Original article

The relationship of peritumoral lymphatic vessel density, marginal adipose tissue invasion and clinicopathological parameters in breast carcinoma

Mirsad Dorić*, Edina Lazović Salčin, Nina Čamdžić,
Suada Kuskunović-Vlahovljak, Svjetlana Radović, Mirsad Babić

Department of Pathology, Faculty of Medicine, University of Sarajevo, Čekaluša 90, 71000 Sarajevo

Abstract

Objective: To investigate a possible association between peritumoral lymphatic vessel density (LVD) and adipose tissue invasion (ATI) of cancer cells at the tumor margin and their correlation with other prognostic parameters in breast cancer, including lymph node status.

Material and methods: Data of 75 patients with breast carcinoma were evaluated through combination of peritumoral LVD and ATI at the tumor margin and compared with clinicopathological parameters. Peritumoral LVD was assessed by immunostaining for D2-40 using the Chalkley counting method. Marginal ATI was defined as either the presence of more than 20 cancer cells in direct contact with the adipose tissue or as the presence of cancer cells in the adipose tissue.

Cases were evaluated concerning to patient's age, tumor size using the TNM staging system, histological type, histological grade (Nottingham histological score, Elston and Ellis), lymphovascular invasion, lymph node metastasis, estrogen receptor (ER), progesterone receptor (PR), HER2/neu status and Ki-67.

Results: The relationship was identified between LVD and marginal ATI but without statistical significancy (r = -0,207; p=0,113). There was a significant correlation of the marginal ATI with Ki-67 expression (r=0,250; p =0.03). The regression model (all variables according to ATI) showed a significant total effect (p <0.05), where Ki-67 was an only independent indicator of ATI.

Conclusion: The present study suggests that adipose tissue invasion of cancer cells at the tumor margin can be a better predictive biologic indicator of aggressiveness than peritumoral lymphatic vessel density in breast carcinoma.

Keywords: breast carcinoma, peritumoral lymphatic vessel density, marginal adipose tissue invasion.

***Corresponding author**
Mirsad Dorić
Department of Pathology
Faculty of Medicine
University of Sarajevo
Čekaluša 90, 71000 Sarajevo
Bosnia and Herzegovina
E-mail: mirsad.doric@mf.unsa.ba

Introduction

Breast cancer (BC) is the most commonly diagnosed cancer in women. Metastasis is the leading cause of mortality in patients diagnosed with breast cancer (1). Tumor associated lymphatic vessels are considered to be the main route of tumor cells to axillary lymph nodes (2). Some studies have suggested that peritumoral lymphatic vessel density (LVD) is associated with an increased risk of lymph node (LN) metastasis (3,4); however, this conclusion is not supported by all of the published studies (5,6). Tumor-stromal interactions seem to be a fundamental aspect of tumor aggressiveness (7-9). Adipocyte is one of the predominant stromal cell types in the microenvironment of mammary tissue. However, the prognostic significance of adipose tissue invasion (ATI) at the tumor margin has not been fully evaluated in breast cancer, and the biologic characteristics of tumors with ATI are also insufficiently known.

Prognostic factors are essential in BC diagnosis as they allow the identification of high-risk patients, for whom, an adjuvant therapy can improve prognosis (10,11). The traditional prognosis can only identify the group of approximately 30% of patients and their outcome. Therefore, new prognostic markers are required (12,13).

The purpose of this study was to investigate the relationship between LVD using the Chalkley count method and ATI at the tumor margin as predictive markers for the risk of axillary LN metastasis and their relationship to other clinicopathological factors in primary breast cancer patients.

Material and methods

Cases and histopathologic examination

A retrospective analysis was conducted using the data of 75 patients with invasive breast carcinoma by searching the database of the Department of Pathology, Faculty of Medicine in Sarajevo, Bosnia and Herzegovina. All patients with BC underwent partial or total mastectomy with axillary lymph node dissection. No neoadjuvant chemotherapy or radiotherapy was administered before the surgical treatment. Tissues specimens were fixed in 10% buffered formalin, paraffin embedded, processed, and stained with hematoxylin and eosin. All cases were reviewed to confirm the diagnosis and the histopathologic characteristics, including immunostaining for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) status and Ki-67.

