Original Article

ACTIVATED LYMPHOCYTES ASSOCIATED TO THE LYMPHATIC VASCULAR WALL MAY CAUSE OVERESTIMATION OF LYMPHATIC ENDOTHELIAL CELL PROLIFERATION IN BREAST CANCER

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Summary

Proliferation of lymphatic endothelial cells (LEC) is often assessed using the Ki-67 nuclear proliferation marker. The presence of proliferating (activated) lymphocytes associated with the lymphatic vascular wall may obscure the results from evaluation. The main aim of the retrospective study was to compare LEC proliferation in breast cancer to that of non-cancerous mammary tissue in the context of possible Ki-67 positivity of activated lymphocytes. The study was carried out on 23 representative paraffin- embedded tissue samples from invasive breast cancer and 12 representative samples from non-cancerous mammary tissue. LEC proliferation was quantified and compared in the context Ki-67 positivity of lymphocyte infiltrates. In cancer, there was a significant difference at the 0.05 level of the distributions of the total number of Ki-67-stained cell nuclei, and those verified as proliferating lymphatic endothelial cells alone p=0.041. When compared, the median values for LEC proliferation in non-cancerous mammary tissue did not differ from those of LEC proliferation in breast cancer W =154.0; p=0.547; n=20. LEC proliferation was not associated with the lymph-node status (t= - 1.43; p=0.171; n=19). The protocol suggested for LEC proliferation in breast cancer may be more accurate and may eliminate some factors that obscure the overall results from evaluation.

Key words: overestimation, lymphatic endothelial cell proliferation, breast cancer

Hearing only what you want to hear
And knowing only what you've heard..."
Metallica – My Friend of Misery

Introduction

During the last decade there is an increasing interest in lymphangiogenesis. The results from experimental inhibition of lymphangiogenesis in animal models have suggested that lymphangiogenic growth factors facilitate the metastatic spread of tumour cells via the lymphatic system [1, 2]. Lymphangiogenesis is thought to occur in several human tumour types, including malignant melanoma, squamous cell carcinoma, gastric carcinoma, pancreatic endocrine tumours and lung cancer [3, 4, 5, 6, 7, 8]. Several studies have reported association between lymphangiogenesis and the presence of lymph node metastases [9].

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The presence of active lymphangiogenesis in the breast at present seems controversial.

Some authors have demonstrated the presence of lymphangiogenesis in breast cancer [10, 11, 12, 13], others have found no lymphangiogenesis associated to breast cancer [14, 15, 16].

This controversy is to a certain extent due to the differences in the evaluation methods used: lymphatic vascular density (LVD) [14], lymphatic endothelial cell (LEC) proliferation [16] and specific lymphatic marker expression [10].

Apart from the possible differences in the structure of the examined patient populations in the studies mentioned above, controversy in the results concerning LEC proliferation in breast cancer may be attributed to the properties of the markers used (Table 1).

According to the standard methodology for lymphangiogenesis quantification in solid human tumours suggested in 2006 by Van der Auwera et al., the combination of D2-40 /Ki-67 may be used to evaluate LEC proliferation [17]. In breast tissues, D2-40 was found to demonstrate membranous and cytoplasm reaction with LEC of initial, collecting and draining lymphatic vessels, as well as myoepithelial cells of normal ductal and lobular structures of the breast [18].

Stromal lymphocyte infiltration is often associated with breast cancer, and is currently considered to play a key role in cancerous growth and metastases [19]. Ki-67 is a nuclear antigen, which is specifically expressed during the late G1, S, G2, and M phases of the cell cycle [20]. Ki-67 is used to highlight activated proliferating lymphocytes in tissues with severe mononuclear infiltration as in liver allograft rejection [21]. Because lymphocyte infiltration in breast cancer can be associated with lymphatic vascular wall (detected in or around lymphatic vessels), Ki-67 positive lymphocytes might be present, and this presence might lead to misinterpretation of LEC proliferation.

