Lymphangiogenesis assessment and its relation to tumour grade, breast cancer subtype and expression of basal markers

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Lymphangiogenesis is a potential indicator of cancer patients' survival. However, there is no standardisation of methodologies applied to the assessment of lymphatic vessel density. In 156 invasive ductal breast cancers (T ≥ 1/N+/M0), lymphatic and blood vessels were visualised using podoplanin and CD34, respectively. Based on these markers expression, four parameters were assessed: (i) distribution of podoplanin-stained vessels (DPV) – the percentage of fields with at least one lymphatic vessel (a simple method proposed by us), (ii) lymphatic vessel density (LVD), (iii) LVD to microvessel density ratio (LVD/MVD) and (iv) the expression of podoplanin in cancer-associated fibroblasts. Next, we estimated relations between the above-mentioned parameters and: (i) breast cancer subtype, (ii) tumour grade, and (iii) basal markers expression.

We found that intensive lymphangiogenesis, assessed using all studied methods, is positively related to high tumour grade, triple negative or HER2 subtype and expression of basal markers. Whereas, the absence of podoplanin expression in fibroblasts of cancer stroma is related to luminal A subtype, low tumour grade or lack of basal markers expression.

Distribution of podoplanin-stained vessels, assessed by a simple method proposed by us (indicating the percentage of fields with at least one lymphatic vessel), might be used instead of the "hot-spot" method.

Key words: breast cancer, podoplanin, basal subtype, basal markers.

Introduction

Lymphatic vessels comprise an open-ended capillary network that collects lymph from various organs and tissues. The wall of lymphatic vessels is lined by endothelium with no fenestration. Vascular and lymphatic systems are necessary for tumour growth and metastatic spread. Tumour vascularization is a potential indicator of cancer patients' survival [1], while visualization and targeting of lymphatic vessels still remain one of the challenges for oncology. Among known markers of lymphatic vessel endothelium, podoplanin seems to be the most promising [2]. It allows for assessment of microvessel density and demonstration of lymphovascular invasion [2-9]. However, there is no standardized method recommended for assessment of lymphangiogenesis [1].

Authors who reported a relation between lymphatic vessel density and lymph node metastases or other clin-

ico-pathological parameters applied the method recommended by Weidner *et al.* or used the "Chalkley count" [6, 10-15]. Both methods are based on immunohistochemical visualization of blood vessels using specific endothelial markers (e.g. CD34 or CD31). In the first method [10] the assessment of microvessel density is carried out with a light microscope in a single area with the highest microvessel density. Whereas in the "Chalkley count" technique the fixed dots of an eyepiece graticule (12-point), that come into contact with CD31-stained tissue are recorded instead of counting individual microvessels [11, 12]. The above-mentioned technique examines the relative area occupied by microvessels rather than the true vessel count [11, 12].

Additionally, application of podoplanin staining gives an opportunity to analyse its expression in stromal fibroblasts. This parameter was shown to be an indicator of poor outcome [15-17].

The aim of the present study was a comparison of two methods applied for lymphatic vessel density assessment: (i) modified Weidner method [10] and (ii) a technique developed in our laboratory – measuring a percentage of fields with at least one lymphatic vessel. This technique seems to be appropriate for assessment of lymphangiogenesis within tumours because intratumoral lymphatic vessels are relatively sparse (as compared with blood vessels). For this reason, evaluation of fields with vessels in the whole tumour specimen might bring more information than calculating its number in most vascularised fields. To test the two above-mentioned methods ("hot-spot" vs. ours) we analysed relations between lymphangiogenesis and widely accepted prognostic factors, namely breast cancer subtype, grade or expression of basal markers (CK5/6 – cytokeratin 5/6, SMA – smooth muscle actin) [18-21].