Cases were evaluated for patient's age; histologic tumor type; tumor size (using the TNM staging system); tumor grade; LN metastasis; ER; PR; HER2 status; Ki-67 and D2-40. Tumor grade was determined by using Nottingham histological score, Elston and Ellis histologic grading criteria (14). Lymphatic vessel invasion (LVI) was defined as the presence of neoplastic emboli within D2-40 positive endothelium-lined spaces (Figure 1A) in areas adjacent to, but outside the margins of the carcinoma (15).

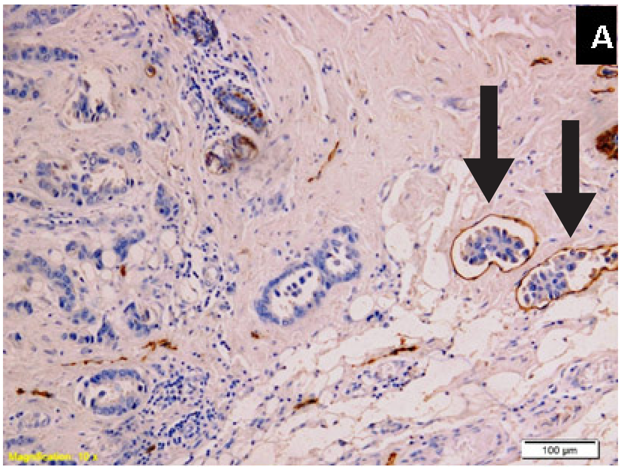

Figure 1A. D2-40 highlighting a lymphatic vessel (arrows) around a tumor embolus (D2-40, ×100)

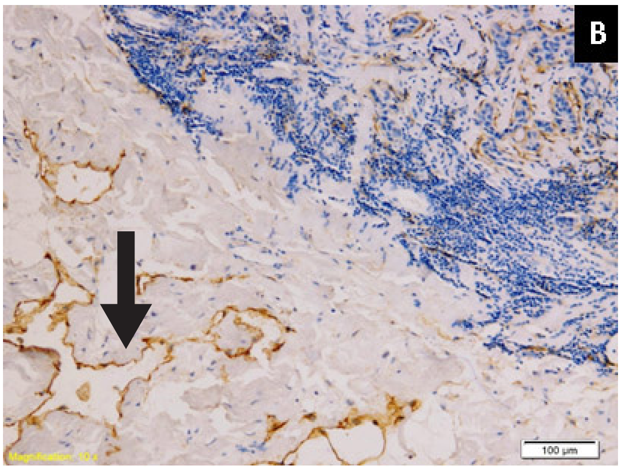

Figure 1B. The dilated peritumoral lymphatic channels (arrow) adjacent to invasive carcinoma of the breast (D2-40, ×100).

One paraffin block with the maximum bulk of tumor with adjacent benign peritumoral tissue was chosen from each case for D2-40 immunohistochemical staining. For the study, 4-μm-thick sections of formalin-fixed, paraffin-embedded tissue were mounted on coated slides. Immunohistochemical analysis using lymphatic endothelial marker D2-40 was performed. The sections were deparaffinized in xylene and rehydrated in a decreasing ethanol series. The sections were incubated with a monoclonal commercially available antibody (clone D2-40, Dako Cytomation; dilution 1:50). Heat-induced epitope retrieval techniques were used for antigen retrieval as follows: citrate buffer (pH 6.0) in a microwave oven (1000 W) for 15 minutes.

The primary antibody against the ER was performed in a humidity chamber in EDTA buffer (pH 9) for 40 min. (clone 1D5, Dako Cytomation; dilution 1:30). The protocols for staining PR and Ki-67 included a microwave antigen retrieval step, 3 times for 5 minutes: anti-PR (clone PgR, Dako Cytomation; dilution 1:30), anti- Ki-67 (clone MIB-1, Dako Cytomation; dilution 1:10). Antigen retrieval for HER2 using HercepTest was performed following the manufacturer's protocol (Dako Cytomation).

