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Relationship of *VEGF* and *p53* expression with other prognostic parameters in breast carcinomas

Meme karsinomlarında VEGF ve p53 ekspresyonunun diğer prognostik parametrelerle ilişkisi

Perihan Özlem Doğan Ulutaş, Sevgi Bakarış, Gülçin Güler Şimşek

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Abstract

Purpose: We aimed to evaluate the relationship between *VEGF* and *p53* immunohistochemical expressions and other clinicopathological prognostic parameters in breast carcinomas.

Materials and methods: Sections prepared from paraffin-embedded blocks diagnosed with a total of 74 primary breast cancers were examined and *VEGF*, *p53*, estrogen, progesterone, *Cerb-B2* and *Ki-67* immunohistochemical stains were applied. The relationship of *VEGF* and *p53* with other immunohistochemical stains and prognostic parameters was investigated.

Results: Statistically significant results were obtained across *VEGF* with lateralization, grade and lymphovascular invasion. Furthermore, while no staining with *VEGF* was observed in any of the normal breast tissues, an increase in *VEGF* expression was observed as the tumor progressed from carcinoma in situ to invasive carcinoma. It was observed that *VEGF* expression increased while the invasive tumor progressed from low grade to moderate grade, whereas *VEGF* expression decreased when it progressed from moderate to high grade.

Statistically significant correlation among *p53* with *Ki-67*, grade, diameter and opposite correlation between *p53* and estrogen was found. There was increased *p53* expressionin the in situ and invasive field of tumor.

Conclusion: Similar *p53* expression rates in in situ and invasive areas of the tumor may be helpful in predicting the behavior of the tumor in the in situ stage and in guiding the treatment. According to our data, the role of VEGF in tumor progression and its relationship with many prognostic factors is evident.

Key words: Breast cancer, *Ki-67*, *p53*, *VEGF.*

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Öz

Amaç: Meme karsinomlarında *VEGF* ve *p53* immünhistokimyasal ekspresyonları ile diğer klinikopatolojik prognostik parametreler arasındaki ilişkiyi değerlendirmeyi amaçladık.

Gereç ve yöntem: Toplam 74 primer meme kanseri tanılı parafin gömülü bloktan hazırlanan kesitler incelendi ve *VEGF, p53*, östrojen, progesteron, *Cerb-B2* ve *Ki-67* immünhistokimyasal boyaları uygulandı. *VEGF* ve *p53*'ün diğer immünhistokimyasal boyalar ve prognostik parametrelerle ilişkisi araştırıldı.

Bulgular: *VEGF* ile lateralizasyon, derece ve lenfovasküler invazyon arasında istatistiksel olarak anlamlı sonuçlar elde edildi. Ayrıca tüm normal meme dokularında *VEGF* ile boyanma görülmezken, tümör in situdan invaziv hale progrese oldukça *VEGF* boyanma yoğunluğunda artış izlendi. İnvaziv tümör düşük dereceden orta dereceye progrese olurken *VEGF* boyanması artarken, orta dereceden yüksek dereceye doğru boyanmada düşüş izlendi.

P53 ile *Ki-67*, derece ve çap arasında istatistiksel olarak anlamlı, *p53* ile östrojen arasında östrojen ile ise ters korelasyon bulundu. Tümörün in situ ve invaziv alanlarında *p53* ekspresyonunda artış izlend**i.**

Sonuç: Tümörün in situ ve invaziv alanlarında benzer *p53* ekspresyon oranlarının izlenmiş olması, in situ evredeki tümörün davranışının tahmin edilmesi ve tedavinin yönlendirilmesinde yardımcı olabilir. Verilerimize göre *VEGF*'ün tümör progresyonundaki rolü ve birçok prognostik faktörle ilişkisi belirgindir.

Anahtar kelimeler: Meme kanseri, *Ki-67*, *p53*, *VEGF*.

