Original Article

Vascular Endothelial Growth Factor Expression by Immunohistochemistry as a Possible Indicator of Prognosis in Invasive Breast Carcinoma of No Special Type

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Abstract

Context: Angiogenesis, the formation of new blood vessels from preexisting vascular network, is essential for tumor growth and spread. Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor. **Aims:** To assess the expression of VEGF in invasive carcinoma of no special type and its correlation with all the known prognostic factors of breast carcinoma. **Settings and Design:** Descriptive. **Materials and Methods:** Mastectomy specimens were studied noting the clinical details. The formalin-fixed tissues were subjected to routine processing and hematoxylin and eosin sections and studied extensively for all the histological prognostic factors. Representative sections from each case with the tumor were subjected to immunohistochemistry (IHC) staining with VEGF, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu) antibodies. **Statistical Analysis Used:** Descriptive statistics, Chi-square tests, contingency table analysis using SPSS for Windows. **Results:** One hundred and twelve cases of invasive carcinoma of special type were studied to evaluate various clinicopathological parameters. The association of VEGF with clinicopathological parameters and all the known prognostic factors was studied to note its significance. VEGF overexpression was observed in 69% of the cases. It was noted that larger tumor size, higher histological grade, lymphovascular invasion, nodal involvement, tumor necrosis, high microvessel density, ER negativity, PR negativity, and HER2/neu positivity had a significant statistical association with VEGF overexpression. **Conclusions:** We conclude that incorporating VEGF as a biomarker along with the known factors into a prognostic index will not only help predict clinical outcome more accurately, but also determines the patient who can be benefited with combinational therapy including anti-VEGF factors.

Keywords: *Breast cancer, prognostic factors, therapy, vascular endothelial growth factor*

Introduction

Breast cancer is the most common malignancy in women and the main cause of cancer-related mortality.[1] Angiogenesis helps cancer spread and develop. Molecular players of angiogenesis have been characterized since early angiogenic investigations, and one of the most prominent stimulating growth factors is vascular endothelial growth factor (VEGF) family. VEGF family is the main controller of angiogenesis, which is vital in breast cancer development. Anti-angiogenic medicines block molecular signaling pathways.[2] In this study, VEGF expression in breast carcinoma was correlated with known prognostic factors, thus helped to increase the existing understanding of this life-threatening illness.

Materials and Methods

The objective of this study is to investigate the clinical and morphological characteristics of invasive cancer of no special and establish a correlation between the expression of VEGF and established prognostic factors. The study was carried out between 2018 and 2021. This study exclusively incorporated cases of invasive breast ductal carcinoma (not otherwise specified, no special type) that were confirmed through histological examination. The study did not include noninvasive ductal carcinomas (IDCs). The statistical evaluation conducted in this study utilized statistical package for the social sciences (SPSS, released 2010, version 22.0. Armonk, NY, USA: IBM Corp) software to perform descriptive statistics, Chi-square testing, and contingency table analysis. Institutional

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Study design

Purposeful sampling was used to examine specimens from mastectomy procedures. The tissues were subjected to routine formalin fixation and subsequent processing, followed by examination of hematoxylin and eosin (H and E)-stained sections to assess histological prognostic factors. Immunohistochemical staining was performed on representative sections of the tumor using antibodies against VEGF, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu).

Positive immunoreactions include the presence of VEGF cytoplasmic precipitate, ER and PR in the nucleus, and HER2/neu that is localized in the membrane. The quantification of labeled cells within the entirety of the tissue present on the slide was performed. The level of VEGF expression in each case was assessed and categorized as mild, moderate, or heavy staining, following the classification outlined in Table 1 [Figure 1g-i].

The Allred (quick) scoring method was employed to assess the expression of ER and PR. This method involved evaluating the proportion of stained cells and the intensity of nuclear staining [Figure 1a and b]. According to the established criteria, invasive malignant cells that exhibited staining in at least 1% of their population were considered immunoreactive for ER and PR. The HER2 test was assessed on a scale ranging from 0 to 3, with a score of 0 or 1 indicating a negative result, a score of 2+ regarded as equivocal, and a score of 3+ reflecting a positive result. For a 3+ score, more than 10% of invasive malignant cells must exhibit strong, full circumferential cytoplasmic membrane staining. The technique of fluorescent *in situ* hybridization was employed to analyze situations that exhibited a HER2 staining intensity of 2+.