The aim of the study was to compare LEC proliferation in breast cancer to that of non-cancerous mammary tissue in the context of possible Ki-67 positivity of activated lymphocytes, mimicking LEC proliferation. A secondary aim of the study was to evaluate the connection between LEC proliferation and lymphocyte proliferation to axillary node status.

Table 1. The most used LEC markers in the reviewed literature and the observed results are presented

LEC marker + proliferation marker	Results observed	Studies
LYVE-1 + Ki-67	Lack of lymphangiogenesis (LEC proliferation)	15,16
	Lymphangiogenesis (LEC proliferation) is present	11, 12 *
D2-40 + Ki-67	Lack of lymphangiogenesis (LEC proliferation)	14
	Lymphangiogenesis (LEC proliferation) is present	13

* PCNA instead of Ki-67

Patients and Methods

Patients

The retrospective study was carried out on 23 representative formalin-fixed paraffin-embedded tissue materials from patients operated on for invasive breast cancer (one tissue sample from each patient) and 12 representative formalin-fixed paraffin-embedded tissue materials from non-cancerous mammary tissue (one tissue sample from each patient). The materials were retrieved from the archives of the department of general and clinical pathology of the University Hospital "Dr G. Stranski"- Pleven. The samples from the cancer patient group were matched by age to the non-cancer control group. All patients were female. The examined tumours were classified according to the WHO criteria [22] and graded according to the Elston & Ellis criteria [23]. Additional information concerning the patient and control group is displayed in (Table 2).

Immunohistochemistry

Hematoxylin- and eosin-stained sections were initially reviewed and three consecutive, histologic 3 μm sections were prepared for immunohistochemical analysis. Monoclonal antibodies D2-40 (clone D2-20; mouse anti-human; DAKO) and Ki-67 (clone Mib-1; mouse anti-human; DAKO) were used for the initial evaluation of LEC proliferation. Monoclonal

Table 2. Additional information concerning the patient population

	Tumor specimen N=23		Non -cancerous mammary tissues N=15
Age /mean(min - max)	58.4 (030 -78)		65.3 (51 -81)
Histological diagnosis (n,%)	IDC	17 (73.91%)	Non -cancerous mammary tissues 15 (100%)
	ILC	3 (13.045%)	
	Special types	3 (13.045%)	
Tumor size (pT)	T1	11 (47.84%)	
	T2	12 (52.17%)	
	T3	0 (0%)	
	T4	0 (0%)	
Axillary status (N)	N0	10 (43.8%)	
	N1	6 (26.09%)	
	N2	3 (13. 02%)	
	Nx	4 (17.09%)	
Grade (G)	G1	6 (26.09%)	
	G2	13 (56.82%)	
	G3	4 (17.09%)	

antibody CD 20 (clone L26; mouse anti-human; DAKO) and polyclonal CD 3 (rabbit anti-human; DAKO) were used to verify the nature of Ki-67 positivity in cases with lymphocyte infiltration.

A modified protocol based on D2-40 and Ki-67 antibody cocktail and a single detection kit was used. The modification was based on the standard D2-40 protocol provided by the manufacturer (DAKO). Instead of using only D2-40 antibody (according to the original protocol), a D2-40/Ki-67 antibody cocktail was used.

All immunostaining was performed manually. The FLEX EnVISION (DAKO) method was used for the immunohistochemical staining. Heat-induced epitope retrieval was carried out in citrate buffer pH 6.0 for 25 minutes in water bath. Immunohistochemical reactions were developed with 3-3` diaminobenzidine and sections were counterstained with Mayer hematoxylin.

