Impact of Different Tumour Stroma Assessment Methods Regarding Podoplanin Expression on Clinical Outcome in Patients with Invasive Ductal Breast Carcinoma

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Abstract. *Background: Podoplanin, a small mucin-type transmembrane protein has been shown in several studies to be expressed in cancer-associated fibroblasts (CAFs) and affect patient outcome. Materials and Methods: We evaluated podoplanin expression in CAFs in a cohort of 257 patients with invasive ductal breast carcinomas (IDCs) using three different assessment scales based on the number of positive cells alone or in combination with the reaction intensity. Results: Two of the utilized scales yielded prognostic information concerning patients' overall survival (OS), but scores were not independent prognostic factors in the multivariate analysis. On the contrary, two scales based on the combination of cell positivity and reaction intensity had no significant impact on patients' OS, but they were significantly correlated with a greater number of analysed clinicopathological parameters. Conclusion: In summary, podoplanin expression in CAFs may be considered a possible marker of poor prognosis in IDC, however, caution should be taken as the results varied regarding the utilized scales.*

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Podoplanin is an *O*-glycosylated transmembrane glycoprotein, known also under many different synonyms (D2-40, gp38, T1α, PA.A26, gp36, Aggrus, and M2A). Podoplanin was first-identified as a marker of lymphangiogenesis due to its expression in lymphatic vessel endothelium (1-10). Podoplanin expression in different malignancies was intensively investigated in numerous studies (1, 7, 9, 11-16). Moreover, in some of the analysed tumours podoplanin expression was also noted in cancer stroma in cells identified as cancer-associated fibroblasts (CAFs) (17-25).

Recent reports indicate that CAFs and their crosstalk with cancer cells may contribute to cancer cell invasion and metastasis (26, 27). CAFs were shown to have distinct gene expression and properties different from that of normal fibroblasts (28). Moreover, CAFs were shown to secrete numerous proteins such as stromal derived growth factor-1 (SDF-1), matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-3 (MMP-3) and therefore to promote tumour growth, recruitment of endothelial progenitor cells, cancer cell invasion and migration (29-31).

In contrast to normal breast stroma, where podoplanin expression was not present in fibroblasts, only in myoepithelial cells, CAFs of invasive ductal breast carcinoma (IDC) express this glycoprotein widely (17, 25, 32). However, details regarding the molecular mechanisms underlying podoplanin induction in CAFs or their origin in cancerous stroma of different malignancies remain scarce. α-Smooth muscle actin (αSMA) is regarded as a marker of CAFs, although recent

studies have brought new insights into the heterogeneity of CAFs of tumour stroma and no reliable single marker may be implied as accurately defining a CAF (33-35). In the study of Ito *et al*., the tumour-promoting potential of lung adenocarcinoma cell line A549 by podoplanin-positive fibroblasts was shown to be mediated by enhanced Ras homolog gene family, member A (*RhoA*) activity (36).

Recently, podoplanin expression in CAFs was identified as a marker of poor prognosis of IDC (17, 25). Podoplanin-positive CAFs were shown to have differential impact on patient survival dependent on the analysed tumour type (17-25). However, in these studies, different assessment scales were used. Therefore the results of some studies may be difficult to compare and the different scales used for assessment may have impact on the final results, as seen in studies comparing methods for proliferation markers and vascularity assessment (37, 38). These may have a critical impact on future studies concerning utilization of stromal markers in clinical studies based on the expression of various proteins in the tumour stroma. Thus, the aim of this study was the comparison of the recently used scales of immunohistochemical (IHC) expression of studied antigens regarding their correlation with clinicopathological data and prognostic significance in a series of IDC to determine the most eligible method for cancer stroma assessment.

Materials and Methods

Patients and tumours. In this study 257 tissue specimens of IDC sampled before treatment initiation from patients operated on at the Lower Silesian Oncology Center in Wroclaw and the Maria Sklodowska-Curie Memorial Institute in Krakow in 1999-2006 were used. The clinical and pathological data were obtained from the archives of both hospitals (Table I). The mean patient age at diagnosis was 57.5±11.57 (range: 30-84) years. In the follow-up period, patients were observed for 63.21±38.54 (1-141) months. In this time, 55 (21.4%) patients died of their disease and 89 (34.6%) had local or systemic recurrence. All the patients were treated by mastectomy or conservative quadrantectomy followed by axillary lymph node resection. Adjuvant systemic chemotherapy was administered to 227 (88.3%) women. Postoperative tamoxifen therapy was given to 164 (63.8%) patients and 126 (49.0%) women underwent post-surgical radiotherapy.

