Expression of Podoplanin in Non-melanoma Skin Cancers and Actinic Keratosis

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Abstract. *Background/Aim: Recent studies have indicated that expression of podoplanin changes during the neoplastic processes, we therefore aimed at assessing its expression in cancer and stromal cells of basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and actinic keratosis (AK). Materials and Methods: Formalin-fixed paraffin-embedded tissue samples of 134 patients (38 BCC, 57 SCC, 20 AK and from 19 healthy volunteers) were analyzed. Podoplanin-immunoreactivity was detected in 32.1%/44.7%, 70%/20% and 87.7%/79% for BCC, AK and SCC tumour/stroma cells, respectively. Mean podoplanin expression in tumour cells was 1.2±1.8, 1.4±1.1 and 5.6±3.9 for BCC, AK and SCC, respectively. Mean podoplanin expression in stromal cells was 1.5±2.3, 0.65±1.57 and 3.2±2.4 for BCC, AK and SCC, respectively. Podoplanin expression was significantly higher in SCC stromal cells compared to the rest of the analyzed groups ($p<0.001$), suggesting a potential role of podoplanin in the development and progression of this malignancy.*

Podoplanin is a 38-kDa mucin-type transmembrane protein, which consists of 162 amino acids. Podoplanin is expressed in most normal human tissues such as alveolar type I cells in lung, mesothelial cells, osteocytes, osteoblasts, epithelial cells of choroid plexus, myofibroblasts of the breast and salivary glands, and basal keratinocytes (1-4). Its extracellular domain participates in platelet aggregation through platelet aggregation stimulating domain (PLAG). PLAG domain plays a key role in tumor cell-induced platelet aggregation (5-7). Furthermore, in normal as well as tumor cells the intracellular domain was shown to be responsible for interaction with protein kinase C and proteins of the ERM (ezrin, radixin, moesin) family. Connection between ERM proteins and the cytoskeleton facilitates cell motility and invasiveness warranting further tumour progression (1, 5). Moreover, since podoplanin expression has been identified on lymphatic endothelial cells, it is widely used to highlight lymphatics in normal and cancerous tissues, as well as to detect cancer cell lymphatic invasion (8, 9). Podoplanin has been detected in a variety of cancers, including lung adenocarcinoma, breast cancer, oral, esophageal and laryngeal squamous cell carcinomas, the central nervous system, ovarian and germ cell tumors, but its role in cancerogenesis is still being investigated (10-14). Its expression seems to be connected with higher aggressiveness, increased metastatic potential and poor patient prognosis (2, 10, 11, 13).

Excessive exposure to ultraviolet radiation (UV) and increasing number of organ transplant recipients are linked with higher incidence of basal cell carcinoma (BCC), actinic keratosis (AK) and squamous cell carcinoma (SCC), the most common skin malignancies in clinical practice (15). It should be kept in mind that AK, being a part of a multi-step carcinogenesis process, represents an early stage in a continuum that leads from carcinoma *in situ* to invasive SCC (16). An analogous cascade of carcinogenic events occurs during oral tumorigenesis, in which podoplanin expression was shown to increase with the severity of the dysplasia of oral leukoplakia and dedifferentiation of oral squamous cell carcinomas (17). Recent data indicate, that loss of podoplanin expression in keratinocytes had no impact on their adhesion, migration and proliferation, nor affected healing of skin wounds inflicted to podoplanin-deficient mice (18). Nevertheless, the role and behaviour of podoplanin in skin carcinogenesis still remains unclear, and have not been explored to date. Therefore, the current study

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Key Words: Podoplanin, skin cancer, basal cell carcinoma, squamous cell carcinoma, actinic keratosis, skin cancerogenesis.

Table I. *Characteristics of the studied group.*

	SCC	BCC	AK	Controls
Number of cases	57	38	20	19
Age (years)				
Range	37-100	35-85	40-85	21-82
Mean±SD	72.4±13.2	66.5±13.3	71.4±11.9	62.5±14.4
Gender, n (%)				
Female	24 (42.1)	27 (71.0)	16 (80)	9 (47.4)
Male	33 (57.9)	11 (29.0)	4 (20)	10 (52.6)
Sun exposure, n (%)				
Uncovered	52 (91.2)	25 (65.8)	14 (70)	18 (94.7)
Covered	5 (8.8)	13 (34.2)	6 (30)	1 (5.3)
Localization, n (%)				
Face	30 (52.6)	21 (55.3)	10 (50)	15 (78.9)
Lip	11 (19.3)	1 (2.6)	2 (10)	-
Ear	7 (12.3)	2 (5.3)	1 (5)	-
Head	5 (8.8)	1 (2.6)	1 (5)	3 (15.8)
Upper extremities	3 (5.3)	3 (7.9)	0 (0)	-
Lowerextremities	0 (0)	1 (2.6)	5 (25)	-
Trunk	1 (1.7)	9 (23.7)	1 (5)	1 (5.3)
Disease duration (months)				
Range	1-240	6-360	6-84	-
Mean±SD	22.6±34.9	36.4±57.6	26.4±18.3	-
Diameter (mm)				
Range	5-75	2-75	4-30	-
Mean±SD	18.5±13.8	15.2±15.2	13.1±7.6	-

SCC, Squamous cell carcinoma; BCC, basal cell carcinoma; AK, actinic keratosis; SD, standard deviation.

was undertaken to evaluate podoplanin expression in tumor and stromal cells of non-melanoma skin cancers (NMSC) as well as in AK. Another aim of the study was to also estimate the eventual relationship between podoplanin-immunoreactivity and lymphatic vessel density as well as some clinical parameters, including the type of the tumor.