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Perihan Özlem Doğan Ulutaş, M.D. Department of Pathology, University of Health Sciences Ankara Atatürk Sanatorium Training and Research Hospital, Ankara, Turkey, e-mail: tadby@hotmail.com (https://orcid.org/0000-0003-4945-6183) (Corresponding Author)

Sevgi Bakarış, Prof. Department of Pathology, University of Health Science Adana City Training and Research Hospital, Adana, Turkey, e-mail: sevgi.bakaris@gmail.com (https://orcid.org/0000-0002-3165-0650)

Gülçin Güler Şimşek, Prof. Department of Pathology, University of Health Sciences Ankara Gulhane Training and Research Hospital, Ankara, Turkey, e-mail: drgulcinguler@gmail.com (https://orcid.org/0000-0001-7710-4631)

Introduction

Breast cancer is the most common cancer in women worldwide. It is the most frequent cause of cancer death in women (15.5% of total) [1]. Invasion and metastasis of breast cancer involves multi-step process and each step includes numerous biological factors whether they have diagnostic or prognostic potential. Available prognostic factors and clinicopathological parameters often indicate that how patients respond to different adjuvant chemotherapy and hormonal therapy. Several immunohistochemical markers including estrogen receptor (*ER*), progesterone receptor (*PR*), *HER2/neu* (*Cerb-B2*) and *Ki-67*, are used routinely to instruct the clinic about the prognosis of cancer and the response to therapy of patient. But new markers are required to determine new diagnostic and therapeutic parameters and better understanding of the therapy resistance.

One of the most studied ones is *p53*. It is a protein that coded by a tumor suppressor gene. While most of the studies [2] claimed that the *p53* mutation has a prognostic significance in the breast cancer, some studies did not support it clearly [3].

Studies [4] promote that angiogenesis and lymphangiogenesis plays an important role in tumor growth of breast cancer. Among the known pro-angiogenic molecules, *vascular endothelial growth factor* (*VEGF*) plays a key role. Some studies supported that *VEGF* could be a prognostic marker in breast carcinoma patients [5], but some didn't [6].

In the present study we searched *VEGF* expression in normal breast tissue, atypical hyperplasia, carcinoma in situ (CIS) and invaziv areas of the patients with breast carcinoma by using IHC staining. Also we analyzed the relationship of VEGF and *p53* with 4 other immunohistochemical markers (*ER, PR, Cerb-B2, Ki-67*) and prognostic parameters.

Materials and methods

A total of 74 formalin-fixed, paraffin-embedded blocks of primary breast cancer specimens were included. 3 of the materials were radical mastectomy, 68 of them were modified radical mastectomy, 3 of them were simple mastectomy and 3 of them were partial mastectomy. There were 63 invasive ductal carcinomas, 4 pure invasive lobular carcinomas, 4 metaplastic carcinomas and 3 mucinous carcinomas. The average age of patients was 53.6.

57 of 74 cases had normal breast tissue, 13 of 74 cases had atypical hyperplasia areas and 30 of 74 cases had CIS fields (27 cases ductal carcinoma in situ and 3 cases lobular carcinoma in situ), accompanying the invasive area. The tumor grade was determined by histological examination of H&E stained preparations according to Bloom-Richardson System, Nottingham modification [7]. Cases were divided into three groups according to tumor size (≤2 cm=1. group, 2-5 cm=2. group, >5 cm=3. group) considering TNM staging system and divided into four groups according to nodal status (no nodal involvement=1, 1-3 nodal involvement=2, 4-9 nodal involvement=3, ≥10 nodal involvement=4). Information about personal and tumoral details reported in Table 1.

Immunohistochemistry

Four μm-thick sections were mounted onto poly-l-lysine coated slides from formalin-fixed and parafin-embedded tissue blocks and immunohistochemistry for *ER, PR, Cerb-B2, Ki-67, VEGF* and *p53* was performed to all cases. The listed antibodies were used: Monoclonal Rabbit Anti-human Estrogen Receptor α clone EP1 (DAKO, Code IS084), Monoclonal Mouse Anti-human Progesterone Receptor clone PgR636 (DAKO, Code IS068), Polyclonal Rabbit Anti-human *Cerb-B2* oncoprotein (DAKO, Cat A0485), Monoclonal Mouse Anti-human *Ki-67* antigen clone MIB-1 (DAKO, Code IS626), Monoclonal Mouse Anti-human *p53* protein Clone DO-7 (DAKO, IS616) and Anti-*VEGF* Rabbit Polyclonal Antibody (Biogenex, Code AR-483-5R).