Tissue samples were fixed in 10% buffered formalin, dehydratated and embedded in paraffin. Haematoxylin and eosin-stained (H&E) preparations were made to verify the diagnosis and to assess the grade of malignancy according to Elston and Ellis by two independent pathologists (39).

IHC. IHC was conducted as described previously (17). Briefly, IHC was performed on 4-μm-thick paraffin sections. Target Retrieval Solution, pH 9 (97˚C, 20 min) and a PT Link Rinse Station were used to de-paraffinize the sections and retrieve the antigens. The sections were then washed in TBS/0.05% Tween buffer. Endogenous peroxidase was blocked using EnVision FLEX Peroxidase-Blocking Reagent (incubation 5 min at room temperature, RT). Subsequently, the sections were then washed in TBS/0.05% Tween and primary antibodies directed against podoplanin (D2-40; ready-to-use), Ki-67 (MIB-1; 1:100), oestrogen receptor (ER, clone 1D5; 1:100), progesterone receptor (PR, clone 636; 1:100) were applied and incubated at RT for 20 min in an automated staining platform (Link48 Autostainer) to ensure repeatable reaction conditions. After washing the sections in TBS/0.05% Tween, EnVision FLEX/horseradisch peroxidase (HRP) secondary antibodies were applied (20 min at RT). Sections were then washed in TBS/0.05% Tween and EnVision FLEX the substrate for peroxidase, diaminobenzidine, was applied and the sections were incubated for 10 min at RT. Finally, the sections were counterstained with Mayer's haematoxylin, dehydrated in alcohol (70%, 96%, 99.8%) and xylene and then mounted using SUB-X Mounting Medium.

Human epidermal growth factor receptor 2 (HER2) expression was examined using a HercepTest™ kit, following the procedure recommended by the manufacturer. In cases of equivocal IHC results (+2) HER2 FISH pharmDx™ Kit was utilized to determine the *HER2* amplification status. All the antibodies, reagents and equipment were obtained from Dako Cytomation (Glostrup, Denmark).

Negative controls were performed by omitting the primary antibody, whereas tumour sections known to have high expression of the analysed marker were used as positive control. Podoplanin expression in lymphatic vessel endothelium served as an internal control.

Table I. Patients' and tumour characteristics.

Parameter	Number	%
Age		
≤50 years	72	28.0
>50 years	185	72.0
Menopausal status		
Pre-	85	33.1
Post-	172	66.9
Tumour size		
T1	126	49.0
T2	114	44.4
T3	13	5.0
T4	4	1.6
Lymph nodes		
Negative	103	40.1
Positive	154	59.1
Grade		
G1	20	7.8
G2	147	57.2
G3	90	35.0
ER		
Positive	185	66.9
Negative	72	33.1
PR		
Positive	162	63.0
Negative	95	37.0
HER2		
Positive	44	17.1
Negative	213	82.9
Ki-67		
≤25%	166	64.6
>25%	91	35.4

ER, Estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.

Table II. *Modification of the semi-quantitative immunoreactive score (IRS) scale of Remmele and Stegner for assessment of tumour stroma (40). In each case the IRS was calculated by multiplying the score for the positive area (0-4) and that for the intensity of the colour reaction (0-3), yielding a final score of 0 to 12 (Σ=A×B). The scale of Kawase et al. and Yamanashi et al. are also summarized below.*

	Modified IRS	
A (area)	B (intensity)	
0, No positive area 1, Up to 10% of positive area 2, 11% to 50% of positive area 3, 51% to 80% of positive area 4, >80% of positive area	0, No colour reaction 1, Low colour intensity 2, Reaction colour of moderate intensity 3, Intense reaction colour	
	Scale of Kawase *et al*. (20)	
0 (Negative) Podoplanin-positive stromal area/overall stromal area, ×100 magnification ≤10%	1 (Positive) Podoplanin-positive stromal area/overall stromal area, ×100 magnification 11-50%	2 (Positive) Podoplanin-positive stromal area/overall stromal area, ×100 magnification >51%
	Scale of Yamanashi *et al*. (21)	
Negative (Group B)	Positive (Group A)	
Staining intensity in tumour is less than that seen in the lymphatic endothelium in <30% of overall stromal area	Staining intensity in tumour is equal to or stronger than that seen in the lymphatic endothelium in ≥30% of overall stromal area	