Two observers conducted measurements of MVD. Every individual engaged in the task of slide counting conducted their counts autonomously. A microvessel possessing an open lumen was delineated. Hotspot screening was employed to examine the H and E and VEGF IHC slides. Low-power scanning of the slides was used to identify microvessel hot spots [Figure 1d]. Using an appropriately

Table 1: The scoring system used for the staining patterns of vascular endothelial growth factor immunostain

Score	Staining pattern (% of positive tumor cells)	Positivity
Score 0	Negative	Negative
Score 1+	Weak or mild staining 5%–10%	Negative
Score 2+	Moderate staining <25%	Negative
Score 3+	Strong staining 25%–50%	Positive
Score 4+	Highly strong positivity >50%	Positive

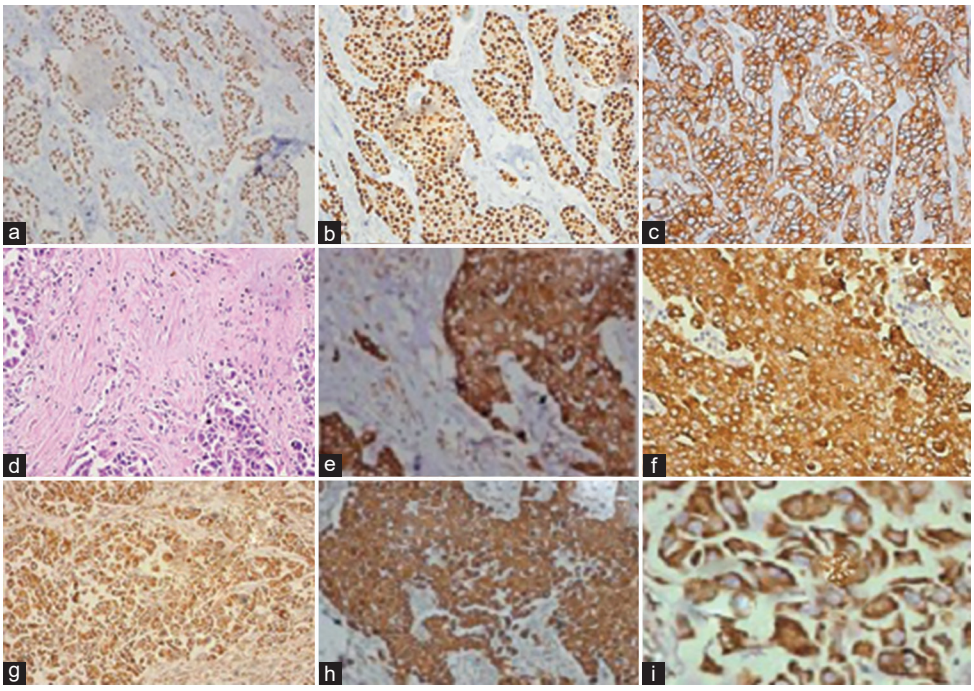

Figure 1: (a) Estrogen receptor (ER) – Tumor cells showing nuclear positivity with ER immunostain (ER IHC, ×100), (b) Progesterone receptor (PR) – Tumor cells showing nuclear positivity with PR immunostain (PR IHC, ×200), (c) Human epidermal growth factor receptor 2 (HER-2/neu) – Tumor cells showing membrane positivity with HER-2/neu immunostaining (HER-2/neu IHC, ×200), (d) Microvessel density-Section showing endothelial lined spaces (microvessels) (hematoxylin and eosin [H and E], ×100, (e) Microvessel density-Section showing endothelial lined space (microvessels) (vascular endothelial growth factor [VEGF] IHC, ×200), (f) VEGF IHC staining displaying strong staining intensity of tumor cells (VEGF IHC, ×100), (g) VEGF IHC staining showing week intensity of tumor cells (VEGF IHC, ×200), (h) VEGF IHC staining showing moderate intensity of tumor cells (VEGF, ×100), (i) VEGF IHC staining showing strong intensity of tumor cells (VEGF IHC, ×400)

expanded field of view (×40 objective lens), microvessels were counted. The mean vascular density (MVD) of the sample was calculated as the average of the vascular density measurements obtained from the ten most vascularized regions observed under a magnification of ×400.