Scoring

VEGF protein expression was mainly observed in the cytoplasm of tumor cells, a case of hemangioma accepted as positive control and the stromal cells of normal breast tissues were accepted as internal positive control. Staining with *VEGF* was categorized semiquantitatively on the basis of percentage of positive tumor cells as follows: 0=no immonureactivity; 1=<10% tumor cells stained; 2=10-50% tumor cells stained; and 3=>50% tumor cells

Table 1. Formations about cases

		n (%)
Age	≤40 years	13 (17.6)
	>40 years	61 (82.4)
Menopause	premenopausal	29 (39.2)
Status	postmenopausal	45 (60.8)
Lateralization	Right	30 (40.5)
	Left	44 (59.5)
Grade	1	5 (6.8)
	2	40 (54.1)
	3	29 (39.1)
Size	1	11 (14.9)
	2	43 (58.1)
	3	20 (27)
Lymphnode metastasis	1	14 (18.9)
	2	25 (33.8)
	3	17 (23)
	4	18 (24.3)
Lymphovascular invasion	Yes	67 (90.5)
	No	7 (9.5)

n:number

stained. Staining intensity was scored as follows:0 (negative); 1 (weak); 2 (moderate); 3 (strong). The immunohistochemical score (IHS) was calculated by multiplication the quantity score with the staining intensity score, and ranged from 0 to 9 [8]. Patients were categorized into four groups: negative/no (IHS 0), low immunoreactivity (IHS 1-3), moderate immunoreactivity (IHS 4-6) and high immunoreactivity (IHS >6).

A high grade brain tumor with known positivity was used as a positive control for *p53*. Nuclear staining was based on. No staining in tumor cells: 0, below 10% (cut off value) staining:1, 10-50% staining:2, more than 50% staining:3. Then we evaluated as; 0 and 1:negative, 2 and 3:positive [9].

Cases were accepted as positive for *ER* and *PR* if nuclear immunoreactivity was present in ≥10% of tumor cells [10].

The *Cerb-B2* was scored as 0 (negative), 1+, 2+, 3+ in accordance with the recommendations of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) [11]. A case known as positive in our institute was used as positive control.

For *Ki-67* proliferation index, cases were considered as positive if nuclear immunoreactivity was present in >15% of tumor cells [12], then divided into 3 groups as negative/low, moderate and high [13]. Germinal centers of a reactive lymph node were used as positive control.

Olympus BX51 light microscope, including x4, x10, x20, x40, x100 objectives and x10 oculars, was used for microscopic examination. We processed data with “SPSS 12.0 for Windows”. Chi-square test was used to investigate association between *VEGF*, *p53* and other routine immunhistochemical markers (*ER, PR, Cerb-B2*, *Ki-67*) and prognostic parameters (age, menopausal status, tumor lateralization, grade, size, node status, LVI). We also compared *VEGF* scores between normal breast tissue, atypical ductal hyperplasia, ductal carcinoma in situ (DCIS) and invasive areas. The significance level was set to 0.05 and *p* values of <0.05 were considered statistically significant.

The study was approved by Kahramanmaras Sutcu Imam University Non-Invasive Clinical Research Ethics Committee. (Decision No: 2013/06-2 Date: 04.04.2013)

Results

Expression of *VEGF* in normal breast tissue, atypical hyperplasia, in situ and invasive areas