Analysis of IHC section. The IHC sections were evaluated under a BX-41 light microscope (Olympus, Tokyo, Japan) independently by two pathologists who were blinded to the patients' clinical data. In doubtful cases a re-evaluation was performed using a double-headed microscope and the staining was discussed until a consensus was achieved. For podoplanin expression assessment in CAFs, three different scales utilised previously in the literature were used. All the scales described herein are based on semi-quantitative assessment of podoplanin expression in the tumour stroma, which for the purpose of this study, was defined as the tumour area between tumour nests with a margin not exceeding 1 mm outside the tumour invasive front (Table II). As the results of earlier studies have shown that podoplanin is expressed by CAFs, identified upon coexpression of αSMA and vimentin, but not in the stroma of benign tissues and non-transformed stroma, an assumption for this study was made that podoplanin expression noted in the tumour stromal area defined above was exclusively restricted to CAFs (17, 20, 25). Although podoplanin is also expressed in breast myoepithelial cells and lymphatic vessels, these structures are easy distinguishable from podoplanin-positive CAFs due to the high cellular density and weak cellular delineation of the latter (17).

The first method considered was a three-grade system introduced by Kawase *et al*. which was also used in our previous work (17, 20). In this method, the sections are regarded as negative (grade 0) when the ratio of podoplanin-positive stromal area to overall stromal area, observed under ×100 magnification, is less or equal to 10%. Sections are classified as positive when podoplanin expression is found in 11-50% (grade 1) or 51-100% (grade 2) of the tumour stroma.

The second scale was used by Yamanashi *et al*. (21). The authors regarded podoplanin expression as positive when the staining intensity noted in CAFs was equal to or stronger than that seen in the lymphatic endothelium. When the reaction intensity was weaker or absent, the staining was considered as negative. When positive staining was noted in 30% or more of the overall tumour stromal area, the case was considered positive (group A, positive), whereas cases with positive podoplanin staining in less than 30% of the overall tumour stromal area were scored negative (group B, negative).

As the third assessment scale, the semi-quantitative immunoreactive score (IRS) method of Remmele and Stegner was utilized, which has been successfully used for scoring expression of markers in neoplastic cells (40, 41). The scale was originally based on the percentage of positive cells showing reaction in the whole section and the intensity of the colour reaction, therefore similar to that used by Kitano *et al*. (19). For our purposes, the percentage of positive cells was defined as the podoplanin-positive stromal area relative to the overall stromal area in the whole-tissue section as mentioned above (Table II). The scale ranges from 0-12 points, where 0 denotes absence of reaction, 1-2 corresponds to weak reaction, 3-4 to moderate reaction and 6-12 to a pronounced, strong reaction. For the purpose of this study, two cut-off points, IRS 0-2 denoted as weak expression and IRS 0-4 denoting cases with weak and moderate expression, based on statistical analysis and the character of the two other employed scales were utilized.

The Ki-67 antigen was evaluated semi-quantitavely in whole-tissue sections according to tumour cell positivity and encoded as follows: 0, 0% cells stained; 1, 1-10% cells stained; 2, 11-25% cells stained; 3, 26-50% cells stained; and 4, 51-100% cells stained. Similarly, for ER and PR expression assessment, a semi-quantitative four-grade scoring system based on tumour cell positivity was utilized: 0, 0%