Table I. Clinicopathological characteristics of 156 invasive ductal breast cancer patients (T ≥ 1, N+, M0)

Parameter	N (%)
T	
1	18 (11.5)
2	131 (84.0)
≥ 3	7 (4.5)
grade	
1	16 (10.4)
2	57 (37.0)
3	81 (52.6)*
local therapy	
Patey/Madden	153 (98.1)
Halsted	3 (1.9)
chemotherapy	
not administered	10 (6.7)
administered	140 (93.3)**
hormonal therapy	
not administered	48 (31.8)
administered	103 (68.2)***
herceptin	
not administered	144 (95.4)
administered	7 (4.6)***

**grade was not assessed in two cases, data not available for **6 and ***5 cases, respectively*

Material and methods

Patients

One hundred and fifty six invasive ductal breast cancer patients (T ≥ 1, N+, M0) who underwent radical modified mastectomy between 2001 and 2010 at the Department of Surgical Oncology, Centre of Oncology, Cracow Branch, were included into the study. The patho-clinical characteristics of this group (age, grade, tumour size, treatment) are shown in Table I. The mean age of patients was 55.7 ±0.9 (SE) (range: 24-84) years.

The study has been approved by the Local Ethics Committee.

Two pathologists re-examined all archival specimens independently in order to confirm the histological subtype (according to current WHO classification) and tumour grade (assessed using Bloom-Richardson scale in Elston-Ellis modification).

Immunohistochemistry

Sections from tissues fixed in 10% neutral buffered formalin and embedded in paraffin were cut at 4 μm, mounted on SuperFrost® Plus (Menzel-Gläser, Germany) slides, and then dewaxed in xylene and rehydrated through a series of alcohols.

In Table II we presented the sources, clones, as well as the number of IHC-stained cases and cases with marker expression. After antigen retrieval (Target Retrieval Solution, pH = 6.1, 50 min, 96°C, DakoCytomation Denmark A/S, Glostrup, Denmark), slides were incubated overnight with primary antibody at 4°C (for SMA no retrieval was applied). Then sections were incubated with BrightVision (Immunologic, Duiven) and DAB (Vector Laboratories, Burlingame, USA).

Moreover, podoplanin and CD34 were visualised using a double staining procedure: CD34 was detected using BrightVision and VIP (Vector Laboratories, Burlingame, USA), while podoplanin, using BrightVision and DAB. Eventually, slides were counterstained with Mayer's Hematoxylin.

The internal positive controls were cells of normal breast ducts and lobules, positively stained for ERα, PR, SMA, CK5/6 and CK5. Tumour specimen with known strong HER2 (3+) expression (external positive control) was added to each series of slides.

Table II. Immunohistochemical procedures used for visualization of the studied markers

Antigen	Manufacturer	Clone	Dilution	Number of immunopositive cases/ number of IHC-stained cases
podoplanin	CellMarque[1]	D2-40	1 : 100	96/121
CD34	DAKO[2]	QBEnd 10	1 : 50	121/121
ERα	Leica Biosystems[3]	6F11	1 : 100	110/155
PR	Leica Biosystems[3]	PGR/2	1 : 200	
HER2	DAKO[2]	–	1 : 250	26/151
Ki-67	DAKO[2]	MIB-1	1 : 75	137/137
CK5/6	DAKO[2]	D5/16 B4	1 : 50	34/143
CK5	Thermo[4]	XM26	1 : 80	
SMA	Leica Biosystems[3]	αsm-1	1 : 50	19/144

[1]CellMarque, Rocklin, California, USA
[2]DakoCytomation Denmark A/S, Glostrup, Denmark
[3]Leica Biosystems Newcastle Ltd, Newcastle, United Kingdom
[4]Thermo Fisher Scientific, Fremont, USA

IHC evaluation

Microvascular density (MVD) and lymphatic vessel density (LVD) were assessed with digital image analysis using BX41 microscope, DP71 camera and Cell D software (Olympus Europa GmbH, Hamburg, Germany), applying CD34 as a marker of blood vessels and podoplanin as a marker of lymphatic vessels. About 7 fields with most intensive lymphatic vessel density were acquired (10× objective magnification, area 1.46 mm^2 of the specimen field). In each field, vessels were marked manually and then counted automatically. Eventually, LVD and MVD were calculated as the mean number of vessels per mm^2 (modified Weidner method [10, 11, 16]). Finally, the LVD/MVD ratio was calculated. Moreover, the whole tumour specimen was scanned (10× objective), and in each field the absence or percentage of lymphatics (at least one) was recorded. Eventually, the percentage of fields with at least one lymphatic vessel – distribution of podoplanin-stained vessels (DPV) – was calculated. In all the above-mentioned procedures, the large lymphatic vessel both without (Fig. 1A, arrow), and with emboli (Fig. 1B, arrow), and the small ones (Fig. 1A, arrowhead) were included into the vessel count.