For 74 cases, 12 cases (16.2%) had no, 8 (10.8%) had low, 15 (20.3%) had moderate and 39 (52.7%) had high *VEGF* cytoplasmic expression in invasive areas. All of the 57 cases were negative for *VEGF* in the normal breast epithelial cells (Figure 1A). For the 13 cases including atypical hyperplasia component adjacent to the invasive areas; 3 cases (23.1%) had no, 9 cases (69.2%) had low, 1 case (7.7%) had moderate expression for *VEGF* in atypical hyperplasia areas (Figure 1B). For the 30 cases including CIS component adjacent to the invasive area; 9 cases (30%) had no, 6 cases (20%) had low, 7 cases (23.3%) had moderate and 8 cases (26.7%) had high expression of *VEGF* in CIS component (Figure 1B). The *VEGF* expression was associated statistically significant with progression to malignancy (normal breast tissue→atypical hyperplasia→ CIS→invasion). The percentage of moderate and high expression of *VEGF* was observed to increase from normal breast to hyperplasia, CIS and invasion. Also in 74 cases, there was a significant correlation between the *VEGF* staining scores of invasive and in situ components of the tumor. In tumors accompanying in situ components, the staining scores of invasive and in situ components are correlated.

Expression of *p53* in the in situ and invasive areas

For 74 cases, 48 cases (64.8%) had no, 2 cases (2.7%) had staining in tumor cells below 10%, 7 cases (9.4%) had staining in tumor cells 10-50% and 17 cases (22.9%) had staining in over 50% tumor cells with *p53* in invasive areas. In 30 tumors with an in situ component accompanying the invasive area, there was a statistically significant correlation between *p53* staining scores of the invasive and in situ areas (Table 2).

The correlation of *VEGF* with *ER*, *PR*, *Cerb-B2*, *Ki-67* and the other prognostic parameters

VEGF expression was associated with lateralization, grade and lymphovascular invasion as shown in Table 3. High *VEGF* expression was revealed especially in the left breast cancers. An increase was observed from low grade to intermediate grade tumor and then a decline was observed from intermediate grade to high grade tumor for *VEGF* expression (Figure 1C). The cases including lymphovascular invasion had a higher VEGF expression than LVI negative cases. There were no statistically significant associations between *VEGF* and patient's age, menopause status, tumor size, nodal status, *ER*, *PR*, *Cerb-B2*, *Ki-67*, *p53* expressions.

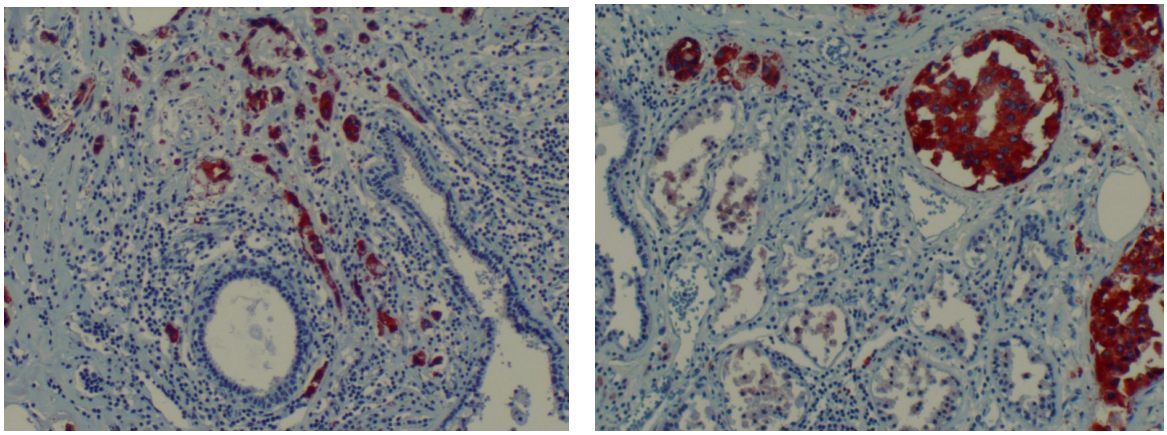

Figure 1. High grade ductal carcinoma case with *VEGF* staining, **A)** high expression of *VEGF* is observed in the tumor cells, while neighboring normal breast tissue is not observed, (x10), **B)** strong positivity is observed with *VEGF* in the invasive and in situ areas, and weak positivity is observed in hyperplasia areas, (x20),

Table 2. Association of *p53* with statistically significant parameters in 74 cases