Tumors were considered positive for ER and PR when at least 1% of the tumor cells showed unequivocal nuclear staining according to ASCO/CAP guidelines (16).

The intensity of HER2 staining was scored as follows: 0, 1+, 2+, or 3+ (17). For the results, we classified scores of 0 and 1+ as negative and score 3+ as positive. Cases scored as 2+ were considered equivocal, and retested using chromogen in situ hybridization (CISH).

The Ki-67-labeling index is the percentage of cells with Ki-67 positive nuclear immunostaining. Negative Ki-67 was defined as less than 15% stain, and positive Ki-67 was ≥ 15%.

The cases were assessed after staining with D2-40, an immunostaining marker for lymphatic endothelium (Figure 1B). In the present study, we applied the Chalkley grid. The Chalkley counting method is recommended by the international consensus report because it is considered to be a simple and acceptable procedure for daily clinical use and produces lower interobserver variability compared to the more frequently used conventional microvessel density method (18, 19). The mean number of lymphatic vessels in three hotspots was calculated in peritumoral areas.

Adipose tissue and marginal adipose tissue invasion were defined according to previous studies (20-22). Marginal adipose tissue invasion was defined as either the presence of more than 20 cancer cells in direct contact with the adipose tissue or as the presence of cancer cells in the adipose tissue (Figure 2.). Doubtful cases were considered negative.

Statistical analysis was performed using SPSS for Windows (Version 23). A two-tailed unpaired t test was performed to identify the differences between the two groups. A one-way ANOVA test was performed for comparing the groups that were used for the t-test. The correlation between the variables was assessed by the Spearman rank sum test, the Pearson linear correlation coefficient and bivariate linear regression analysis. P values <0.05 were considered statistically significant.

Results

The patient age at the time of surgery ranged from 37 to 87 years, with a mean ± SD of 59 ± 11,7 years (median, 61 years). Clinicopathological characteristics of the studied cases are shown in Table 1.

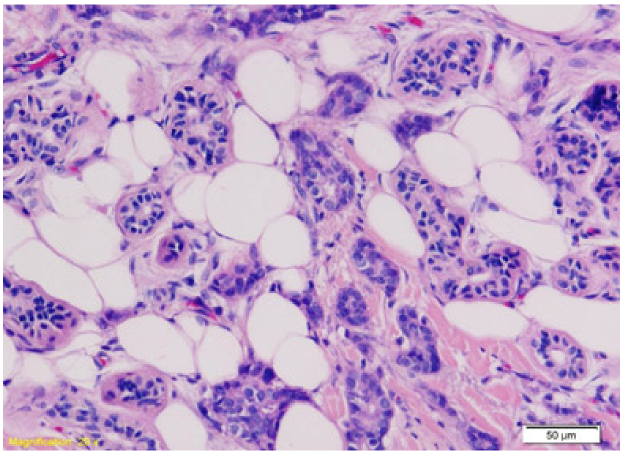

Figure 2A. Ductal carcinoma cells infiltrate the fibroadipose tissues but not the adipose tissues: ATI negative specimen. (H&E, ×200).

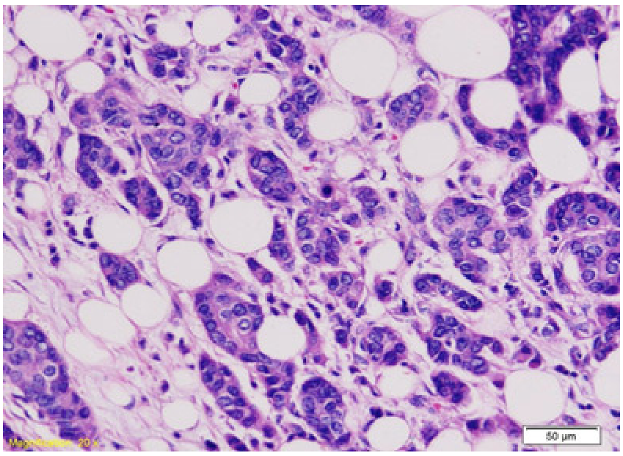

Figure 2B. More than 20 cancer cells are present in the adipose tissue: ATI positive specimen (H&E, ×200).

