The Egyptian Journal of Hospital Medicine (January 2023) Vol. 90, Page 79- 83

High Tumor Levels of Ki-67, VEGF and Endostatin are Associated with Progression of Breast Cancer in Iraqi Women

Rana H. K. Al-Rubaye[1]& Rakad M. Kh AL-Jumaily[2*]

[1]Department of Biology, College of Education for Pure Sciences/ Ibn Al-Haitham, University of Baghdad, Iraq.

[2]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

***Corresponding author:** Rana H. K. Al-Rubaye, **E-mail:** rana.h.k@ihcoedu.uobaghdad.edu.iq

ABSTRACT

Background: Breast cancer (BC) is the most widespread cancer among women worldwide. Its incidence and mortality rates have risen in the previous three decades as a result of changes in risk factor profiles, improved cancer registry, and cancer detection. **Objective:** The study's goals were to establish if Ki-67 could be used as a potential marker in serum of cancer disease patients as well as their interaction with vascular endothelial growth factor (VEGF) and ES in various stages of breast cancer to assess their function in the progression of BC.

Materials and Methods: The levels of Ki-67, VEGF and endostatin (ES) in serum were assessed by commercial enzyme linked immunosorbent assay (ELISA) kits in 60 women diagnosed with breast cancer (age range 33–80 yrs.) and 30 age-matched healthy controls. Two groups of breast cancer patients: groups 1 consisted of stage II (Low level) and groups 2 consisted of patients in stage III and IV (High level).

Results: The results showed a significant increase of Ki-67 and VEGF in BC patients as related to healthy control with increases in patients in advanced stage. The data revealed that the level of ES was much lower in patients with low-stage (stage II) compared to the group of control, but it was significantly higher in women with advanced-stage of BC.

Conclusions: The Ki-67, VEGF, and ES levels in the serum of studied groups may be a good marker in the progression of BC.

Keywords: Ki-67, VEGF, Endostatin, Breast cancer.

INTRODUCTION

Breast cancer (B.C.) is a global problem, ranking top among malignant neoplasms and affecting around 13% of women in their lifetime [(1)]. Breast cancer accounts for one-third of all registered women's cancers in Iraq [(2)].

Ki-67 is a DNA binding protein that is found in most of vertebrates. It's commonly used as a proliferation mark for tumor grading. It's expressed in every kinetic stage of the cell cycle except G0. It is regarded as one of the most dependable indices for determining the degree of malignant tumor cell proliferation [(3)]. Ki-67 has recently received much attention as a promising prognostic, predictive, and therapeutic target in malignant neoplasms such as bladder, lung, cervical, and breast carcinomas [(4)]. In some tumors' tissue, the expression of Ki-67 was used as a clinical setting to study the state of tumor [(5)]. However, it has suggested that Ki-67 have the ability to secrete or leaked out of cancer cells and thus, it can easily be measured in the serum of patients [(6)].

Angiogenesis is the formation of new vasculature from pre-existing ones. Aangiogenesis process is tightly maintaining by the interaction between pro- and anti-angiogenic factors. Many potent promoter angiogenic factors are described since the identification of the master role of growth factor in maintaining the balance of angiogenesis forming [(7)]. VEGF-A is a key regulator of vascular growth among pro-angiogenic factors, and the VEGF family is regarded a key activator of vascular development and angiogenesis. VEGF-A (commonly known as VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) are the most common members of the VEGF family [(8)]. Angiogenesis is a key component of carcinogenesis, development, and metastasis in many human cancers. VEGF is regarded to be a key regulator of angiogenesis, promoting tumor cell proliferation as well as invasion and metastasis. Tumor vascular density, particularly BC, has been shown to be closely related to prognosis [(9)]. However, pathologic angiogenesis can be caused by disturbances in the balance of pro- and anti-angiogenic factors [(10)].