Lymphatic vessel density and expression of markers were evaluated only in the invasive component of the tumours. Expression of estrogen and progesterone receptor (ERα, PR), CK5/6, CK5 as well as SMA were considered positive if > 1% of tumour cells showed immunopositivity.

MIB-1 labelling index (MIB-1 LI) was calculated as the percentage of Ki-67 immunopositive cells. For each slide, between 500 and 1000 cells (at ×400 magnification) were counted in 5-6 fields.

Only tumours with continuous strong membranous HER2 staining (3+) were considered immunopositive.

On the basis of ER, PR, HER2 expression four immunophenotypes were distinguished: (1) luminal A (LA): ER+ or PR+, HER2–, (2) luminal B (LB): ER+ or PR+ and HER2+ (3) HER2 overexpressing (HER2): ER– and PR– and HER2+ and (4) triple-negative phenotype (TNP): ER– and PR– and HER2–.

Statistical analysis

The STATISTICA 9 software (StatSoft, Inc., Tulsa, OK 74104, USA) was used for all calculations. In all statistical procedures, p value < 0.05 was considered significant. Statistical significance of differences between the frequency of events distribution in the investigated categorical variables were found using Pearson χ^2 test (chi-square test for independence). Differences in mean values of continuous variables (age, MIB-1 LI, vascular and lymphatic vessel density) between the two groups were calculated using Student's *t* test, while differences between more than two groups, using one-way ANOVA test. Spearman's correlation was used in the case of continuous variables.

Results

Intratumoral podoplanin expression was found in both large (Fig. 1A arrow) and small vessels (Fig. 1A arrowhead). In some podoplanin-stained vessels emboli were observed (Fig. 1B, arrow). Some vessels were double stained for CD34 and podoplanin (Fig. 1C, arrow). This pattern of staining was found more frequently in vessels with emboli (Fig. 1C, arrow). Additionally, we found weak or strong podoplanin expression in cancer-associated stromal fibroblasts (CAFs) (Fig. 1D) and in myoepithelial cells surrounding non-atypical ducts (Fig. 1E) or *in situ* carcinomas (Fig. 1F, arrow).

Intratumoral lymphatic vessels (with podoplanin expression) were found in 79.3% of cases, while intra-