Table 1. Clinicopathological characteristics of the studied group

	Variable	N	%
	Tumor size (AJCC)		
Tumor size (AJCC)	pT1	41	54,7
	pT2	32	42,7
	pT3	2	2,7
Histological type	Ductal (NOS)	57	76
	Lobular	8	10,7
	Medullary	4	5,3
	Neuroendocrine	3	4
	Mucinous	2	2,7
	Tubular	1	1,3
Grade	G1	23	30,7
	G2	33	44
	G3	19	25,3
ER status	Negative	25	33,3
	Positive	50	66,7
PR status	Negative	27	36
	Positive	48	64
HER2 status	Negative	57	76
	Positive	18	24
Ki-67	Negative	15	20
	Positive	60	80
Lymph node (LN) status	Negative	68	90,7
	Positive	7	9,3
Lymphovascular invasion (LVI)	Absent	46	61,3
	Present	29	38,7
Adipose tissue invasion (ATI)	Absent	15	20
	Present	60	80

Table 2. The relationship between lymph node (LN) status and marginal adipose tissue invasion (ATI) of breast cancer (r=0,046; p=0,696)

LN status		ATI		Total
		present	absent	
Positive within ATI	N	6	1	7
	%	10,0	6,7	9,3
Negative within ATI	N	54	14	68
	%	90,0	93,3	90,7
Total	N	60	15	75
	%	100,0	100,0	100,0

Relationship between ATI and clinicopathologic parameters

Of the 75 cases, 60 (80%) showed marginal ATI. As shown in Table 2, ATI positive cases had a higher incidence of metastases to axillary lymph nodes (6/7 or 85,7%) compared to ATI negative cases (1/7 or 14,3%). Negative axillary lymph nodes were in 54/68 or 79,4% ATI positive and 14/68 or 20,6% ATI negative cases. The relationship between marginal ATI and lymph node status was not statistically significant (r=0,046; p=0,691).

There was a significant correlation of the marginal ATI of cancer cells with Ki-67 expression (r=0,250; p=0,03). However, only a weak correlation with LVI was observed (r=0,136; p=0,244). No statistical differences in age, histologic tumor type, tumor size, nuclear grade, histologic grade, estrogen receptor (ER), progesterone receptor (PR), HER2/neu status were observed (Table 3.).

The regression model (all variables according to ATI) showed a significant total effect ($p<0,05$), where Ki-67 was an only independent indicator of ATI (Table 4.). Cases with Ki-67 positive expression are 6,13 times more likely to have marginal ATI. Also, cases that are LVI positive are 2,6 times more likely to have marginal ATI, but the prediction is not reliable.

Relationship between peritumoral LVD and clinicopathologic parameters

Table 3. shows an association of peritumoral LVD with lymph node status, tumor size, ER, HER2 status, and Ki-67 expression but without statistical significance. We also found no correlation of LVD with age, histologic tumor type, tumor size, nuclear grade, histologic grade and PR.

Regression analysis confirms that neither the whole model (all variables according to LVD) nor the individual variables influence or increase the chance of changing the peritumoral LVD value.

Significance of the combination of ATI and peritumoral LVD

We investigated the relevance of marginal ATI when combined with peritumoral LVD. A marginal correlation of ATI with peritumoral LVD was found (r=-0,207; p=0,075); ATI positive cases have a higher density of peritumoral lymphatic vessels but without statistical significance. No statistically significant association was found between ATI and LVD with lymph node metastasis (Table 4.).

