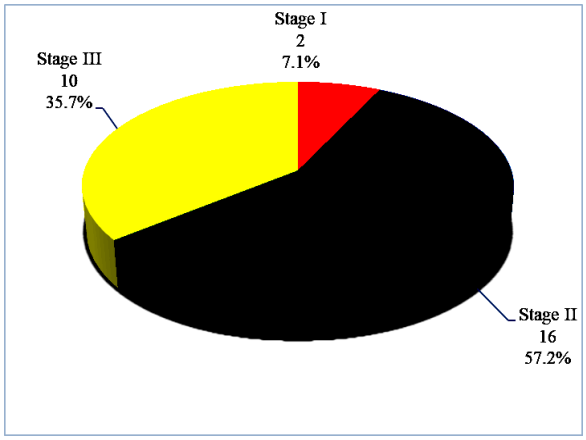

Figure 2. Distribution of studied patients according to the pathological stage.

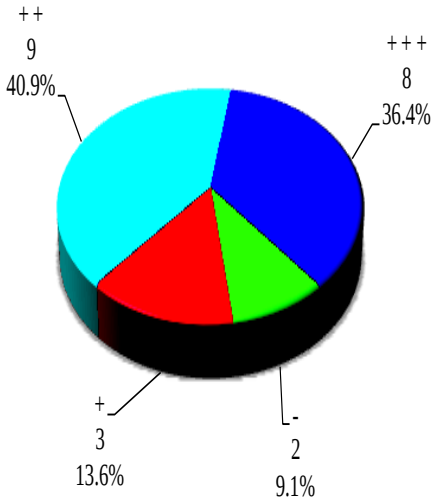

Figure 3 A. Distribution of studied patients according to ER status

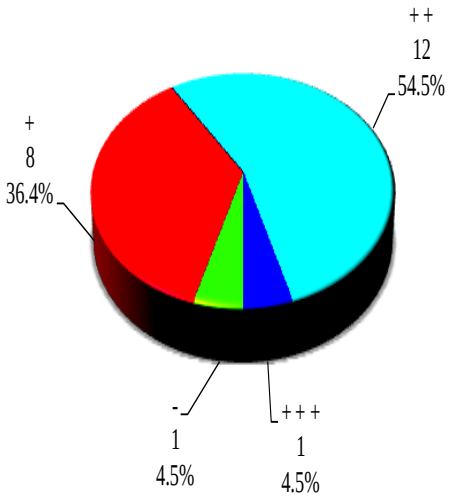

Figure 3 B. Distribution of studied patients according to PR status.

Assessment of IL-8 in breast tumor tissues

Five patients (17.9%) expressed negative staining reaction, 11 patients (39.3%) expressed weak positive (+) reaction, 4 patients (14.3%) expressed moderate (++) positive reaction, while 8 patient (28.6%) expressed strong (+++) positive reaction, Figure 4 (A-C).

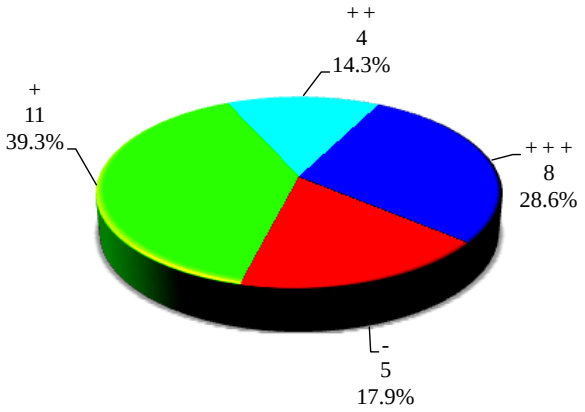

Figure 4A. Distribution of studied patients according to IL-8 level.

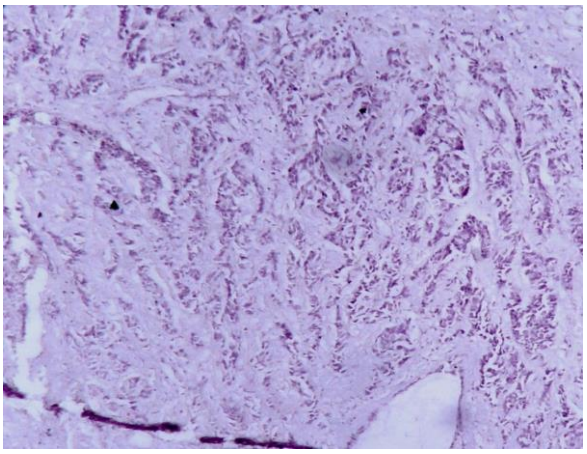

Figure 4B. IHC staining for a case of invasive ductal carcinoma showing negative immunostaining reaction with anti-IL-8 monoclonal antibodies.