Endostatin is created by proteolytic cleavage of collagen XVIII by a set of proteinases such as elastase, matrix metalloproteinases (MMPs) and procathepsin L [(11)]. Endostatin suppresses angiogenesis by binding to VEGFR-1 and VEGF- 2 and preventing VEGF interaction with Flt-1 and Flk-1 and all downstream signaling proceedings [(12)]. This study aimed to identify the relationship between VEGF-A, ES and Ki-67 to evaluate their potential roles in the progression of BC disease.

MATERIALS & METHODS

Study design

A total of 90 Iraqi women participated in this study; 60 women were diagnosed with BC during their attendance at Oncology Teaching Hospital /Medical City/Baghdad. The study extended from November 2020 till April 2021. Age of breast cancer women range from (33–80) years. In addition, 30 apparently healthy women also participated in this study whose ages ranged between 32-75 years. Patients with BC involved in this paper were

Received: 5/8/2022
Accepted: 6/10/2022

categorized into two groups: Group 1: Patients in stage I and stage II was considered (low-level breast cancer patients) and group 2 consisted of patients in stage III and IV (High level). All studied women were handed a questionnaire that asked for age, weight, length, family history, site of breast infection, menstrual cycle status, using of contraceptive pills.

Blood samples collection

Venous blood samples were collected from positive BC patients at the time of diagnosis and control subjects in the sitting position using disposable syringes (5ml). Five milliliters of blood were drawn from each patient through vein puncture and slowly put into disposable serum tubes containing separating gel. After allowing the blood in the gel tubes to coagulate at room temperature (15 minutes), it was centrifuged at 3000 rpm for around 15 minutes. The serum was kept at -20 ℃ for future use.

Measurements of Ki-67, VEGF and Endostatin

Ki-67, VEGF-A and Endostatin were assessed quantitatively in serum samples using commercial enzyme linked immunosorbent assay (ELISA) kits (SunLong Biotech/China). Antibody specific for each parameter has been pre-coated onto a microplate. After that, samples and standards were pipetted into the wells and any present parameters were bound by the antibodies. Then a Horseradish Peroxidase (HRP)-conjugated antibody specific for each parameter was added to microelisa strip plate wells and incubated. Components were washed. Once adding Chromogen Solution, A and B to the wells, the colors develop in response to the number of parameters, turning blue and eventually yellow after the stop solution is added. Finally, the color intensities were measured using an ELISA reader (Mindray/ India) at a wavelength of 450 nm. The concentration was calculated from the standard curve.

Ethics approval:

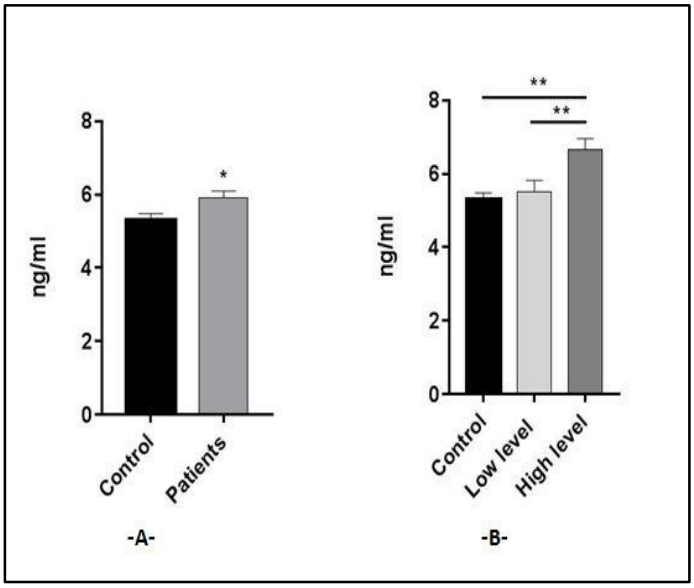
Written informed consents were obtained from all subjects and the study was approved by Ethical Committee (Ref.: CSEC/1120/0082 in November 15, 2020), Department of Biology, College of Science, University of Baghdad. This study was carried out in accordance with the World Medical Association Code of Ethics (Declaration of Helsinki) for studies involving humans.