Immunohistochemical evaluation

Only fine quality samples were evaluated. The samples demonstrating tissue shrinkage over 5%, affecting lymphatic vasculature were discarded. The affected foci in the samples with less than 5% shrinkage and crushing were not evaluated either. The evaluation of the double-stained (two primary antibodies /one detection kit) slides was carried out under x40 objective / x10/eyepiece (total magnification x 400) on an Olympus BX 40 microscope. Lymphatic endothelial cells with brown membrane/cytoplasm and light gray-blue nuclei (only D2-40 positive) were considered non-proliferative, while cells with brown membrane/cytoplasm and dark brown nuclei (both D2-40/Ki-67 positive) were considered proliferative. The lymphatic endothelial cell proliferation was calculated, dividing the number of proliferating (Ki-67 positive) LECs by the total number of LECs, counted on the whole slide (approximately 1.5mm^2). The D2-40/Ki-67 stained slides were evaluated in the context of CD3 and CD20 stained slides in the respective fields.

Ki-67 positivity in proliferating (activated) lymphocytes was evaluated as the number of activated lymphocytes out of a total of 500 lymphocyte cells within an area with highest lymphocyte infiltration under x40 objective / x10 eyepiece (total magnification x400).

Statistics

Statgraphics plus 2.1 software was used for statistical analysis. Normal distribution was examined for the parameters studied. Differences in median values of LEC proliferation in cancerous and non-cancerous tissues were examined using Mann–Whitney U test (parameters were not normally distributed). The

distribution of the values of LEC proliferation alone, and LEC proliferation evaluated for all positive Ki-67 nuclei, associated to lymphatic vascular wall, were compared using paired sample analysis sign test (parameters were not normally distributed). The correlation between "true" LEC proliferation in breast cancer and axillary lymph node status was assessed using Student's t- test. Correlation of proliferating T cells in breast cancer with axillary lymph node status was assessed using Kruskal-Wallis test (parameters were not normally distributed). The significance level was set at $p \leqslant 0.05$ for all tests.

Results

The non-proliferating lympho-vascular endothelium presented with brown-stained cytoplasm membranes and cytoplasm, and readily distinguishable blue/ gray-blue nuclei (hematoxylin counterstained). The proliferating lympho-vascular endothelium presented with brown-stained membranes and cytoplasm and dark brawn stained nuclei (total cellular positivity) (Fig. 1).

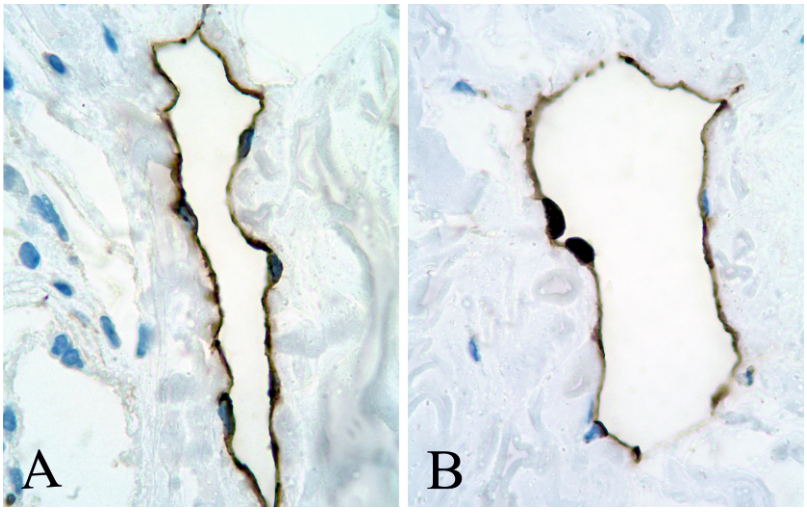

Figure 1. A - Non-proliferating lymphatic endothelium - brown stained cytoplasm membranes and cytoplasm and blue/ gray-blue nuclei; B - Proliferating lympho-vascular endothelium - brown stained membranes and cytoplasm and dark brawn stained nucleus (total cellular positivity) Double immunostaining D2-40/Ki-67; x1000