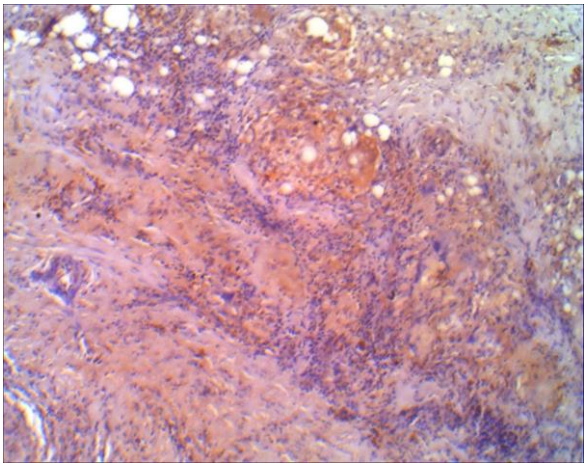

Figure 4C. IHC staining for a case of invasive ductal carcinoma showing positive immunostaining reaction with anti-IL-8 monoclonal antibodies.

Assessment of angiogenesis in breast tumor tissues

Eight patient (28.6%) expressed negative staining reaction, 10 patients (35.7%) expressed weak positive (+) reaction, 7 patients (25.0%) expressed moderate (++) positive reaction, while 3 patients (10.7%) expressed strong (+++) positive reaction, Figure 5 (A-C).

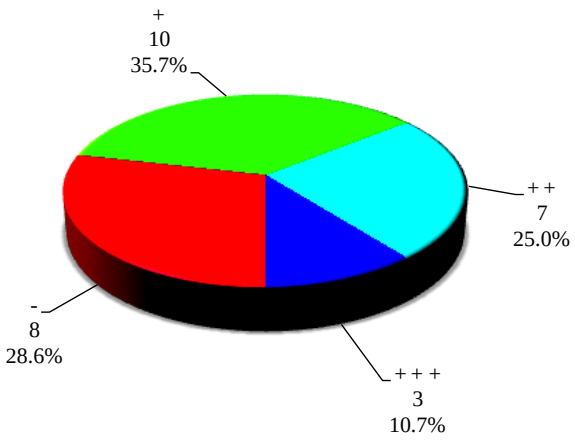

Figure 5A. Distribution of studied patients according to level of angiogenesis (VEGF-A)

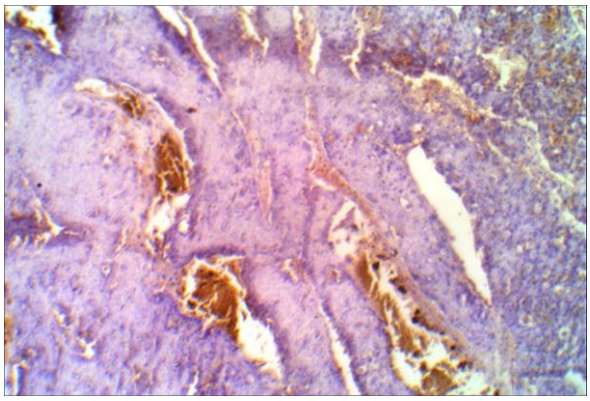

Figure 5B. Immunohistochemistry (IHC) staining for a case of high-grade ductal carcinoma showing negative immunostaining reaction with anti VEGF-A monoclonal antibody (IHC X100).

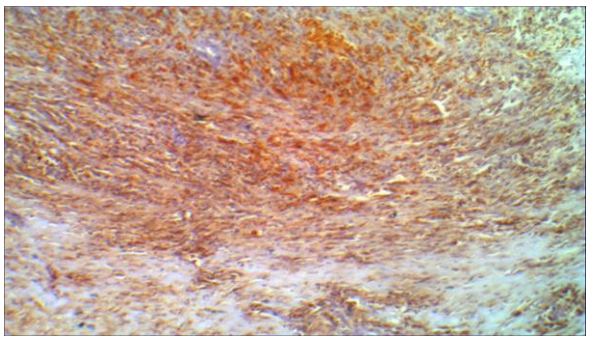

Figure 5 C. Immunohistochemistry (IHC) staining for a case of high-grade ductal carcinoma showing positive immunostaining reaction with anti VEGF-A monoclonal antibody (IHCX100).

Assessment of Serum MMP-9

A significant increase of MMP-9 serum level was found among studied breast cancer patients in

comparison to the control group ($P \leq 0.001$) (Table 1).

Table 1. Comparison between breast cancer patient group and control group according to serum MMP-9 level

	Patients (n = 26)	Control (n = 10)	*P* value
MMP-9 (ng/ml)			
Min. – Max.	110.90 – 294.10	17.53 – 77.98	<0.001
Mean ± SD.	206.34 ± 71.54	45.37 ± 22.02	

*Student t-test. Statistically significant at $P \leq 0.05$.