Statistical analyses

The obtained data were statistically analyzed using SPSS version 23. Differences with P values <0.05 were considered to be statistically significant. The data were expressed as Mean ± SE. Statistical comparison between groups were analyzed using an analysis of variance (ANOVA) .

RESULTS

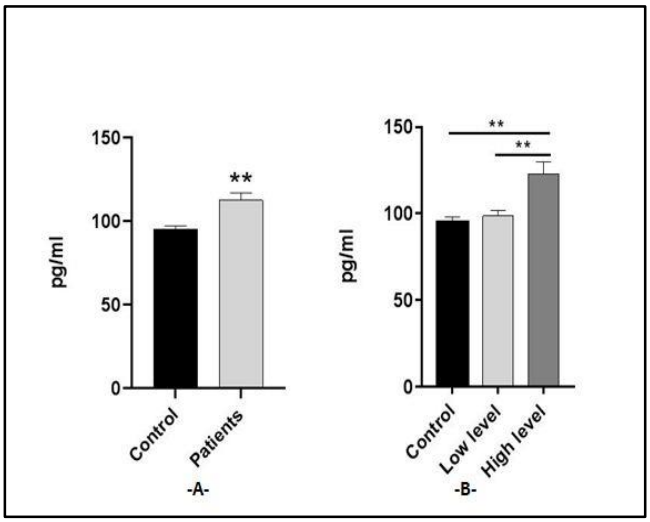
Ki-67 level showed slightly significant ($p \leq 0.05$) increase in patients with BC compared to control group (Figure 1–A). Also, a highly significant increase ($p \leq 0.001$) were demonstrated in patients at high level stage compared to patients at low level stage and control groups, but no significant ($p > 0.05$) difference were noticed between patients at low level stage and control as illustrated in figure (1-B).



A: Ki-67 level in the breast cancer as compared to control. B: Ki-67 level in the early (low level) and advanced (high level) breast cancer in compared to a control group. *Refers to significant (P≤0.05). ** Refers to significant (P≤0.001). Values represent the Mean ±SE.

Figure (1): Blood levels of Ki-67 in patients with breast cancer compared to healthy controls

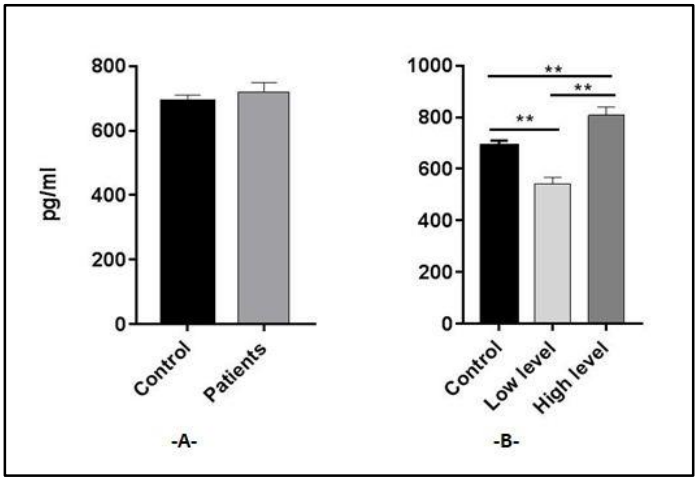
Our results showed that the expression of VEGF-A was significantly increased (P≤0.001) in serum samples in BC patients in comparison with that seen in healthy subjects as shown in figure (2-A). Also, the results showed a significant increase (P≤0.001) in VEGF-A levels in women with advanced breast cancer (high levels stage) than in low level stage and control. While, no significant difference (P>0.05) was detected between low level stage and control group as shown in figure (2-B).