Prognostic Factor	Groups	Case n (%)	*p53* immunoreactivity score**				*p* value
			Negative n (%)	<10% n (%)	10-50% n (%)	>50% n (%)	
Type	Ductal	63 (85.1)	40 (54.1)	2 (2.7)	5 (6.8)	16 (21.6)	0.699
	Lobular	4 (5.4)	4 (5.4)	0 (0)	0 (0)	0 (0)	
	Mucinous	3 (4.1)	2 (2.7)	0 (0)	1 (1.4)	0 (0)	
	Metaplastic	4 (5.4)	2 (2.7)	0 (0)	1 (1.4)	1 (1.4)	
Grade	1	5 (6.8)	5 (6.8)	0 (0)	0 (0)	0 (0)	**0.002***
	2	40 (54.1)	32 (43.2)	2 (2.7)	2 (2.7)	4 (5.4)	
	3	29 (39.2)	11 (14.9)	0 (0)	5 (6.8)	13 (17.6)	
Size	≤2 cm	11 (14.9)	7 (9.5)	2 (2.7)	0 (0)	2 (2.7)	**0.027***
	>2; ≤5 cm	43 (58.1)	30 (40.5)	0 (0)	4 (5.4)	9 (12.2)	
	>5 cm	20 (27)	11 (14.9)	0 (0)	3 (4.1)	6 (8.1)	
ER	Negative	25 (33.8)	11 (14.9)	0 (0)	3 (4.1)	11 (14.9)	**0.011***
	Positive	49 (66.2)	37 (50)	2 (2.7)	4 (5.4)	6 (8.1)	
p53	Negative	21 (70)	21 (70)	0 (0)	0 (0)	0 (0)	**0.000***
in situ***	<10%	1 (3.3)	0 (0)	1 (3.3)	0 (0)	0 (0)	
	10-50%	5 (16.7)	0 (0)	0 (0)	3 (10)	2 (6.7)	
	>50%	3 (10)	0 (0)	0 (0)	0 (0)	3 (10)	
Ki-67	Negative/Low	13 (17.6)	12 (16.2)	0 (0)	1 (1.4)	0 (0)	**0.005***
	Moderate	28 (37.8)	21 (28.4)	1 (1.4)	4 (5.4)	2 (2.7)	
	High	33 (44.6)	15 (20.3)	1 (1.4)	2 (2.7)	15 (20.3)	

*Parameters with statistically significant correlation
***p53* immunoreactivity score in invasive carcinoma areas
****p53* immunoreactivity score in in situ areas

Table 3. Association of *VEGF* with statistically significant parameters in 74 cases

Prog Fac.	Groups	Case n (%)	*VEGF* immunoreactivity score, n (%)				*p* value
			Negative	Low	Moderate	High	
Type	Ductal	63 (85.1)	12 (16.2)	6 (8.1)	14 (18.9)	31 (41.9)	0.597
	Lobular	4 (5.4)	0 (0)	1 (1.4)	1 (1.4)	2 (2.7)	
	Mucinous	3 (4.1)	0 (0)	0 (0)	0 (0)	3 (4.1)	
	Metap	4 (5.4)	0 (0)	1 (1.4)	0 (0)	3 (4.1)	
Later	Right	30 (40.5)	9 (12.2)	2 (2.7)	3 (4.1)	16 (21.6)	**0.025***
	Left	44 (59.5)	3 (4.1)	6 (8.1)	12 (16.2)	23 (31.1)	
Grade	1	5 (6.8)	0 (0)	1 (1.4)	3 (4.1)	1 (1.4)	**0.019***
	2	40 (54.1)	3 (4.1)	3 (4.1)	9 (12.2)	25 (33.8)	
	3	29 (39.2)	9 (12.2)	4 (5.4)	3 (4.1)	13 (17.6)	
LVI	No	7 (9.5)	4 (5.4)	2 (2.7)	0 (0)	1 (1.4)	**0.003***
	Yes	67 (90.5)	8 (10.8)	6 (8.1)	15 (20.3)	38 (51.4)	

Later: ateralization, LVI: lymphovascular invasion, n: number
*Parameters with statistically significant correlation