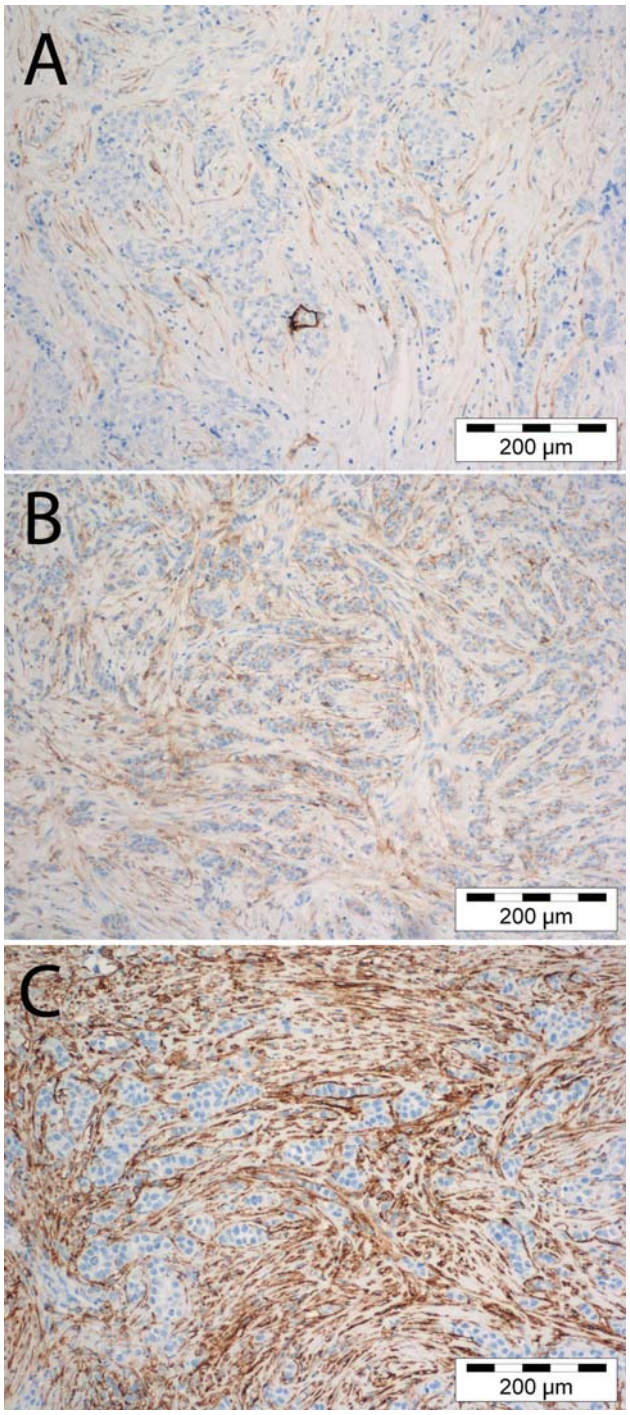

Figure 1. *Examples of podoplanin expression in cancer-assoviated fibroblasts of invasive ductal breast carcinoma presenting different expression intensities: weak (A), moderate (B) and strong (C).*

cells stained; 1, 1-10% cells stained; 2, 11-50% cells stained; 3, 51-100% cells stained. ER and PR sections scoring 1 and higher were regarded as positive according to the criteria of the 11th St. Gallen conference (42).

Statistical analysis. The statistical analysis was performed using the Prism 5.0 and Statistica 10.0 software (GraphPad, La Jolla, CA, USA and StatSoft, Krakow, Poland, respectively). Correlations between clinicopathological parameters and expression of the studied markers were analysed by Fisher's exact test. The Mann-Whitney *U*-test was used to compare the groups of data that failed to satisfy the assumptions of the parametric test. Correlations between the scores of the examined assessment scales were tested using Spearman's correlation test. Significance of differences of the overall survival (OS) and event free survival (EFS) times were determined by the Mantel-Cox log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. For each variable, the hazard ratio and 95% confidence interval (95% CI) were estimated. In all the analyses, results were considered statistically significant when $p<0.05$.

Results

According to the scale of Kawase *et al*., 60 (23.3%) cases were scored as negative, whereas 67 (26.1%) were scored as grade 1 and 130 (50.6%) were scored as grade 2 (20). Using the scale of Yamanashi *et al*., 118 (45.9%) cases were regarded as negative (group B) and 139 (54.1%) were regarded as positive (group A) (21). Using the modified IRS, 82 (31.9%) cases showed weak podoplanin expression (IRS 0-2), 41 (15.9%) showed moderate expression (IRS 3-4), whereas in 134 (52.2%) cases, strong podoplanin expression was noted (Figure 1). When the non-categorized scores were analysed using Spearman's correlation test, strong positive correlations between all the scores of the utilized assessment scales were noted (Table III).

Fisher's exact test was utilized to analyse the significance of podoplanin expression using the different assessment scales (Table IV). For all the employed scales, higher podoplanin expression in CAFs was significantly associated with higher grade of malignancy and high proliferation rate of cancer cells measured by the expression of Ki-67 antigen. Different results for the scales were found regarding associations with primary tumour size, ER, HER2 and Ki-67 expression (Table IV). Higher (IRS 6-12) podoplanin expression in CAFs was associated with larger primary tumour size ($p=0.0340$), ER-negativity ($p<0.0008$), PR-negativity ($p=0.0382$) and HER2-positivity ($p=0.0482$). When weak (IRS 0-2 *vs*. 3-12) podoplanin expression in CAFs was used as the cut-off point, no association with PR expression status was noted. However, podoplanin positivity (IRS 3-12) was significantly associated with larger primary tumour size ($p=0.0077$), ER-negativity ($p=0.0043$) and HER2-positivity ($p=0.0337$). Cases regarded as positive according to scale of Yamanashi *et al*. were characterized by ER-negativity and HER2-positivity ($p=0.0054$ and $p=0.0076$, respectively). Borderline significant correlations with patients' pathological data were found, as cases characterized by podoplanin expression in CAFs were shown to be associated with larger primary tumour sizes ($p=0.0052$). No associations were noted with patient age, menopausal status and presence of lymph node metastases.