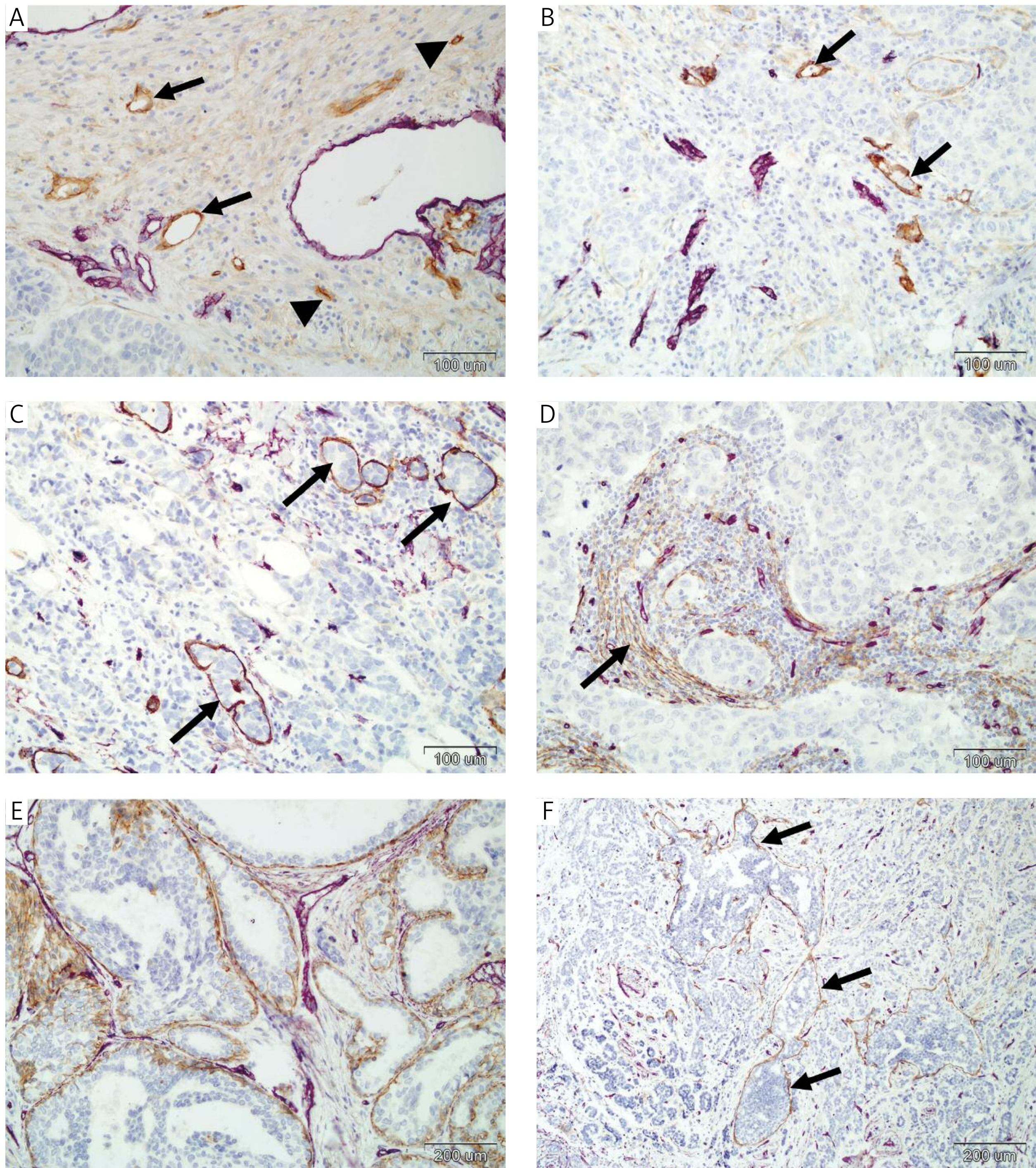

Fig. 1. Expression of podoplanin in ductal breast carcinomas. A – lymphatic vessels with lumen (arrow) and without lumen (arrowhead) expressing podoplanin (brown colour) as well as blood vessels characterised by expression of CD34 (violet colour); B – emboli (arrow) in podoplanin-stained vessels; C – co-expression of podoplanin and CD34 in endothelium of the lymphatic vessel (arrow) with emboli; D – brown-stained, podoplanin expressing (arrow) cancer-associated fibroblasts; E – expression of podoplanin in myoepithelial cells of non-atypical ducts; F – podoplanin-stained myoepithelial cells surrounding carcinoma *in situ* (arrow)

tumoral blood vessels (with CD34 expression) in 100% of cases (Table II). The mean values and standard errors of DPV, LVD and LVD/MVD are presented in Table III. Median values and ranges of DPV, LVD, MVD and LVD/MVD were: 57.1 (0-100), 1.7 (0-21.1), 65.3 (26.9-227.4) and 3.1 (0-42.4), respectively. The mean value for MVD was 72.7 ±2.7 (SE).

We found a statistically significant positive correlation between parameters used for lymphangiogenesis assessment (LVD, DPV, LVD/MVD) ($p = 0.000$).

Table III. Relations between clinical and histological parameters and mean values of DPV, LVD, LVD/MVD, and the frequency of podoplanin expression in cancer-associated fibroblasts (CAF)