Table 4. Regression model: all variables according to ATI

	OR	95% C.I.for OR	p
Age	0,981	0,920-1,045	0,699
pT (tumor size)	1,987	0,518-7,625	0,675
Histological type	1,210	0,713-2,052	0,755
grade	1,284	0,378-4,366	0,643
LVI	2,586	0,515-12,989	0,239
LN	0,697	0,045-10,712	0,691
ER	1,295	0,133-1,580	0,540
PR	0,266	0,025-2,800	0,810
HER-2	0,814	0,161-4,106	0,787
Ki-67	6,130	2,863-13,557	0,030
LVD	0,480	0,194-1,187	0,113
Constant	1,580		0,937

Table 3. The results of ATI and LVD association with clinicopathological parameters in 75 breast cancer patients

Parameters	ATI		LVD	
	Correlation Coefficient	Sig. (2-tailed)	Correlation Coefficient	Sig. (2-tailed)
age	-0,088	0,452	-0,090	0,441
pT (tumor size)	0,031	0,792	0,170	0,144
Histological type	-0,023	0,847	0,091	0,437
grade	-0,056	0,632	0,037	0,751
LVI	0,136	0,244	-0,038	0,745
LN	0,046	0,696	-0,114	0,331
ER	-0,071	0,547	0,159	0,173
PR	-0,028	0,813	0,056	0,634
HER-2	-0,031	0,790	-0,136	0,243
Ki-67	0,250	0,031	0,186	0,111

Discussion

Axillary lymph node status at time of diagnosis is the most significant and durable prognostic factor in breast cancer patients (23). The same studies reported a significant increase in peritumoral LVD (6,8), in which some of lymphatic vessels containing tumor emboli (8). A consensus seems to exist with regard to increased density of peritumoral lymphatic vessels that might be sufficient for tumor cell transit to lymph node.

The results of this study showed an association of peritumoral LVD with lymph node status, tumor size, ER, HER2 status, and Ki-67 expression, but without statistical significance. Zhang et al. in the meta-analysis of 13 studies, involving 1029 breast cancer cases, showed LVD positive correlation with LN metastasis (24). The evidence of our study is limited because the study included relatively small sample size and different types of tumors, which could have led to confounding factors and selection bias.

The contradicting results about the role of peritumoral LVD in tumor progression may be due to differences in patient selection, sample size, method, and/or the types of tumors included in the analyses. Moreover, the different LVD counting methods and the varied dilutions of antibodies could have affected the conclusions (24).

In this study conducted on 75 cases, 60 cases (80%) showed presence of ATI which is almost identical to the results of Yamaguchi et al. with 79 % ATI. Yamaguchi et al. previously reported the prognostic effect of marginal adipose tissue invasion in invasive ductal carcinoma of the breast (20). Although the results of our study show that ATI positive cases have higher incidence of metastases to axillary lymph nodes (6/7 or 85.7%) compared to ATI negative cases (1/7 or 14.3%), the relationship between marginal ATI and LN status was not statistically significant ($r = 0.046$; $p = 0.691$). This can be explained by that the randomized cases of our study had a significantly lower number of cases with metastases to the axillary lymph nodes (7/75), compared to the study by Yamaguchi et al. (106/199).

The prognostic significance of adipose tissue invasion of cancer cells at the tumor margins has not been fully evaluated. Logistic regression analysis (all variables according to ATI) showed a significant total effect ($p < 0.05$), where Ki-67 was an only independent indicator of ATI. Cases with Ki-67 positive expression are 6.13 times more likely to have marginal ATI. Although the prognostic role of Ki-67 is a matter of debate, in recent years, evidence has shown that the Ki-67 labeling index is an independent prognostic factor for the survival and recurrence of tumors. These studies examined more than 4600 cases and proved that Ki-67 labeling index is a significant prognostic factor (25). In a survey by Kermani et al. there was a negative correlation between Ki-67 and ER and PR (26). Cell proliferation plays an important role in the clinical behavior of invasive BC.