Table III. *Correlations between the different assessment scales used in the study. Significant p-values are given in bold.*

Assessment scale	Scale of Kawase *et al*. (20)	IRS stroma scale	Scale of Yamanashi *et al*. (21)
Scale of Kawase *et al*.		r=0.84, ***p*<0.0001**	r=0.89, ***p*<0.0001**
IRS stroma scale			r=0.85, ***p*<0.0001**
Scale of Yamanashi *et al*.			

Table IV. *Correlations between podoplanin (D2-40) expression by cancer-associated fibroblasts and clinicopathological characteristics in patients with invasive ductal carcinoma of the breast. Significant p-values are given in bold.*

Characteristic	No.	Scale of Kawase *et al*., n (%)		*p*-Value	IRS stroma score, n (%)		*p*-Value	IRS stroma score, n (%)		*p*-Value	Scale of Yamanashi *et al*., n (%)		*p*-Value
		Neg.	Pos.		0-2	3-12		0-4	6-12		Neg.	Pos.	
Age													
≤50	72	19 (26.4)	53 (73.6)	0.5124	25 (34.7)	47 (65.3)	0.5542	40 (55.5)	32 (45.5)	0.1289	40 (55.5)	32 (45.5)	0.0696
>50	185	41 (22.2)	144 (77.8)		57 (30.8)	128 (69.2)		83 (44.9)	102 (55.1)		78 (42.2)	107 (57.8)	
Menopausal status													
Pre	85	20 (23.5)	65 (76.5)	1.0000	27 (31.8)	58 (68.2)	1.0000	45 (52.9)	40 (47.1)	0.2889	45 (52.9)	40 (47.1)	0.1432
Post	172	65 (37.8)	132 (62.2)		55 (29.7)	117 (70.3)		78 (45.3)	94 (54.7)		73 (42.4)	99 (57.6)	
Tumour size													
T1	126	39 (30.9)	87 (69.1)	**0.0052**	50 (39.7)	76 (61.3)	**0.0077**	69 (54.8)	57 (45.2)	**0.0340**	65 (51.6)	61 (48.4)	0.0806
T2-T4	131	21 (16.0)	110 (84.0)		31 (23.7)	100 (76.3)		54 (41.2)	77 (58.8)		53 (40.5)	78 (59.5)	
Lymph nodes													
Negative	103	26 (25.2)	77 (74.8)	0.6520	34 (33.0)	69 (77.0)	0.7857	51 (49.5)	52 (50.5)	0.7032	45 (43.7)	73 (56.3)	0.6101
Positive	154	34 (25.3)	120 (74.7)		48 (31.2)	106 (68.8)		72 (46.8)	82 (53.2)		73 (47.4)	81 (52.6)	
Grade													
G1, G2	167	50 (42.7)	117 (57.3)	**0.0006**	68 (40.7)	99 (59.3)	**<0.0001**	95 (56.8)	62 (43.2)	**<0.0001**	89 (53.3)	29 (46.7)	**0.0016**
G3	90	10 (11.1)	80 (88.9)		14 (15.5)	76 (84.5)		72 (80.0)	28 (20.0)		78 (86.7)	61 (13.3)	
ER													
Positive	185	47 (34.1)	138 (65.9)	0.2517	68 (36.7)	117 (63.3)	**0.0043**	101 (54.6)	84 (45.4)	**0.0008**	95 (51.2)	90 (48.8)	**0.0054**
Negative	72	13 (18.1)	59 (81.9)		13 (18.1)	59 (71.9)		22 (30.5)	50 (69.5)		23 (31.9)	49 (68.1)	
PR													
Positive	162	39 (24.1)	123 (75.9)	0.7618	57 (35.2)	105 (64.8)	0.1661	86 (53.1)	76 (46.9)	**0.0382**	81 (50.0)	81 (50.0)	0.0932
Negative	95	21 (22.1)	74 (77.9)		25 (26.3)	70 (73.7)		37 (38.9)	58 (61.1)		37 (38.9)	58 (61.1)	
HER2													
Positive	44	6 (13.6)	38 (86.4)	0.1176	8 (18.1)	36 (81.9)	**0.0337**	15 (34.1)	29 (65.9)	**0.0482**	12 (27.3)	36 (72.7)	**0.0076**
Negative	213	54 (25.3)	159 (74.7)		74 (34.7)	139 (65.3)		108 (50.1)	105 (49.9)		106 (49.7)	107 (50.3)	
Ki-67													
≤25%	166	50 (30.1)	116 (69.9)	**0.0004**	69 (41.6)	97 (58.4)	**<0.0001**	95 (57.2)	71 (42.8)	**<0.0001**	92 (55.4)	74 (44.6)	**<0.0001**
>25%	91	10 (10.9)	81 (89.1)		13 (14.3)	78 (85.7)		28 (30.7)	63 (69.3)		26 (28.6)	65 (71.4)	

IRS, Immunoreactive score; Neg., negative; Pos., positive; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.