Results

The study was carried out at a tertiary care pathology department between 2018 and 2021. A total of 112 cases of invasive cancer were studied. The age distribution of the patients in the study ranged from 32 to 80 years, with a calculated mean age of 51.9. Among those in the study, 75% were identified as postmenopausal, while the remaining 25% were classified as premenopausal. The study revealed that 50% of primiparous women were into the age range of 20–24 years, whereas 41% and 9% belonged to the age groups of 15–19 and 25–29, respectively. A majority of individuals, specifically 52%, presented with right-sided tumors, whereas the remaining 48% exhibited left-sided tumors. The majority of the tumors were located in the upper outer quadrant, with the upper inner quadrant, lower outer quadrant, and lower inner quadrant following in descending order of prevalence. All four breast quadrants were affected in one case. Lymphocytic infiltration, necrosis, lymphovascular invasion (LVI), and desmoplasia were observed in 66% of the cases. The median MVD on H and E sections was 4.60/HPF and 4.70/HPF on VEGF-stained sections. The incidence of Grade 3 tumors (52%) exceeded that of Grade 2 tumors (48%). The majority of cases (71%) were classified as pT2, followed by pT3 and pT4. A total of 45% of patients did not have lymph node metastases, 39% in lymph nodes 1–3 (N1), 9% in lymph nodes 4–9 (N2), and 7% in lymph nodes >10 axillary (N3). Of the cases, 30.3% were HER2/neu-positive and 37.5% were ER/PR-positive. The distribution of tumor subtypes in the study population was as follows: 32.1% were classified as Luminal A, 5.9% as Luminal B, 37.5% as triple negative, and 25% as HER2/neu positive. A total of 69% of the patients exhibited overexpression of VEGF. A total of 48.7% of participants achieved a score of 3 or higher, while 51.3% of participants achieved a score of 4 or higher. The overexpression of VEGF as shown in Figure 1e-h was found to be associated with several factors including greater tumor size, lymph node metastases, higher histological grade, LVI, necrosis, high microvessel density (MVD), triple negative tumors, and HER2/neu positivity; the overexpression of VEGF showed an inverse correlation with the positivity of ER and PR, as demonstrated in Table 2.

The measurement of microvascular density (MVD) was conducted on sections stained with H and E as well as VEGF using the hot spot technique. The median microvessel density (MVD) seen on H and E-stained sections was 4.60 high-power fields (HPF), while on VEGF-stained sections, it was 4.70 HPF.

Discussion

Angiogenesis is carefully regulated by endogenous activators and inhibitors. There are thirty endogenous proangiogenic factors. Angiogenesis, lymphangiogenesis, and vasculogenesis are all dependent on the VEGF family. Vascular formation is significantly regulated by VEGF. Anti-angiogenic drugs may target VEGF, which is expressed in tumors of the colon, breast, cervix, lungs, prostate, and stomach.[3] Lymph node metastases, necrosis of the tumor, and LVI are unfavorable prognostic factors that are associated with a higher histologic grade. ER expression was higher in patients aged 40 and older [Figure 1a]. ER expression was associated with benign neoplasms, necrosis, and a decreased incidence of metastatic axillary lymph nodes, as well as a diminished histological grade and tumor size. For prognosis and long-term responsiveness to cancer-specific treatments, the presence of this receptor must be identified.[4,5]

This study established an association between PR expression [Figure 1b] and histologic grade, tumor size, and the presence of metastatic axillary lymph nodes, revealing a correlation between these variables. The predictive efficacy of PR in the decade of the 1990s diminished. Individuals with ER-negative status who exhibit a response to hormone treatment have led to renewed interest in comprehending the range of responses to this treatment.[6,7]