B: Breast cancer at different stages and control. Data are presented as Mean ± standard error (SE). **P<0.001 vs control.

Figure (2): Expression of VEGF-A at serum level. A: Breast cancer and control

No significant difference in ES serum level in breast cancer women when compared to control group. Furthermore, the ES level in BC patients was highly significant P value (P≤0.001) lower in patients with BC at low level (stage II) while ES level increased significantly(P≤0.001) in women with BC at high level when compared to healthy group, and there was significant difference (P≤0.001) between ES level in patient groups (Figure 3A & B).



A: Breast cancer and control. B: Breast cancer at different stages and control. Data are presented as Mean ± standard error (SE). **P<0.001 vs control.

Figure 3: Expression of ES at serum level.

Table (1) showed that the levels of serum VEGF showed highly significant (p ≤ 0.001) increase (176.71 ± 13.99 pg/ml) in patients with metastatic breast cancer (stage IV) compared to the two groups (stage II and III) while no significant differences (P>0.05) were observed between patients in stage II and stage III. Also, there was significantly increase (p ≤ 0.001) in serum endostatin level in breast cancer patients in stage IV in comparison with patients in stage II and III. While no significant differences (P>0.05) were observed between patients in stage II and stage III. The results revealed significant (p ≤ 0.001) increase in serum ki 67 level in breast cancer patients in stage IV in comparison with patients in stage II and III. While there was no statistically significant difference in ki-67 levels between stage III and stage II patients.

Table 1: Comparison of serum markers in different stages of breast cancer.

Stages / Parameters	Stage II	Stage III	Stage IV	P value
	Mean ± SE			
Ki-67 (ng/ml)	5.60 ± 0.21b	6.15 ± 0.15b	7.68 ± 0.28a	0.001*
VEGF-A (pg/ml)	98.70 ± 3.12 b	105.74 ± 3.29 b	176.71 ± 13.99 a	0.001*
ES (pg/ml)	542.81 ± 23.75b	556.57 ± 21.81b	1058.34 ± 43.68a	0.001*

Different small letters refer to significant differences (P≤0.001) within-stages comparison. Similar letters refer to non-significant differences within-stages comparison.

DISCUSSION

Breast cancer detection at an early clinical stage is critical for disease management. Patients with breast cancer can benefit greatly from early detection and treatment, which improves their prognosis and long-term survival. Therefore, it is important and crucial to explore for biomarkers for early disease diagnosis and prognosis. Ki-67 level showed slightly significant increase in patients with breast cancer compared to control group. Also, a highly significant increase was demonstrated in patients at high level stage compared to patients at low level stage and healthy group, but there were no differences between BC patients at low level stage and control. Ki-67 expression is frequently employed as a biomarker of tumor aggressiveness because it's a highly connected with the proliferation of intrinsic cell populations in cancer diseases [13]. However, in healthy breast tissue, very low of Ki-67 levels (<3%) have been recorded [14]. On the other hand, the expression of Ki-67 was increased significantly in malignant tissue [15]. Cell proliferation, through altered expression and/or activity of cell cycle-related proteins, plays an important role in the initiation and progression of cancer. Thus, measurement of cellular proliferation protein may provide useful information about disease status [16]. In accordance with our results, many studies indicated that a higher Ki-67 level significantly was associated with increasing tumor

size and positive lymph nodes [17]. **Ragab *et al*.** [18] revealed a high correlation between the expression of Ki-67 and some breast cancer features such as tumor grade, lymph node status and tumor size. Also, it's reported that block of the expression of Ki-67 by injection of antisense oligonucleotides leads to inhibition of the cell proliferation [19]. In addition, we assessed the relationship between Ki-67 expression and various stages of breast cancer. The results revealed significant increase in serum ki-67 level in BC patients in stage IV in comparison with patients in stage II and III. This result is in accordance with **Ren *et al*.** [20] who referred to significant increase in serum ki-67 level in lymph node metastasis BC patients. **Warth *et al.* [21]** showed that Ki-67 expression is elevated significantly in advanced stages of lung cancer and indicated that Ki-67 can work as a predictor for tumor development. Ki-67 expression has a significant impact on all stages of carcinogenesis, including tumor genesis, development, and metastasis, as well as treatment susceptibility [22]. It has stated that the aberrant proliferation of Ki-67 is associated with many tumor features including tumor differentiation, invasion, metastasis, tumor progression and patient survival [23].