Univariate analysis revealed that podoplanin-positive cases according to the binomial scale of Yamanashi *et al*. were characterized by significantly shorter patient OS (p=0.0411; Figure 2) and EFS (p=0.0255). In addition, podoplanin-positive cases by the scale of Kawase *et al*. had shorter OS as compared to podoplanin-negative cases (p=0.0355; Figure 2). No significant impact on patient OS and EFS was noted when patients survival was analysed with regard to both cut-off points of the IRS. Of note, borderline significantly shorter EFS was noted for cases scoring IRS 6-12 (p=0.0538), as compared to cases scoring IRS 0-4 (Table V). From the analysed clinical and pathological factors, the presence of lymph node metastases and G3 grade were also associated with poor OS (p=0.0272 and p=0.0116, respectively). Higher Ki-67 antigen expression and G3 malignancy were significantly associated with shorter EFS (p=0.0099 and p=0.0089, respectively).

Factors showing significant impact on patients' OS were entered in the multivariate analysis using the Cox proportional hazard model. Two separate analyses were performed. In the

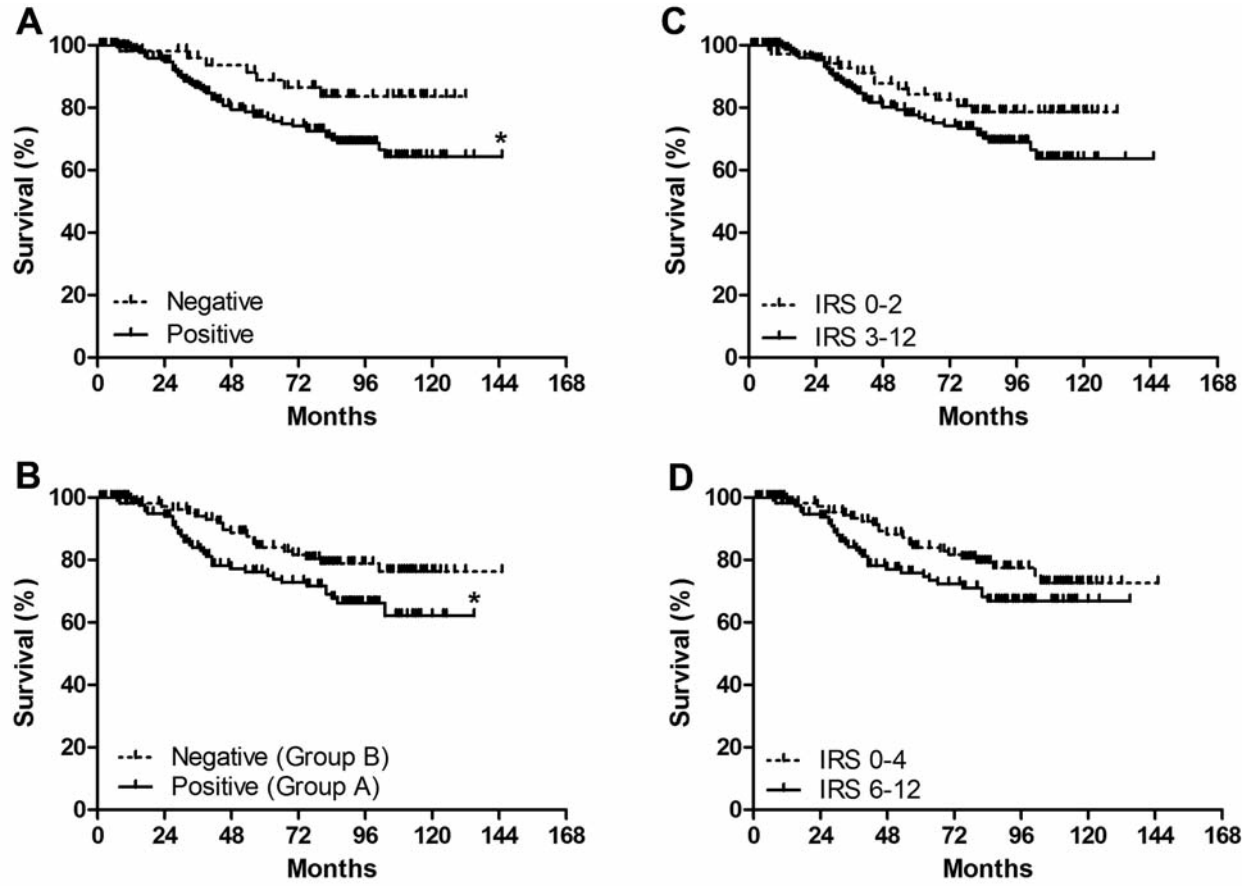

Figure 2. *Kaplan-Meier survival curves based on podoplanin assessment in the tumour stroma according to Kawase et al. (20) (A), Yamanashi et al. (21) (B) and immunoreactive score (C, D).*

first multivariate analysis podoplanin expression according to Kawase *et al*., presence of lymph node metastases and malignancy grade were entered into the statistical model, whereas in the second model, the scale of Kawase *et al*. was replaced with the scale of Yamanashi *et al*. (Table VI). Multivariate analysis revealed that only G3 malignancy grade was an independent prognostic factor of poor prognosis in both analyses (Table VI).

Discussion

We and Schoppmann *et al*. have recently shown that podoplanin expression in CAFs of IDC is an unfavorable marker of poor prognosis (17, 25). Similar results for non-small cell lung cancer, breast cancer and intra-hepatic carcinoma were obtained by other research groups, whereas podoplanin expression in CAFs of colorectal carcinoma was associated with longer survival (18-21, 23, 24). Moreover, podoplanin expression in CAFs of cervical cancer did not yield any prognostic significance (22). In these studies, different assessment scales were used for podoplanin quantification in the tumour stroma, therefore we analysed its