In general, when intraductal carcinoma cells invade the breast stroma, the cells initially penetrate the fibrous tissue, followed by the fibroadipose tissue and, finally, the adipose tissue. Marginal ATI may lead to a larger contact area between cancer cells and the peritumoral functional lymphatic endothelium, increasing the chances for lymphovascular invasion (20). The results of our study showed that LVI positive cases are 2.6 times more likely to have marginal ATI but the prediction is not reliable.

Marginal adipose tissue invasion is probably a time-dependent pathologic factor, like the nodal status, tumor size, and peritumoral vascular invasion (27). Conversely, some types of breast carcinoma, including the lobular type, display much more interaction with fat tissue than other types in tumor development (28).

In exploration for the potential prognostic indicators of breast carcinoma, attention of our study has been focused on the relevance of marginal ATI when combined with peritumoral LVD. To our knowledge, this is the first study to investigate the relationship between LVD and ATI of cancer cells at the tumor margin as a predictive marker for the risk of axillary lymph node metastasis. A marginal correlation of ATI with peritumoral LVD was found ($r = -0.207$, $p=0,075$); ATI positive cases have a higher density of peritumoral lymphatic vessels but without statistical significance. No statistically significant association was found between ATI and LVD with lymph node metastasis. To determine the relevance of marginal ATI when combined with peritumoral LVD and its prognostic impact in BC patients will require further studies with a larger sample size.

Conclusion

A reliable estimation of different prognostic factors in BC patients is required for the selection of an optimal therapeutic strategy. The findings of this study suggests that marginal adipose tissue invasion of cancer cells can be a better predictive factor of aggressiveness than peritumoral lymphatic vessel density in breast carcinoma. More studies and investigations are required for substantiating the significance of ATI and its influence on the prognosis of breast cancer.

Declaration of interest

The authors declare no conflict of interest for this study.

References

[1] Ran S, Volk L, Hall K, Flister MJ. Lymphangiogenesis and lymphatic metastasis in breast cancer. Pathophysiology. 2010; 17: 229–251.

[2] Nathanson SD, Zarbo RJ, Wachna DL, et al. Microvessels that predict axillary lymph node metastases in patients with breast cancer. Arch Surg. 2000; 135: 586-593.

[3] El-Gohary Y. M, et al. Prognostic signifcance of intratumoral and peritumoral lymphatic density and blood vessel density in invasive breast carcinomas. American journal of clinical pathology. 2008; 129: 578–586.

[4] Mohammed R. A, Ellis I. O, Elsheikh S, Paish E. C, & Martin S, G. Lymphatic and angiogenic characteristics in breast cancer: morphometric analysis and prognostic implications. Breast Cancer Res Treat. 2009; 113: 261–273.

[5] Zhao, Y. C, et al. Peritumoral lymphangiogenesis induced by vascular endothelial growth factor C and D promotes lymph node metastasis in breast cancer patients. World journal of surgical oncology. 2012; 10: 165.

[6] van der Schaft, D. W, et al. Absence of lymphangiogenesis in ductal breast cancer at the primary tumor site. Cancer letters. 2007; 254: 128–136.

[7] Acs G, Dumoff KL, Solin LJ, et al. Extensive retraction artifact correlates with lymphatic invasion and nodal metastasis and predicts poor outcome in early stage breast carcinoma. Am J Surg Pathol. 2007; 31: 129-140.

[8] Agarwal B, Saxena R, Morimiya A, et al. Lymphangiogenesis does not occur in breast cancer. Am J Surg Pathol. 2005; 29: 1449-1455.

[9] Kimijima I, Ohtake T, Sagara H, et al. Scattered fat invasion: an indicator for poor prognosis in premenopausal, and for positive estrogen receptor in postmenopausal breast cancer patients. Oncology. 2000; 59: 25-30.

[10] Arens N, Bleyl U, Hildenbrand R. HER2/neu, p53, Ki67, and hormone receptors do not change during neoadjuvant chemotherapy in breast cancer. 2005; 446 : 489-496.