	DPV (%)	LVD (vessels/mm²) modified Weidner [1995] method	LVD/MVD	Podoplanin in CAF	
	(N) Mean ±SE	(N) Mean ±SE	(N) Mean ±SE	0, N (%)	1, N (%)
Total	(114) 53.7 ±3.8	(121) 3.0 ±0.3	(120) 4.8 ±0.6	68 (61.3)	43 (38.7)
Age					
≤ 50	(40) 58.7 ±6.4	(43) 2.9 ±0.5	(43) 4.4 ±0.7	21 (61.8)	13 (38.2)
> 50	(74) 50.9 ±4.7	(78) 3.1 ±0.4	(77) 5.1 ±0.9	47 (61.0)	30 (39.0)
pT					
1	(11) 58.2 ±13.6	(12) 3.8 ±1.3	(12) 5.7 ±2.1	8 (80.0)	2 (20.0)
2	(96) 53.6 ±4.0	(103) 3.0 ±0.4	(102) 4.9 ±0.7	57 (59.4)	39 (40.6)
≥ 3	(7) 48.0 ±18.1	(6) 1.9 ±0.8	(6) 2.3 ±1.0	3 (60.0)	2 (40.0)
Grade					
1	(8) 36.4 ±12.2	(12) 1.0 ±0.4	(11) 1.7 ±0.5	8 (88.9)	1 (11.1)
2	(34) 34.9 ±5.9	(36) 2.0 ±0.5	(36) 3.4 ±0.9	32 (86.5)	5 (13.5)
3	(70) 64.3 ±4.8[a]	(71) 3.9 ±0.5[b]	(71) 6.0 ±0.9[c]	28 (44.4)	35 (55.6)[a]
Subtype					
LA	(62) 40.3 ±4.9	(68) 1.8 ±0.3	(67) 2.8 ±0.5	48 (75.0)	16 (25.0)
LB	(11) 57.1 ±12.2	(13) 3.0 ±1.0	(13) 4.7 ±1.8	8 (61.5)	5 (38.5)
HER2	(9) 85.9 ±9.3	(9) 6.9 ±1.8	(9) 14.6 ±4.5	1 (14.3)	6 (85.7)
TNP	(30) 67.2 ±6.7[d]	(29) 4.6 ±0.8[a]	(29) 6.4 ±1.2[a]	10 (38.5)	16 (61.5)[a]
CK 5/6 or SMA					
0	(75) 46.8 ±4.6	(81) 2.1 ±0.3	(81) 3.1 ±0.4	52 (69.3)	23 (30.7)
1	(34) 72.0 ±6.0[e]	(34) 5.6 ±0.9[a]	(34) 9.4 ±1.7[a]	10 (33.3)	20 (66.7)[d]

[a]p = 0.000, [b]p = 0.005, [c]p = 0.043, [d]p = 0.001, [e]p = 0.002
Expression of markers: 0 – no expression, 1 – expression. Breast cancer subtype: LA – ER+/PR+/HER2–, LB – ER+/PR+/HER2+, HER2 – ER–/PR–/HER2+, TNP (triple negative phenotype) – ER–/PR–/HER2–. LVD – Lymphatic vessel density, DPV – distribution of podoplanin stained vessels, MVD – microvascular density, CK5/6 – cytokeratin 5/6, SMA – smooth muscle actin

Moreover a significant relation between intensity of podoplanin staining in stromal fibroblasts and lymphangiogenesis assessed using DPV, LVD and LVD/MVD was found ($p = 0.000$). No correlation between MVD and DPV or LVD was found and no relation between MVD and the expression of podoplanin in CAF was noted ($p > 0.05$).

We found significantly higher LPV, DPV and LVD/MVD for high grade (G3) tumours (vs. low grade ones), for HER2 and TNP carcinomas (vs. luminal A subtype) and for basal marker expressing tumours (vs. basal marker-negative cancers) ($p < 0.05$) (Table III). No relation between MVD and the above-mentioned parameters was found ($p > 0.05$).

Weak or no expression of podoplanin in cancer-associated fibroblasts was noted more frequently in low grade (G1-G2) tumours, luminal A carcinomas and tumours negative for basal markers (vs. G3, LB or HER2 or TNP, carcinomas with basal markers expression, respectively) (Table III).

There was a significant positive correlation between MIB-1 LI and MVD or LVD/MVD (but not between MIB-1 LI and DPV or LVD) ($p = 0.013$ and $p = 0.042$, respectively).

Discussion

We found podoplanin expression in vessels located both intratumorally and in non-neoplastic areas surrounding tumour tissue. Moreover, expression of this marker was noted in myoepithelial cells and in stromal cancer-associated fibroblasts, what is in accordance with other authors studies [2-9, 12-17]. Selected vessels were double-stained with CD34 and podoplanin. This pattern of staining is not surprising as CD34 was found to be expressed both in blood and lymphatic vessels [22].