expression on a subset of IDC cases using three different assessment methods. Currently, to our knowledge no consensus concerning cancerous stroma assessment methods exists. Highly-significant correlations observed between the scores obtained using the scales should produce comparable results regarding patient clinicopathological factors and survival, but as shown by the results of this study, the statistical analysis revealed differences among the utilized scales. Of note, strong correlations between the scores obtained using different scales should be analysed with caution, as the scale of Yamanashi *et al*. in such analyses may provide only approximate results due to its binomial values entered in the analysis.

In the current, as well as in previous work of our group, we have shown that the scale first introduced by Kawase *et al*. in the study of 177 lung adenocarcinoma cases, yielded prognostic impact as compared to the IRS for stroma (17, 20). Unfortunately, the results of this study showed that by using such a scoring approach, little information concerning possible correlations with other clinicopathological data may be obtained, as podoplanin expression in CAFs was only found to

Table V. *Univariate survival analysis in 257 patients with invascive ductal breast carcinoma. Significant p-values are given in bold.*

Clinicopathological parameter	Overall survival			Event-free survival		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Kawase et al. (Pos. *vs*. Neg.) (20)	1.929	1.046-3.560	**0.0355**	0.3233	0.7860-2.075	0.3233
CAF IRS (0-2 *vs*. 3-12)	1.558	0.8885-2.734	0.1217	1.245	0.7944-1.950	0.3396
CAF IRS (0-4 *vs*. 6-12)	1.571	0.9229-2.674	0.0960	1.521	0.9932-2.331	0.0538
Yamanashi et al. (Pos. *vs*. Neg.) (21)	1.739	1.023-2.955	**0.0411**	1.623	1.623-2.483	**0.0255**
Age (≤50 yrs. *vs*. >50 yrs.)	1.082	0.6097-1.921	0.7871	1.180	0.7433-1.874	0.4822
Menopausal status (Pre *vs*. Post)	1.131	0.6544-1.955	0.6588	1.035	0.6664-1.607	0.8785
Tumour size (T1 *vs*. T2-4)	1.460	0.8581-2.483	0.1629	1.300	0.8453-1.999	0.2323
Lymph nodes (Pos. *vs*. Neg.)	1.863	1.072-3.235	**0.0272**	1.344	0.8660-2.087	0.1872
Grade (G1,G2 *vs*. G3)	2.107	1.181-3.760	**0.0116**	1.868	1.169-2.985	**0.0089**
Ki-67 (≤25% *vs*. >25%)	1.700	0.9562-3.022	0.0707	1.837	1.157-2.916	**0.0099**

HR, Hazard ratio; CI, confidence interval; IRS, immunoreactive score; Neg., negative; Pos., positive; CAF, cancer-associated fibroblasts.