Our results showed that VEGF-A expression was significantly increased in serum of BC patients, in comparison with that seen in healthy controls. This finding agrees with **Iovino *et al.*** [24] who observed that concentrations of VEGF was increased significantly in the serum of breast cancer patients. This increase may be due to increase of the expression of VEGF-A in cancer cells. The vast majority of solid tumors in human, including breast cancers, express VEGF-A and their receptors. Different cell types produced VEGF-like stromal cells (monocytes, macrophages, and fibroblasts) as well as tumor cells [25]. Vascular endothelial growth factor-A (VEGF-A) is a mitogen and survival factor for endothelial cells that also acts as a key promoter of angiogenesis. It has the ability to bind and activate many tyrosine kinase receptors such as vascular endothelial growth factor receptors (VEGFR1 and VEGFR2). The VEGF/VEGF-receptor pathway activates signaling cascades that enhance endothelial cell growth, proliferation, migration, and differentiation. This potent cytokine also increases microvessel permeability, and activates proteolitic enzymes implicated in tumor invasiveness [26, 27]. However, tumor vascularization, progression, and invasion are all enhanced by VEGF. Also, these results suggested that the level of VEGF-A was associated with their TNM-staging. According to **Hao *et al.*** [28] results, significant higher levels of VEGF-A were found among patients in stage IV. These results showed compatibility with **Ghosh *et al.*** [29] who demonstrated that the high expression of VEG was found in metastatic breast cancer and associated with higher pathologic stage as well as worse prognosis compared to low VEGF secreting tumors.

Our data illustrated that there was a highly significant decrease in ES level in patients with BC at low level (stage II) while ES level was increased significantly in women with BC at high level when compared to control group, and there was significant difference between ES level in patient groups. These results are in line with previous study that indicated that the expression of ES decreased significantly in hepatocellular carcinomas cells [30]. Moreover, this decrease is associated with increasing tumor size, modifications in the histological architecture of cancer patients as well as higher vascular support. Our results also agree with **The *et al.*** [31] who illustrated a decreased level of ES in pre-operative breast cancer patients compared to the post-operative groups. However, the variability in the expression of ES could reflect different angiogenic phenotypes of the tumors.

The results of the present study demonstrated a highly significant increased levels of ES in breast cancer women in high-level stage compared to control and low-level stage. Elevation of ES levels were observed in many human cancers, such as lung cancer and renal carcinoma as well as breast cancer [32]. However, many studies revealed that the effect of ES depends on two factors: length of exposure and type of growth factor [33]. Increase ES levels in patients with metastatic breast cancer compared to those without metastasis in our study indicated that levels of serum ES may be involved in the progression of BC. This finding agrees with the result of **Bachelot *et al.*** [34] who established a significant correlation between ES concentrations and metastatic breast cancer patients.

CONCLUSION

The Ki-67, VEGF, and ES levels in the serum of studied groups may be a good marker in the progression of BC.