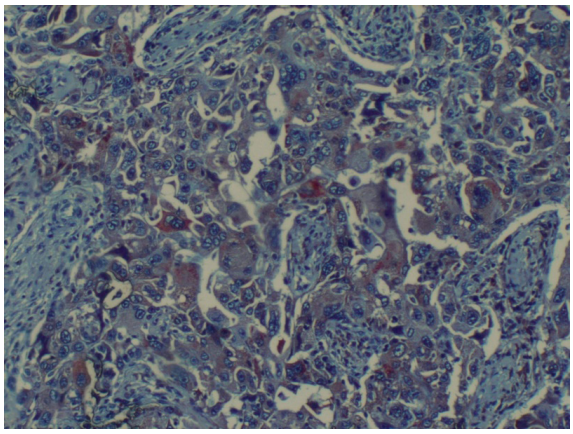

Figure 1. High grade ductal carcinoma case with *VEGF* staining, **C)** Weak staining pattern with *VEGF* in high grade invasive ductal carcinoma case (x20)

The correlation of *p53* with *ER*, *PR*, *Cerb-B2*, *Ki-67* and the other prognostic parameters

P53 expression was associated with diameter, grade, *Ki-67* positivity and significant opposite correlation was found between *ER* positivity and *p53* expression as shown in Table 2. There were no statistically significant associations between *p53* and patient's age, menopause status, lateralization, nodal status, *LVI, PR* and *Cerb-B2*.

Discussion

Many parameters have been used to determine prognosis in breast cancer. However, these parameters were not sufficient to show the prognosis. Therefore, it has become the focus of researchers to find new biological markers that can help guide the treatment. This study was made for this purpose.

Inactivation of function by loss of both alleles (loss of heterozygous) or point mutations of the *p53* tumor suppressor gene plays an important role in tumor development. While normal *p53* protein can not be detected by IHC, mutant *p53* can be detected mostly [2]. Done et al. [14] emphasized that *p53* expression occurs before the invasive phase in the breast, it can be used to rate DCIS and that *p53* expression may be a marker for the prevention and treatment of invasion while the tumor is still non-invasive. Liu et al. [9] showed that IDC cases, including DCIS domains, *p53* immunoreactivity increased in both in situ and invasive domains, but there was no significant staining difference between the two. The present study has also supported these findings, and there is a statistically significant correlation (p=0.000). In the study, although the relationship of *p53* with age is not statistically significant, high expression pattern was found in patients over the 40 years old. Also the correlation of *p53* overexpression with tumor grade, diameter and *Ki-67* staining percentage was significant (p=0.002, p=0.027 and p=0.005, respectively). While all well-differentiated tumors (5 cases) were stained negative with *p53*, 62.1% of poorly differentiated cases (29 cases) were stained positively with *p53* (Figure 2). Sirvent et al. [15] found that a negative relationship between *p53* and both *ER* and *PR*. A significant opposite correlation (p=0.011) was found between *p53* expression and *ER* in this study. But no significant correlation was found between *PR* and *p53* (p=0.530).

As a result, *p53* overexpression, which can be detected before the invasive carcinoma phase, can be used as a marker for the transition from in situ carcinoma to invasive carcinoma. If the results obtained in the present study are support by larger studies, it can help to predict the behavior of the tumor and direct the treatment while in situ phase. The more expression of *p53* in the tumors which are bigger than 2 cm, poorly differentiated, *ER* negative and has a high proliferative index, indicates that it may be a good prognostic marker.

Studies in recent years showed that angiogenesis was essential for tumor growth, invasion and metastasis [16] and have focused specifically on the *VEGF* family. *VEGF* system, a part of platelet-derived growth factor gene family, includes 5 growth factor and 3 tyrosine kinase receptor which have different roles in physiological and pathological angiogenesis.