The overexpression of HER2 has been identified as a negative prognostic indicator and has the potential to serve as a predictive marker for therapy response. The overexpression of HER-2/neu as shown in Figure 1c was found to be associated with unfavorable prognostic factors, including higher histologic grade, larger tumor size, axillary lymph node involvement by metastatic tumor, LVI, and necrosis. Ponzone *et al*.[8] discovered comparable findings. In this study, we observed an inverse relationship between the expression of ER and PR and the overexpression of HER2; Lal *et al*.[9] reported comparable findings.

Patients were subtyped into Luminal A, Luminal B, HER2/neu positive, and basal-like, based on the expression of ER, PR, and HER2/neu. The majority of cases exhibited triple negative subtype (37.5%), while Luminal A subtype accounted for 32.1% of cases. HER2/neu positive subtype was observed in 25% of cases, and Luminal B subtype was the least prevalent, accounting for 5.9% of cases.

The classification of breast cancer subtypes based on biomarkers may exhibit variations across different ethnicities, methods of classification, biomarker profiles, antibody clones, laboratory methodologies, and assessment criteria. A total of 21 cases exhibited triple negativity. The majority of the observed malignancies had poor differentiation, accompanied by the presence of positive axillary lymph nodes upon initial diagnosis. In addition, all patients presented with tumors of significant magnitude,

Table 2: Correlating vascular endothelial growth factor expression with other known prognostic factors of breast cancer

Clinicopathological parameters	Total patients, *n* (%)	VEGF expression		*P*	Significance (*P*<0.05)
		Positive, *n* (%)	Negative, *n* (%)		
Age					
31–40	16 (14)	14 (87.5)	2 (12.5)	0.475	No
41–50	30 (27)	22 (73)	8 (27)		
51–60	52 (46)	34 (65)	18 (35)		
61–70	12 (11)	6 (50)	6 (50)		
71–80	2 (2)	0	2 (100)		
Menopause					
Postmenopausal	84 (75)	52 (62)	32 (38)	0.09	No
Premenopausal	28 (25)	24 (86)	4 (14)		
Histopathological grade					
Grade 1	0	0	0	0.027	Yes
Grade 2	54 (48.3)	30 (55)	24 (45)		
Grade 3	58 (51.7)	48 (82.8)	10 (17.2)		
Necrosis					
Yes	54 (48.3)	46 (85)	16 (15)	0.015	Yes
No	58 (51.7)	32 (56)	26 (44)		
Desmoplasia					
Yes	76 (67.8)	50 (65.8)	26 (34.2)	0.362	No
No	38 (32.2)	28 (77.8)	8 (22.2)		
Lymphocytic infiltration					
Yes	40 (35.8)	24 (60)	16 (40)	0.452	No
No	72 (64.2)	46 (63.8)	26 (36.1)		
LVI					
Yes	58 (51.7)	50 (85.7)	8 (14.3)	0.009	Yes
No	54 (48.3)	28 (51.8)	26 (48.1)		
Tumor size (cm)					
<2	4 (3)	0	4 (100)	0.006	Yes
2–5	84 (75)	54 (63.1)	30 (36.9)		
>5	12 (22)	24 (100)	0		
Lymph node status					
N0	50 (44.6)	22 (44)	28 (56)	0.002	Yes
N1	44 (39.2)	40 (90.9)	4 (9.1)		
N2	10 (9.2)	8 (80)	2 (20)		
N3	8 (7)	8 (100)	0		
ER					
Positive	42 (37.5)	18 (42.9)	24 (57.1)	0.001	Inverse significance (yes)
Negative	70 (62.5)	60 (85.7)	10 (14.3)		
PR					
Positive	42 (37.5)	18 (42.9)	24 (57.1)	0.001	Inverse significance (yes)
Negative	70 (62.5)	60 (85.7)	10 (14.3)		
HER2/neu					
Positive	34 (30.3)	32 (94.1)	2 (5.9)	0.009	Yes
Negative	78 (69.7)	46 (59)	46 (41)		
Molecular subtypes					
Luminal A	26 (32.1)	14 (38.9)	22 (61.1)	0.001	Yes
Luminal B	6 (5.9)	4 (66.7)	2 (33.3)		
Triple negative	42 (37)	32 (76.2)	10 (23.8)		
HER2/neu positive	28 (25)	28 (100)	0		