REFERENCES

1. **Cirmena G, Ferrando L, Ravera F**, ***et al.*** (**2022**)**:** Plasma Cell-Free DNA Integrity Assessed by Automated Electrophoresis Predicts the Achievement of Pathologic Complete Response to Neoadjuvant Chemotherapy in Patients with Breast Cancer. JCO Precision. Oncology, 6: e2100198.
2. **Ali C, Lafta F, Al Sayyid M e*t al.* (2020):** BRCA1 Gene Expression is Down Regulated in Both Familial and Sporadic Breast Cancer Cases in Baghdad-Iraq. Iraqi Journal of Science, 61, (1): 34-41.
3. **Sun X, Kaufman P** (**2018**)**:** Ki-67: more than a proliferation marker. Chromosoma, 127 (2): 175-186.
4. **Menon S, Guruvayoorappan C, Sakthivel K *et al.*** (**2019**)**:** Ki-67 protein as a tumour proliferation marker. Clinica Chimica Acta., 491: 39-45.
5. **Honma N, Horii R, Iwase T *et al.*** (**2015**)**:** Ki-67 evaluation at the hottest spot predicts clinical outcome of patients with hormone receptor-positive/HER2-negative breast cancer

treated with adjuvant tamoxifen monotherapy. Breast cancer, 22 (1): 71-78.