Correlation between different studied parameters and angiogenic factors (IL-8, VEGF-A, MMP-9 levels)

No significant correlation was found between angiogenic factors and each of the studied clinicopathological parameters: age, histological type, histological grade, pathological stage, vascular invasion, and hormonal receptor status (positive or negative); estrogen receptor (ER) and progesterone receptor (PR)

Correlation between IL-8 and VEGF-A levels in breast tissue

A significant positive correlation was found between IL-8 and VEGF-A *P* <0.001) Table 2.

Table 2. Correlation between IL-8 and VEGF-A levels in breast tissue.

	IL8 level	
	r_s	**P* Value
VEGF-A level	0.906	<0.001

$_{rs}$ spearman correlation. *statistically significant at $P \leq 0.005$

Correlation between MMP-9, IL-8 and VEGF-A

A significant positive correlation was observed between the serum levels of MMP-9 and both of angiogenesis and IL-8 level in breast tumor tissues studied breast cancer patients (p≤ 0.001) Table 3.

Table 3. Correlation between serum MMP-9, IL-8, and VEGF-A in breast tumor tissue.

	MMP-9 level	
	r_s	**P* Value
VEGF-A level	0.920	<0.001
IL-8 level	0.953	<0.001

$_{rs}$ spearman correlation. *statistically significant at $P \leq 0.005$

Discussion

Angiogenesis is a fundamental step that transitions benign tumors into malignant tumors within TME as tumors require a rich vascular supply, which provides oxygen and nutrients necessary for growth.[27] It was observed that rapidly growing tumors have rich vascular supply to facilitate malignant cells invasion.[28]

The aim of the current study was to evaluate the angiogenesis in breast cancer TME through assessment of the angiogenic factors IL-8, VEGF-A and MMP-9 as well as the prognostic value of MMP-9. To achieve this goal, 28 female Egyptian breast cancer patients scheduled for modified radical mastectomy were included in the study. IL-8 and VEGF-A were immunohistochemically assessed in tumor tissue samples and MMP-9 systemic production was measured using ELISA technique.

According to our results, tumor tissue samples seemed to exhibit increased concentrations of IL-8 as IL-8 expression was observed in 82.1% of the total examined tumor tissue samples. This finding is expected regarding IL-8 as it has a vital role in modulating tumor microenvironment, tumor growth and tumor progression through the regulation of angiogenesis.[29]

Increased expression of IL-8 in tumor tissue samples is in accordance with the observation that increased expression of IL-8 and /or its receptors has been characterized in cancer cells; endothelial cell, infiltrating neutrophils and tumor associated macrophages suggesting that IL-8 may function as a significant regulatory factor within TME.[30] In agreement with our findings, a study by Deng et al., 2021[31]

concluded that IL-8 overexpression promotes the migration of tumor cells and tumor growth via mitogen activated protein kinase (MAPK) signaling pathways.

Considering IL-8 as an important chemotactic factor, the detected IL-8 expression in breast tumor tissue samples of our study could be responsible for the inflammatory infiltrates such as neutrophils and macrophages within the tumor microenvironment. Such inflammatory infiltrates interact with numerous cellular and molecular components of the surrounding stroma resulting in more tumor progression and metastasis[32] In this context, a study by Chenn et al., 2003,[33] found that the inflammatory infiltrates have been associated with enhanced tumor growth and low survival rate due to the release of angiogenic factors by neutrophils and macrophages. They also found that infiltrating macrophage density was positively correlated with tumor IL-8 mRNA expression and intratumor microvessel counts but negatively correlated with patients' survival. Recently a study by Bukowski et al., 2020,[34] also demonstrated that resistance to chemotherapeutic drugs in human breast cancer cells was associated with autocrine production of different growth factors, including IL-1, IL-4, IL-6, and IL-8.

The positive staining reaction detected among the majority (71.4%) of breast tumor tissue samples of our study indicated that considerable amount of VEGF-A, as an angiogenic parameter, could occurred to compensate the lack of oxygen and nutrients within the TME through formation of new blood vessels from the pre-existing one. In support to such finding, a study by Lee et al., 2015,[35] reported that VEGF family represents the most important component in the angiogenic pathway and a study by Haibe et al., 2020,[36] showed that high levels of circulating VEGF are indicator of poor prognosis in several cancers, including breast cancer.

Consistent with our results a study by Shera et al., 2019,[37] observed over expression of VEGF in breast cancer which also correlated with tumor grade and stage. In addition, a study by Alumn et al., 2015,[38] reported overexpression of VEGF in malignant breast tumors in comparison to normal breast tissue and its expression was positivity associated with aggressive tumors. These findings confirmed VEGF important role in the pathogenesis of breast carcinoma and its implication in different stages of tumor growth.