VEGF: Vascular endothelial growth factor, LVI: Lymphovascular invasion, ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2

exceeding 2 cm in diameter. The findings of this study are similar to those reported in a study conducted by Ovcaricek *et al.*[10] Triple negative breast cancers demonstrate a heightened level of aggressive clinicopathological features

and a reduced duration of relapse-free survival. The formation of tumors necessitates the production of a vascular stroma by malignant cells.[11] The process of angiogenesis initiates within an environment of *in situ* breast cancer. The process of tumor development and metastasis necessitates the establishment of new blood vessels, known as neovascularization, before invasion.[12] The initial research investigation carried out by Weidner *et al*.[13] demonstrated the significance of tumor neovascularization as a prognostic indicator in individuals diagnosed with invasive breast cancer. Since then, several studies have presented divergent results concerning the prognostic significance of microvessel density (MVD). Some investigations have demonstrated a negative correlation between survival rates and MVD, while others have failed to establish any link.[12]

In the present study, the median microvessel density (MVD) observed in Figure 1d and e was 4.70 HPF when stained with VEGF to identify endothelial cells. Tumors that were larger in size, had metastasized to lymph nodes, and exhibited poor differentiation were shown to have a greater microvessel density (MVD). The findings of our study about MVD exhibit a resemblance to the research conducted by Shivakumar *et al*.[14] The expression of VEGF is detected using immunohistochemical staining, which reveals its presence in the cytoplasm of tumor cells. Numerous studies have employed diverse scoring techniques for VEGF due to the absence of a standardized approach.

The expression of VEGF in breast IDC tumors is infrequent. The expression of VEGF exhibited a range of 53%–88% in several investigations. In the present study, the expression of VEGF was found to be 69%. Okada *et al*.[15] discovered a comparable expression status. Lee *et al*.[16] and Konecny *et al*.[17] have observed a higher level of expression in comparison, while Almumen *et al*.[18] and Shankar *et al*.[19] have reported a lower level of expression in contrast to our study.

Similar to various researches, our study was unable to establish any correlation between age and the expression of VEGF. In the course of our research, it was observed that a majority of patients who tested positive for VEGF were in the premenopausal age This was in resemblance to the studies conducted by Comsa *et al*.[20] Grade 3 tumors include higher expression of VEGF compared to Grade 2 tumors. This statement aligns with the findings of Linderholm *et al*.[21] Several studies, including those conducted by Konecny *et al*.,[17] Mylona *et al*.,[22] and Almumen *et al*.,[18] observed an increase in VEGF expression associated with higher tumor grade. However, it is important to note that these findings did not reach statistical significance.

In a manner akin to the findings of Raica *et al*.,[23] the expression of VEGF was found to be associated with LVI.

Multiple studies have demonstrated that the size of a tumor is a significant prognostic indicator in cases of breast cancer, as it is associated with a higher likelihood of axillary lymph node metastasis and worse overall survival rates.[24,25] In the current investigation, it was shown that 71% of tumors had T2 or T3 characteristics at the time of diagnosis. The data presented in this study indicate that a majority of women sought medical attention when their tumors had reached a greater size.

A correlation has been established between tumor size and overexpression of VEGF, as observed in previous studies by Linderholm *et al*.,[21] Konecny *et al*.,[17] and Shankar *et al*.[19] However, Valković *et al*.[26] and Mylona *et al*.[22] did not find any correlation in cases of IDC. The use of various thresholds for VEGF expression in the studies resulted in several inconsistencies.