We observed the presence of intratumoral lymphatic vessels in 79.3% of cases. Other researchers found them in 85% of studied cases [6]. The range of LVD found

in our study (0-21.1) was similar to ranges reported by other authors (0-10.57 [12], 0-31 [13], 0-45 [6]). Additional parameters evaluated in this study (DPV, LVD/MVD) are presented for the first time.

The mean value of MVD found by us was within the range of values reported by other authors (discussed in [23, 24]). We confirmed no correlation between LVD (or DPV) and MVD [25].

Using all applied methods (DLV, LVD and LVD/MVD) we found significantly higher lymphatic vessel density (DPV, LVD/MVD) in high grade tumours, TNP or HER2 overexpressing carcinomas and in tumours expressing basal markers (CK5/6 or SMA). This observation suggests that distribution of podoplanin-stained vessels (DPV), very simple method developed by us, might be used interchangeably with the "hot-spot" method advocated by Weidner [10, 11, 13, 15]. The latter is time-consuming and characterized by high intra- and interobserver variability [1]. A significant positive correlation between DLV, LVD and LVD/ MVD confirms the above-mentioned statement.

Other authors using the "hot-spot" method [6, 13] or "Chalkley count" [12] reported ambiguous results. Those who found a relation between lymphatic vessel density (assessed using Weidner "hot-spot" method) and nodal status [6] did not find any relation with age, tumour size or grade, while those who found a relation with grade and age [13], reported no significant relation with tumour size, histology or ER expression [13]. Authors [12] who applied "Chalkley count" for microvessel density assessment did not notice any relation with breast cancer subtype [12]. In our opinion, "Chalkley count" might be less appropriate for assessment of tumour lymphangiogenesis, because intratumoral lymphatic vessels are relatively sparse (as compared to blood vessels) [6, 12, 13] and hence this method might be not sensitive enough.

Another parameter significantly related to tumour grade, breast cancer subtype and expression of CK5/6 or SMA was podoplanin expression in cancer-associated fibroblasts. Other authors found similar relations between the above-mentioned parameter and grade, ER/PR/HER2 expression and tumour cells proliferation [16-18]. This result suggests the existence of a specific subpopulation of stroma cancer-associated fibroblasts with podoplanin expression that may stimulate (or may be associated with) aggressive behaviour of cancer cells. On the other hand, the relation between podoplanin expression in fibroblasts of cancer stroma and lymphatic vessel density (LVD, DLV, LVD/MVD) found in our study might suggest involvement of these fibroblasts in lymphangiogenesis.

The fact that high lymphatic vessel density is related to high tumour grade, TNP or HER2 subtype and expression of basal markers as well as the relation between lack of podoplanin expression in fibroblasts of cancer stroma and luminal A subtype, low tumour grade or lack of basal markers expression, may confirm the suggestion that podoplanin stimulates endothelial-mesenchymal transition, cell migration and invasiveness [26-29].

Moreover, in some reports comedo necrosis was found more frequently in basal-like breast carcinomas [21]. It is possible that hypoxia (near necrotic areas) might induce secretion of VEGF that stimulates angio- and lymphangiogenesis. Hence, higher lymphatic vessel density (or distribution of podoplanin-stained vessels or LVD/MVD ratio) found in our study for TNP and carcinomas with basal marker expression confirms this hypothesis.

Conclusions

1. All methods used in our study for assessment of lymphangiogenesis (LVD, DLV, LVD/MVD) were correlated to each other and to parameters indicating aggressive tumour behaviour (high grade, TNP, HER2 subtype, basal marker expression), hence they might be used equivalently.
2. DPV (distribution of podoplanin-stained vessels) – a simple parameter developed by us, indicating the percentage of fields with at least one lymphatic vessel, might be used instead of the "hot-spot" method. Our method is easier, less time-consuming and might offer high intra- and interobserver reproducibility (other researchers should confirm its usefulness).
3. Lack of podoplanin expression in cancer-associated fibroblasts is related to luminal A subtype, low tumour grade or lack of basal markers expression. This result indicates the need for further studies aimed at explaining possible interactions between cancer cells and podoplanin expressing stromal fibroblasts, as well as characterizing cancer-associated fibroblasts.

The study was supported by the Polish Ministry of Science and Higher Education; grant number NN 401 096 137.

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