When the lymphoendothelial proliferation was matched with CD3/CD20 staining, in 6 (30%) cases, the lymphatic endothelial cells, initially considered as proliferating, appeared to be CD3 positive /Ki-67 positive. The immunophenotype expression designated the activated T-lymphocytes as adherent to the lymphatic vascular wall (Fig. 2). The median value of the total number of Ki-67 stained nuclei (including CD 3 positive /Ki-67 positive) was 1.15%, range 0.0-8.2%, while the median value for true proliferating lymphatic endothelial cells (Ki-67 positive/associated to D2-40 positive vessel wall) was 0.8%, range 0.0 – 3.4%. When medians were compared, it was found that 6 cases of Ki-67 positive/ CD 3 negative / D2-40 positive cells had median values lower than the median values of CD 3 positive /Ki-67 positive cells, associated to positive lymphatic vascular wall (D2-40 positive). In 14 cases the median values were the same, and there was no case of higher median values. At the $p \leqslant 0.05$ level, the two distributions were found to be significantly different p=0.041.

The calculated median percentage of proliferating LECs, associated to breast cancer, was 0.8% (range 0.0-3.4%), n=20. The median percentage of proliferating LECs in non-cancerous mammary parenchyma was 1.1% (range 0.0-2.7%) n=15. When compared, the median LEC proliferation in non-cancerous mammary tissue did not differ from that in LEC proliferation in breast cancer W=154.0; p=0.547.

No significant difference between the mean values of LEC proliferation was observed in lymph-node positive and lymph-node negative patients (Table 3).

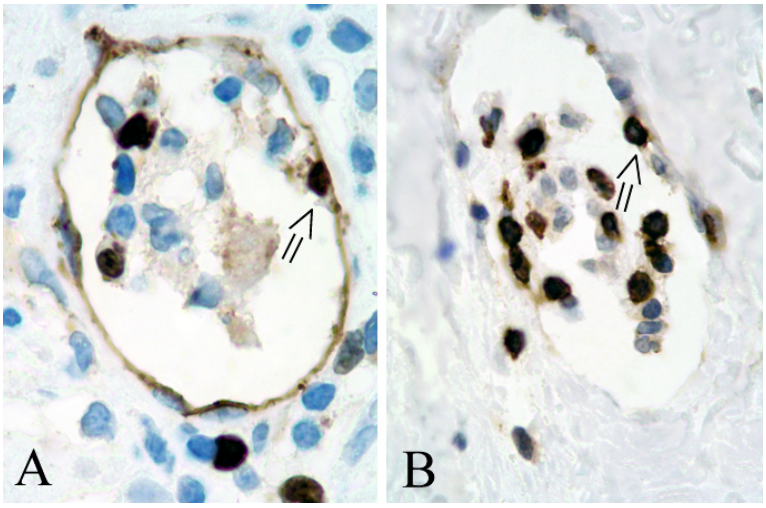

Figure 2. A - Ki-67 positive cellular nuclei in a lymphatic vessel D2-40/Ki-67; x1000; B - The Ki-67 positive cells were found to be predominantly presented by CD 3 positive T lymhocytes The arrowed Ki-67 cell was found to be T lymphocyte. CD 3; x1000

Table 3. Relation of true LEC proliferation in breast cancer to axillary lymph node status

Axillary status (N)	Number	Mean±SD	Student's t test; p
Negative	9	0.63±0.073	t=-1.43
Positive	10	1.21±0.99	p=0.171

Invasion of lymphatic vessels by tumor cells was detected in three (15%) of the examined cases of invasive breast cancer. No case of proliferation activity of lymphatic endothelium was observed in the lymphatic vessels, invaded by the tumor (Fig. 3).