Table VI. *Multivariate Cox proportional hazard survival analysis in 257 patients with invasive ductal breast carcinoma. Significant p-values are given in bold.*

Clinicopathological parameter	Overall survival – Kawase *et al*. (20)			Overall survival – Yamanashi *et al*. (21)		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Kawase et al. (Pos. *vs*. Neg.)	0.6434	0.2184-1.894	0.4236	---	---	---
Yamanashi et al. (Pos. *vs*. Neg.)	---	---	---	0.8430	0.3288-2.161	0.7223
Lymph nodes (Pos. *vs*. Neg.)	2.1113	0.694-6.420	0.1878	2.0798	0.6838-6.325	**0.0418**
Grade (G1,G2 *vs*. G3)	2.6574	1.009-6.997	**0.0478**	2.4716	0.9635-6.3404	**0.0029**

HR, Hazard ratio; CI, confidence interval; IRS, immunoreactive score; Neg., negative; Pos., positive.

be significantly associated with larger primary tumour size, G3 malignancy and higher cancer cell proliferation.

Although the modified IRS for stroma did not reveal any significant differences in patient survival, it produced the most significant associations with patient clinicopathological factors (primary tumour size, malignancy grade, ER, PR, HER2 and Ki-67 antigen expression status) when the cut-off point was set between the moderate (IRS 0-4) and strong (IRS 6-12) expression. A scale very similar to the presented IRS for stroma was utilized by Kitano *et al*. (19). In this study, the authors found that 29% of IDC cases expressed podoplanin in CAFs, whereas using a comparable cut-off point for positivity, we noted strong podoplanin expression in 52.2% of the examined cases. Such a discrepancy may be caused not only by the cut-off point itself, but also by intra-observer variability and type of section (whole-tissue sections or tissue microarray) which were assessed. In contrast to our study, Kitano *et al*. assessed podoplanin not in whole-tissue sections, but in 2-mm tissue microarray punches (19).

This cut-off point of the IRS for stroma (weak and moderate *vs*. strong expression) in our opinion reflects more the scale utilized by Yamanashi *et al*., which, as described earlier, divided the study cohort based on the reaction intensity and area of cells showing podoplanin expression into positive cases and negative cases (showing no or weak podoplanin expression) (21). Of note, this approach seems to be the most strict of the scales tested in this study. Similarly to the scale introduced by Kawase *et al*., it showed prognostic impact regarding patient OS (20, 21).

In our research, we did not directly compare the assessment scales used on whole-tissue sections of lung adenocarcinoma and intrahepatic carcinoma or tissue microarray of cervical uterine carcinoma, as in our opinion, the scale of Kawase *et al*. and the IRS for stroma (IRS 0-2 *vs*. 3-12) may produce comparable results (18, 22, 23). Nevertheless, we found the IRS for stroma superior to that used by Kawase *et al*., and the three studies using the 10% cut-off value for positivity, as it not only took into account the percentage of positive cells with colour reaction, but also the reaction intensity itself. Moreover, due to its linearity of its values this renders the IRS for stroma most useful, when one compares the expression of the studied antigen in cancer cells and CAFs simultaneously, as both these cell types may be successfully assessed using such an approach (40, 41). Comparisons between these cell types seem to be

more problematic using the scale of Yamanashi *et al*., as difficulties may occur in setting a cut-off point for the reaction intensity observed in both cell types (21). This may limit the usefulness of this scale for clinical trials based on assessment of stromal markers other than podoplanin stained sections such as tenascin-C or α-SMA, as no internal positive control could be defined (43, 44).

Although, this study has revealed differences in patient outcome and associations with clinical and pathological parameters, caution should be exercised as all of the utilized scales are semi-quantitative and do not involve auxiliary equipment (*e.g*. computer-assisted image analysis) for the assessment of tumour stroma. Moreover, it seems that IDC is the most easily assessable tumour because podoplanin expression in tumour cells is rarely noted (17, 25). Additionally, podoplanin expression in myoepithelial cells and lymphatic endothelial cells may be distinguished due to their distinct morphology. Moreover, the proportion of these cells does not exceed 10%. Therefore, it seems that interference of these cell types in the stromal assessment may be, in our opinion, regarded as marginal.

In summary, we have shown, we believe for the first time, that different approaches for evaluation of podoplanin expression in CAFs may yield significant differences concerning patients' clinical outcomes. These may cause difficulties in comparison of studies dealing with expression of markers in the stromal compartment of different malignancies. Therefore a consensus regarding scales utilized for stromal marker quantification should be achieved, to minimize the differences among such studies and better-identify potential targets and cut-off points for use in future anticancer therapies.

Conflicts of Interest

The Authors have no conflict of interest to declare.

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