[11] Molino A, Micciolo R, Turazza M, Bonetti F, Piubello Q, Bonetti A. Ki-67 immunostaining in 322 primary breast cancers: associations with clinical and pathological variables and prognosis. Int J Cancer. 1997; 74 (4) : 433-7.

[12] Lu X, Gu Y, Ding Y, Song W, Mao J, Tan J. Correlation of ER, PgR, HER-2/neu, p53, and VEGF with clinical characteristics and prognosis in Chinese women with invasive breast cancer. Breast J. 2008; 14 (3) : 308-10.

[13] Saha Roy S, Vadlamudi R.K. Role of estrogen receptor signaling in breast cancer metastasis. Int J Breast Cancer. 2012; 2012 : 654698.

[14] Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991; 19 : 403-10.

[15] deMascarel I, MacGrogan G, Debled M, Sierankowski G, Brouste V, Mathoulin-Pelissier- S, et al. D2-40 in breast cancer: should we detect more vascular emboli? Mod Pathol 2009; 22 : 216-22.

[16] Deyarmin B1, Kane JL, Valente AL, van Laar R, Gallagher C, Shriver CD, Ellsworth RE. Effect of ASCO/CAP guidelines for determining ER status on molecular subtype. Ann Surg Oncol. 2013 Jan; 20(1): 87-93.

[17] Antonio C. Wolff, M. Elizabeth Hale Hammond, Kimberly H. Allison, Brittany E. Harvey, Pamela B. Mangu, John M.S. Bartlett. Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. J Clin Oncol. 2018 July; 36 (20): 2105-2122.

[18] Hansen S, Sørensen FB, Vach W, Grabau DA, Bak M, Rose C. Microvessel density compared with the Chalkley count in a prognostic study of angiogenesis in breast cancer patients. Histopathology. 2004 May; 44(5): 428-36.

[19] Dhakal HP1, Bassarova A, Naume B, Synnestvedt M, Borgen E, Kaaresen R, Schlichting E, Wiedswang G, Giercksky KE, Nesland JM. Breast carcinoma vascularity: a comparison of manual microvessel count and Chalkley count. Histol Histopathol. 2009 Aug; 24(8): 1049-59. doi: 10.14670/HH-24.1049.

[20] Yamaguchi J, Ohtani H, Nakamura K, Shimokawa I, Kanematsu T. Prognostic impact of marginal adipose tissue invasion in ductal carcinoma of the breast. Am J Clin Pathol 2008; 130(3): 382–388.

[21] Kuba S, Ohtani H, Yamaguchi J, et al. Incomplete inside-out growth pattern in invasive breast carcinoma: association with lymph vessel invasion and recurrence-free survival. Virchows Arch 2011; 458(2): 159–169.

[22] Moriuchi H, Yamaguchi J, Hayashi H, Ohtani H, Shimokawa I, Abiru H, Okada H, Eguchi S. Cancer Cell Interaction with Adipose Tissue: Correlation with the Finding of Spiculation at Mammography. Radiology. 2016 Apr; 279(1): 56-64.

[23] Donegan WL. Tumor-related prognostic factors for breast cancer. CA Cancer J Clin. 1997; 47: 28-51.

[24] Zhang S, Yi S, Zhang D, et al. Intratumoral and peritumoral lymphatic vessel density both correlate with lymph node metastasis in breast cancer. Nature. January 2017; 7: 40364, DOI: 10.1038/srep40364.

[25] Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol. 2000; 182 (3) : 311-22 .

[26] Kermani T. A, Kermani I. A, Faham Z. et al. Ki-67 status in patients with primary breast cancer and its relationship with other prognostic factors. Biomedical Research and Therapy 2019; 6(2): 2986-2991.

[27] Rakha EA, El-Sayed ME, Green AR, Lee AH, Robertson JF, Ellis IO. Prognostic markers in triple-negative breast cancer. Cancer 2007; 109(1): 25–32.

[28] Iyengar P, Espina V, Williams TW, et al. Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/ stroma microenvironment. J Clin Invest 2005; 115(5): 1163–1176.