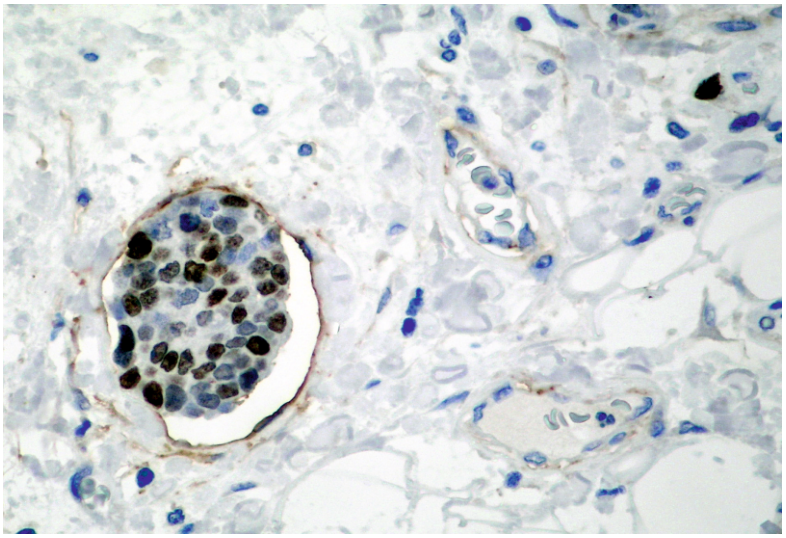

Figure 3. Lymphatic vessels with tumour embolus. Proliferation activity is observed only in the tumour cells of the embolus D2-40/Ki-67; x400

Inflammatory infiltrates were found in 19 (95%) of the studied cases. Such infiltrates were found around disintegrated normal ducto-lobular units, invaded by the tumor or the areas adjacent to the tumor invasion margins (Fig. 4). The lymphocyte infiltrate appeared to be presented predominantly by T (CD3 positive) cells with only rare, focal B cell (CD20 positive) aggregations. The intralobular stroma of non-cancerous, non-inflammatory breast was found to contain only few B and T lymphocytes.

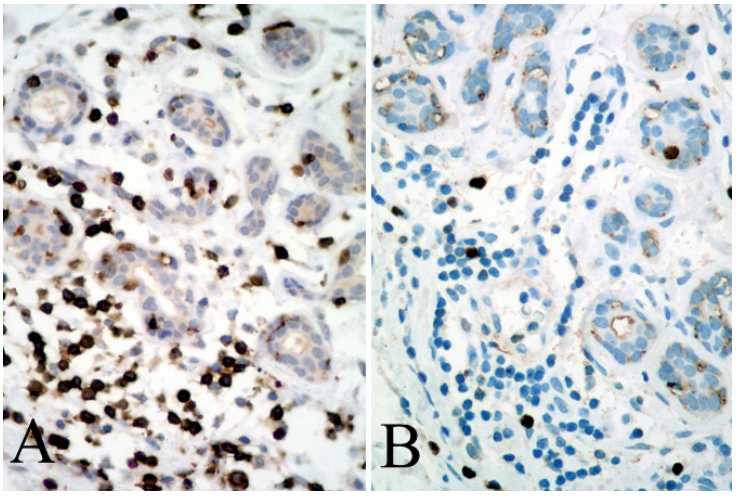

Figure 4. A - Lymphocyte infiltrates observed around disintegrated normal ducto-lobular units adjacent to the tumor invasion front CD 3 x1000; B - Proliferating lymphocytes are seen in the focus of lymphocyte infiltration Ki-67/D2-40; x1000

The median values of proliferating activated T cells associated to the tumor, observed in node negative patients, was not significantly different from those observed in node-positive patients (Table 4).

Table 4. Relation of proliferating T cells in breast cancer to axillary lymph node status

Axillary status (N)	Number	Median	Range	Kruskal-Wallis test; p
Negative	11	2.2	0.0 to 23.2	X^2=0.11
Positive	11	2.4	0.0 to 3.6	p=0.741

Discussion

The application of D2-40 / Ki-67 cocktail based on the application of a single detection kit was found to be effective and useful, although it requires only finest 3 µm thick high quality tissue samples since any artificial tissue shrinkage and crushing of the lymphatic endothelia seriously impede the interpretation of the positivity observed (true/artificial), thus affecting the overall evaluation of results. The modified protocol was found to provide sufficient quality of slide staining in 20 out of 23 (86.96%) of the samples studied and allowed for evaluation of lymphatic endothelial cell proliferation. To exclude possible "false" LEC proliferation (adhesion of activated T cells to the lymphatic vessel wall), the evaluation should be made in accordance to CD3/CD20 stained consequent control slides (or single slide stained for the three markers, if applicable).