The present study establishes a correlation between the expression of VEGF and the presence of node-positive tumors. Several studies have reported a correlation between the expression of VEGF and the presence of lymph node involvement, specifically Almumen *et al*.,[18] Shankar *et al*.,[19] Valković *et al*.,[26] and Yavuz *et al*.[27] have all identified this association in their respective investigations. The study conducted by Konecny *et al*.[17] revealed a significant association between the overexpression of VEGF in patients with positive lymph nodes and a negative prognosis in terms of survival.

The formation of tumors necessitates the production of a vascular stroma by malignant cells. The process of angiogenesis initiates within the context of *in situ* breast cancer. The process of tumor development and metastasis necessitates the establishment of new blood vessels, known as neovascularization, subsequent to invasion. The initial study conducted by Weidner *et al*.[13] demonstrated the prognostic value of tumor neovascularization in individuals diagnosed with invasive breast cancer. Subsequent research has yielded mixed results regarding the prognostic significance of microvessel density (MVD), with certain studies reporting an adverse correlation between survival outcomes and MVD, while others have found no discernible relationship.

According to the study conducted by Lee *et al*.[16] it was observed that tumors expressing VEGF exhibited a higher microvessel density (MVD). VEGF-rich and VEGF-deficient, utilizing the intensity of anti-VEGF antibody staining.[28] Their findings revealed a correlation between microvessel density (MVD) and the overexpression of VEGF. The expression of VEGF was found to be positively correlated with an increase in MVD, as observed in our study. The study conducted by Bolat *et al*.[29] demonstrated a significant positive correlation between microvessel density (MVD) and VEGF, along with other prognostic factors such as lymph node metastases, tumor size, and IDC grade.

According to Konecny *et al*., there is an inverse correlation between the expression of VEGF and the status

of estrogen and PRs. The current study and the research conducted by Okada *et al*.[15] yielded similar findings. HER2/neu-positive tumors are aggressive and have a high risk of progression and metastasis. The utilization of anti-HER2 medications that have been approved by the Food and Drug Administration aids in the mitigation of these potential hazards. Several clinical studies are currently investigating the combined use of chemotherapy, anti-HER2 medicines, and anti-VEGF therapies to enhance the overall survival and reduce the risk of relapse in breast cancer patients.

The statistical correlation between the expression of VEGF and HER2/neu has been confirmed by Lee *et al*.[16] A lack of relationship between VEGF and HER2/neu overexpression was observed by Okada *et al*.[15] and Yavuz *et al*.[27] The presence of a positive association between the overexpression of HER2/neu and the production of VEGF suggests that VEGF plays a role in the aggressive nature of HER2/neu and provides evidence for the potential effectiveness of combination therapies targeting both HER2/neu and VEGF in the treatment of breast tumors that overexpress HER2/neu. The HER2 subtype of breast cancer is recognized as aggressive, frequently leading to the spread of cancer cells to the lymph nodes and resulting in an unfavorable prognosis. The aggressive nature of cancer cells can potentially be elucidated by the production of VEGF-C.[30] The aforementioned results provide evidence of the therapeutic significance of HER2 and VEGF-C expression, suggesting that the inhibition of HER2 could potentially reduce tumor growth and the spread of cancer cells through lymphatic vessels.

The findings from both *in vitro* and *in vivo* studies indicate that FOXP3 exerts a suppressive effect on angiogenesis in breast cancer. The transcription of VEGF is suppressed by the FOXP3 protein, resulting in a reduction in angiogenesis in breast cancer. The findings of our study have revealed the presence of a previously unidentified regulator of VEGF in breast cancer. In addition, our research has provided insights into the cancer-suppressing function of FOXP3, as supported by previous literature.[31] The study conducted by Bakr *et al*.[32] investigates the role of miRNA-373 in breast cancer patients and its interaction with target genes, VEGF, and cyclin D1. The findings suggest that miRNA-373, an oncomir that specifically targets VEGF and cyclin D1, holds potential as a biomarker for the identification and prognosis of breast cancer.

Conclusions

Incorporating VEGF as a biomarker along with known characteristics into a prognostic score helps predict clinical outcome more precisely and identifies patients who may benefit from anti-VEGF medication.

Ethical approval

Institutional review board ethical approval was obtained.

Informed consent

All patients gave consent for evaluation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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