6. **Neumann S, Schuettler J, Frenz M** *et al*. (**2017**)**:** Investigation of serum Ki-67 as a biomarker in tumor-bearing dogs. Research in veterinary science, 110: 16-21.
7. **Teleanu R, Chircov C, Grumezescu A *et al.*** (**2019**)**:** Tumor angiogenesis and anti-angiogenic strategies for cancer treatment. Journal of clinical medicine, 9 (1): 84.
8. **Chen B, Zhang Y, Chen S *et al.*** (**2021**)**:** The role of vascular endothelial growth factor in ischemic stroke. Die Pharmazie-An International Journal of Pharmaceutical Sciences, 76 (4): 127-131.
9. **Li S, Wang L, Meng Y *et al.*** (**2017**)**:** Increased levels of LAPTM4B, VEGF and survivin are correlated with tumor progression and poor prognosis in breast cancer patients. ncotarget, 8 (25): 41282.
10. **Lugano R, Ramachandran M, and Dimberg, A** (**2020**)**:** Tumor angiogenesis: causes, consequences, challenges and opportunities. Cellular and Molecular Life Sciences, 77 (9): 1745-1770.
11. **Wu T, Duan X, Hu T *et al.*** (**2020**)**:** Effect of endostatin on Wnt pathway of stem-like cells in bladder cancer in tumor microenvironment. Molecular Biology Reports, 47 (5): 3937-3948.
12. **Walia A, Yang J, Huang Y *et al.*** (**2015**)**:** Endostatin's emerging roles in angiogenesis, lymphangiogenesis, disease, and clinical applications. Biochimica et Biophysica Acta (BBA)-General Subjects, 1850 (12): 2422-2438.
13. **Horie K, Yamamoto H, Karube K *et al.*** (**2019**): Cyclin A is a reliable proliferation marker in endometrial cancer cell lines. Oncology letters, 17 (5): 4455-4462.
14. **Huh S, Oh H, Peterson M *et al.* (2016):** The proliferative activity of mammary epithelial cells in normal tissue predicts breast cancer risk in premenopausal women. Cancer research, 76 (7): 1926-1934.
15. **Hu H, Liu H, Zhang J *et al.*** (**2012**)**:** Clinical significance of Smac and Ki-67 expression in pancreatic cancer. Hepato-gastroenterology, 59 (120): 2640-2643.
16. **Feitelson M, Arzumanyan A, Kulathinal R *et al.*** (**2015**)**:** Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. Seminars in cancer biology, 35: S25-S54.
17. **Petrelli F, Viale G, Cabiddu M *et al.* (2015):** Prognostic value of different cut-off levels of Ki-67 in breast cancer: a systematic review and meta-analysis of 64,196 patients. Breast cancer research and treatment, 153 (3): 477-491.
18. **Ragab H, Samy N, Afify M *et al.* (2018):** Assessment of Ki-67 as a potential biomarker in patients with breast cancer. Journal of Genetic Engineering and Biotechnology, 16 (2): 479-484.
19. **Schlüter C, Duchrow M, Wohlenberg C *et al.*** (**1993**)**:** The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. The Journal of cell biology, 123 (3): 513-522.
20. **Ren A, Wei M, Yang Y *et al.*** (**2020**)**:** Detection and value of serum antigen KI-67 (ki67) in clinical diagnosis of breast cancer patients. Xi bao yu fen zi Mian yi xue za zhi= Chinese Journal of Cellular and Molecular Immunology, 36 (12): 1124-1128.
21. **Warth A, Cortis J, Soltermann A *et al.* (2014):** Tumour cell proliferation (Ki-67) in non-small cell lung cancer: a critical reappraisal of its prognostic role. British journal of cancer, 11 (6): 1222-1229.
22. **Mrouj K, Singh P, Sobecki M *et al.*** (**2019**)**:** Ki-67 promotes sequential stages of tumourigenesis by enabling cellular plasticity. doi: https://doi.org/10.1101/712380.
23. **Krüger K, Stefansson I, Collett K *et al*.** (**2013**)**:** Microvessel proliferation by co-expression of endothelial nestin and Ki-67 is associated with a basal-like phenotype and aggressive features in breast cancer. The Breast, 22 (3): 282-288.
24. **Iovino F, Ferraraccio F, Orditura M *et al*.** (**2008**)**:** Serum vascular endothelial growth factor (VEGF) levels correlate with tumor VEGF and p53 overexpression in endocrine positive primary breast cancer. Cancer investigation, 26 (3): 250-255.
25. **Moghaddam S, Amini A, Morris D *et al.*** (**2012**)**:** Significance of vascular endothelial growth factor in growth and peritoneal dissemination of ovarian cancer. Cancer and Metastasis Reviews, 31 (1): 143-162.
26. **Matkowski R, Gisterek I, Suder E *et al.*** (**2006**)**:** Correlation between vascular endothelial growth factor and c-met expressions in breast carcinoma. Journal of Clinical Oncology, 24 (18_suppl): 10621-10621.
27. **Lee S, Jeong D, Han Y *et al.*** (**2015**)**:** Pivotal role of vascular endothelial growth factor pathway in tumor angiogenesis. Annals of surgical treatment and research, 89 (1): 1-8.
28. **Hao L, Zhang C, Qiu Y *et al*.** (**2007**)**:** Recombination of CXCR4, VEGF, and MMP-9 predicting lymph node metastasis in human breast cancer. Cancer letters, 253 (1): 34-42.
29. **Ghosh S, Sullivan C, Zerkowski M *et al.*** (**2008**)**:** High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. Human pathology, 39 (12): 1835-1843.
30. **Musso O, Rehn M, Théret N *et al.*** (2001)**:** Tumor progression is associated with a significant decrease in the expression of the endostatin precursor collagen XVIII in human hepatocellular carcinomas. Cancer research, 61 (1): 45-49.
31. **Teh S, Hill A, Lee A *et al*.** (**2004**)**:** Raised plasma endostatin levels correlate inversely with breast cancer angiogenesis. Journal of Surgical Research, 116 (1): 165-171.
32. **Wang Z, Zhu Z, Xiao X *et al*.** (**2015**)**:** Correlation of serum levels of endostatin with tumor stage in gastric cancer: a systematic review and meta-analysis. doi: 10.1155/2015/623939.
33. **Li C, Harris M, Venema V *et al.*** (**2005**)**:** Endostatin induces acute endothelial nitric oxide and prostacyclin release. Biochemical and biophysical research communications, 329 (3): 873-878.
34. **Bachelot T, Ratel D, Ménétrier-Caux C *et al.*** (**2006**)**:** Autoantibodies to endostatin in patients with breast cancer: correlation to endostatin levels and clinical outcome. British journal of cancer, 94 (7): 1066-1070.