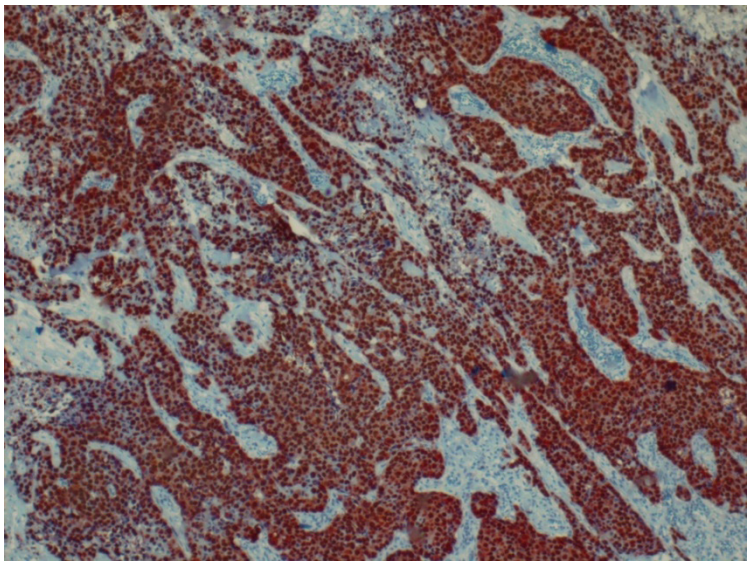

Figure 2. Strong staining with *p53* in a case of high grade invasive ductal carcinoma (x10)

It was identified that the members of family had significant influence on cell survival, mitogenesis, migration, differentiation, vascular permeability, mobilization and cancer development [17]. Although many angiogenic factors have been identified, *VEGF-A/VEGF* is the most potent stimulant and key regulator for tumor angiogenesis, particularly for invasive breast cancer [5]. *VEGF* has been shown to be increased in many cancers such as ovarian [18], lung [19], kidney and bladder [20] cancers.

Angiogenesis starts with the beginning of hyperplasia and increases from CIS to invasive carcinoma [21]. Some studies that targetted to show the change of *VEGF* expression during this progression are available. In some investigations, an increase of *VEGF* in ductal CIS have been noted compared with normal ducts [22]. Wang et al. [23] reported that *VEGF* was low in ductal atypical hyperplasia but significantly increased in ductal CIS and was even higher in invasive ductal carcinoma. Carpenter et al. [21] noted that *VEGF* staining intensity of ductal epithelium increased during the progression from normal to hyperplastic to ductal CIS. In addition to these studies, we compared *VEGF* expression in normal breast tissue, hyperplasia, CIS and invasive areas by immunohistochemistry. In our investigation, normal ducts had no *VEGF* staining. The expression of *VEGF* started in hyperplasia and increased with the progression to malignancy. This evidence shows that the first significant increase in angiogenesis occurs in the phase of atypical hyperplasia. Also we found an interesting correlation between *VEGF* and grade unlike the studies that noted a correlation [23] or noted an inverse correlation [24]. In our investigation, high staining with *VEGF* increased from G1 tumors to G2 tumors and then decreased from G2 to G3 tumors. While low expression was most often observed in G3 tumors, moderate expression was most often observed in G2 tumors. These indications show that the more tumor differentiation decreases and the solid component of the tumor increases, some other angiogenic factors may come into play except *VEGF*. The beginning of *VEGF* staining in hyperplasia stage and the correlation between grade and *VEGF*, can change the direction of the antiangiogenic therapy. In the present study, *VEGF* was not significantly associated with patient's age, menopause status, tumor size, nodal status, *ER*, *PR, Cerb-B2* and *Ki-67* expressions.

A limitation of our study, was the small number of hyperplasia and CIS components.

In conclusion, evaluation of *VEGF i*n breast cancer helps in the selection of patients who could benefit from such therapy. Our study shows that *VEGF* staining starts in hyperplasia phase and increases with the progression to malignancy, but poorly differentiated tumors with great solid component have low *VEGF* expression. More comprehensive studies may result in benefit for breast cancer patients.

Conflict of interest: No conflict of interest was declared by the authors.

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Author Contribution

P.O.D.U.: Conceptualization, data curation, formal analysis, investigation, writing-original draft

S.B.: Conceptualization, data curation, formal analysis, methodology, project administration, supervision, writing-original draft

G.G.S.: Validation, writing review and editing.