Regarding MMP-9, the higher MMP-9 serum levels detected in the patient group is devoted to its role in degrading extracellular matrix to form new blood vessels among those patients.[20,39] In agreement with this result, studies by Pego et al., 2018,[40] and Candido et al., 2016,[41] stated that MMP-9 is important in the TME because it aids tumor invasion, metastasis, and angiogenesis, as well as modulating extracellular matrix remodeling. MMP-9 can also trigger signaling pathways in breast cancer cells that activate genes involved in migration and invasion.[42] A study carried out by Hajazimian et al., 2020,[43] identified overexpression of some matrix metalloproteinases in advanced mammary tumors. In addition, a study by Hsieh et al., 2019,[44] reported that high levels of MMP-9 have been observed in the blood, urine, and tissues of patients with breast cancer.

According to our finding, MMP-9 serum levels, as an important angiogenic factor, could be considered as a noninvasive prognostic factor of metastasis, cancer cell migration within TME. Consistent with this finding, a study by Joseph et al., 2020,[20] demonstrated that MMP-9 expression was an independent prognostic factor associated with shorter breast cancer patients survival. It was found that MMP-9 influences the activity of cancer cells by modulating the bioavailability of growth factors and the function of cell-surface receptors.[44]

Regarding the correlation between Il-8 level and the clinicopathological parameters of the patients under the current study, no significant correlations were found between Il-8 level and any of the studied clinicopathological parameters: age, histological type, histological grade, pathological stage, vascular invasion, and hormonal receptor status; estrogen receptor (ER) and progesterone receptor (PR).

Consistent with our results, a study by Milovanovic et al., 2013,[45] observed that there was no significant relationship between IL-

8/MMP-2/MMP-9 expression and available clinicopathological parameters (patient age, menopausal status, tumor size and tumor grade. Another study by Derin et al., 2007,[46] in breast cancer patients stated that none of the prognostic parameters were correlated with serum IL-8 levels. However, they found that serum IL-8 levels were elevated in patients with metastatic breast carcinoma. A study by Zuccari et al., 2012,[22] reported that when IL-8 expression was assessed, in 72 women with mammary neoplasia, by immunohistochemistry, IL-8 expression levels were significantly correlated with clinical-pathological findings.

In the present study, no significant correlation was found between the level of VEGF-A and each of the studied clinicopathological parameters: age, histological type, histological grade, pathological stage, vascular invasion, and hormonal receptor status (ER and PR). However, a significant positive correlation was found between both angiogenic factors IL-8 and VEGF-A levels in the tumor tissue samples (p= 0.008). This result could be explained according to the notion that IL-8 acts as an end product of the vascular endothelial factor pathway, a key pathway for angiogenesis.[47,48] In accordance with the previous result Martin et al., 2009,[49] found that IL8, a potent proangiogenic and inflammatory chemokine, up-regulates VEGF mRNA and protein levels in endothelial cells by acting on its cognate receptor, CXCR2, and this results in the autocrine activation of VEGFR2.

Our study also revealed that there was a significant positive correlation between serum levels of MMP-9 and both IL-8 and VEGF-A in the studied tumor tissue samples (p= <0.001). This result was expected regarding the role of both MMP-9 and VEGF in the process of angiogenesis as two main players associating with each other. In addition, IL-8 is known as an immune regulatory factor within the TME.[50]

Zhu et al., 2019,[51] stated that MMP-9 level, and VEGF expression were positively correlated in patients with retinoblastoma. Zhang and his coworkers (2014)[52] compared the levels of VEGF and MMP-9 among IDC patients, fibroadenoma patients and healthy adults. Their observations indicated that VEGF induces high expression levels of MMP-9 by the continuous activation of MAPK pathway. They also demonstrated that the levels of VEGF and MMP-9 in IDC patients were significantly higher than those in other two groups. Regarding the correlation between serum MMP9 and IL-8 expression levels, a study by Reis et al., 2012,[53] demonstrated that the overexpression of MMP-9 and higher expression of IL-8 were related to unfavorable prognostic factors of urothelial bladder cancer and tumor recurrence.

In conclusion, our study findings suggest that serum levels of MMP-9 can be considered as a potent predictor for angiogenesis which may influence the breast cancer severity, progression, and metastasis.

Author Contributions

SAA, contributed to the study conception and design, interpretation of the results and revision of the manuscript. GF, MZ, contributed to pathological examination of the tumor samples. SAB, AI, ME, contributed to performance of the practical part of the study. YH, MH, contributed to providing the tumor and blood samples of the patients. YS, EMO, contributed to data collection, statistical analysis of the results and writing the manuscript draft. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee, Medical Research Institute, Alexandria University (April 2013).

Informed consent

An informed consent was obtained from each patient participating in the study according to the Ethical Committee of Medical Research Institute.

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