The insignificant difference between LEC proliferation found in non-cancerous mammary tissue and breast cancer associated lymphatic vessels supports the assumption that active endothelial cell proliferation is absent in breast carcinoma. It seems that lymphatic endothelium in non-cancerous breast tissues is characterized by a low proliferation rate, and this rate is observed in breast tumors too. This finding suggests that the invasion of lymph vessels into the breast tumor masses in breast carcinomas depends not only on LEC proliferation and lymphatic vascular sprouting.

The lack of association of LEC proliferation to lymph node metastases further supports the idea that LEC proliferation is not crucial for the spread of the primary tumor.

It should be noted that LEC proliferation index in the present study was calculated as the percentage of Ki-67 positive LEC nuclei from all LEC nuclei found on the histological slide. Only 5 of the slides from the tumor group contained 100 or more LEC. This low count was different from a protocol suggested in literature [11]. The evaluation of LEC proliferation in samples containing only a few lymphatic vessels may distort the overall results from evaluation. Two possible solutions of the problem are: the application of a large-section technique, or evaluating two or more samples from each patient.

According to the results reported in popular medical on-line sources, this study is the first to discuss the effect of presence of activated (proliferating) T lymphocytes on LEC proliferation evaluation. According to the currently available data, results from LEC proliferation studies may be obscured by the presence of activated lymphocytes adjacent to the lymphatic vessel wall. In such cases the inflammatory cell might be inaccurately considered as proliferating lymphatic vascular endothelium. The suggested algorithm for quantification of lymphangiogenesis in solid tumors does not designate the possible confusion that may arise in foci of T lymphocyte infiltration and the presence of activated T-cells associated to the lymphatic vascular wall [17]. The present study suggests that researchers should be aware of this possibility, since it may lead to significant deviations in the results and overestimation of LEC proliferation in breast cancer. Automated quantification of LEC proliferation in these circumstances is not recommendable. Since the significant difference observed in LEC proliferation may depend on the patient population (extent of inflammatory reaction) it should be retested and studied in larger populations to better understand the risk of overestimation. The presence of tumor-cell lymphatic vascular invasion may also result in such misinterpretation but in the cases studied tumor embolic positivity was readily distinguishable from LEC proliferation.

In addition to the fact that proliferating T-lymphocytes may obscure the results from LEC proliferation, they were found to be associated to destruction of peritumor tissues, and entrapped by the tumor ductulobular units. The lack of association of activated lymphocytes to the axillary status in breast cancer demonstrated no correlation between proliferating lymphocytes and breast cancer progress. It appears that immune response may play a key role in tumor development as suggested by DeNardo [19] but this role needs to be further investigated.

Finally, the use of the term "lymphangiogenesis" may need reconsideration since it refers to a general term that does not express the morphological substrate that is estimated. In the present study we studied the LEC proliferation, which is supposed to correlate with active lymphatic vascularisation of the tumor (sprouting). The estimation of growth factors levels illustrates the stimuli inside the studied tissues, but does not take into account the actual proliferation. Lymphatic vascular density estimates tumor vascularization rather than lymphangiogenesis, since lymphatic vessels might be not only proliferating inside the tumor but may also be entrapped by the growing tumor.

Conclusion

The suggested protocol for LEC proliferation in breast cancer eliminates some factors that obscure the overall results. It may result in a more standardized approach, which in its turn would allow for a more accurate and reproducible evaluation of LEC proliferation and its correlation with tumor development and prognosis in breast cancer.

According to the results from the present study, the extent of LEC proliferation in breast cancer was not higher as compared to non-cancerous mammary tissues, and was not associated to lymph node metastases.

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