Materials and Methods

Patients. Tissue samples used in our study were obtained from 115 patients (67 females, 48 males) between 35 and 100 years of age (mean=68.8±13.8 years) suffering from skin tumors treated atthe Department of Dermatology, Venerology and Allergology of Wroclaw Medical University in the years 2004-2012. All tumors (including 38 BCC, 20 AK and 57 SCC) were excised with conventional surgery. Non-lesional skin samples of 19 age-matched healthy volunteers (mean=62.5±14.4 years; range=21-82 years) collected during corrective plastic surgeryserved as a control. The detailed characteristic of the studied group is given in the Table I.

Tissue samples were immersed in 10% buffered formalin and embedded in paraffin blocks. Alternate sections were stained with hematoxylin and eosin (H&E) and used for further histological analysis. All sections were classified and independently evaluated by two observers, experienced in dermatopathology. Among the samples of BCC, 18 sBCC (superficial type of BCC) and 20 nBCC (nodular type of BCC) were diagnosed. SCC were staged according to the Broder's and Clark's scales and finally sub-typed as keratotic (n=47) and akeratotic (n=10). Moreover, all BCC and SCC samples were also grouped according to the depth and strength of inflammatory infiltrate (Table II). This study was approved by the Ethics Committee of Wroclaw Medical University (protocol number-KB – 735/2012).

Immunohistochemistry. The 4-μm-thick sections were de-paraffinized and antigen retrieval wasperformed in Target Retrieval Solution (pH9, 97˚C, 20 min). Then, the sections were rinsed in TBS/0.05% Tween buffer for 3 min. Endogenous peroxidase activity was blocked using EnVision FLEX Peroxidase-Blocking Reagent. In the next steps sections were washed in TBS/0.05% Tween buffer and incubated with a primary antibody directed against podoplanin (monoclonal mouse anti-human D2-40, clone D2-40, #IR072) in accordance with the manufacturer's instructions in Link48 Autostainer. Following this EnVision FLEX/HRP secondary antibodies were applied (20 min in room temperature) with 3,3'-diaminobenzidine (DAB) utilized as the peroxidase substrate. Finally the sections were counterstained with Mayer's haematoxylin, dehydrated in alcohols (70%, 96% and 99.8%) and xylene and mounted in the SUB-X Mounting Medium. The antibodies, reagents and equipment were obtained from Dako Cytomation (Glostrup, Denmark).

Immunostained sections were assessed under magnification of ×100 and ×200 and digitally imaged for evaluation of podoplanin expression in neoplastic and stromal, and lymphatic vessel density. For the evaluation podoplanin expression intensity the semiquantitative immunoreactive score (IRS) of Remmele and Stegner was used (Table III) (19). The method takes into account both the intensity of the staining (colour) reaction and the percentage of positive cells. The final score (IRS) represents the product of these two values, ranging from 0 to 12 points: no expression 0 points, weak expression 1-2 points, moderate expression 3-4 points, strong expression 6-12 points. Lymphatic vessel density (LVD) was counted in all podoplanin-immunostained slides in tumor stroma and tumour mass. All sections were scanned in the intratumoral and peritumoral area under ×100 magnification, to find three hot spots with the greatest number of lymphatic vessels. Then, total amount of lymphatic vessels in each area were counted and average score for each section was evaluated.

Statistical analysis. All data were statistically analyzed with the Statistica 10 software (StatSoft, Inc, Tulsa, OK, USA). Student's *t*-test for independent variables, Mann–Whitney *U*-test, univariate analysis of variance with post hoc test and χ^2 test were used where appropriate. Correlations between parameters were measured with Spearman's rank correlation test. Results with *p*-values less than 0.05 were treated as statistically significant.

Results

Podoplanin-immunoreactivity was detectedin 32.1%/44.7%, 70%/20% and 87.7%/79% for BCC, AK and SCC tumour/stroma cells, respectively. Positive immunoreactivity was found in 10 healthy skin sections (52.6%). In healthy skins, podoplanin expression was observed in epidermal basal layer, in peripheral cells of the sebaceous glands, and in the outer hair root sheath, no expression was found in dermis.

Table II. *The histological parameters of evaluated squamous cell cancers (SCC) and basal cell cancers (BCC).*

Histological parameters	Number of patients with sBCC/nBCC	Number of patients with SCC keratic/akeratotic
Broders scale		
G1 (>75 % of keratinized cells)	-	7 (12.3)
G2 (50-75 % of keratinized cells)	-	18 (31.6)
G3 (25-50 % of keratinized cells)	-	22 (38.6)
G4 (< 25 % of keratinized cells)	-	10 (17.5)
Depth of neoplastic infiltration according Clark scale		
I- invasion into the epidermis	-	0
II- invasion into the papillary dermis	-	1 (1.7)
III- invasion to the junction of the papillary and reticular dermis	-	3 (5.3)
IV- invasion into the reticular dermis	-	21 (36.8)
V- invasion into the subcutaneous fat	-	32 (56.1)
Density of inflammatory infiltration		
Mild inflammatory infiltration	8 (21.0)	9 (15.8)
Moderate inflammatory infiltration	8 (21.0)	29 (50.8)
Massive inflammatory infiltration	22 (57.9)	19 (33.3)
Depth of inflammatory infiltration		
Dermis	23 (60.5)	13 (22.8)
Subcutis	15 (39.5)	44 (77.2)

sBCC, Superficial basal cell carcinoma; nBCC, nodular basal cell carcinoma.

The mean expression of podoplanin in tumour cells was scored as 1.2±1.8, 1.4±1.1 and 5.6±3.9 for BCC, AK and SCC, respectively. These results were compared to epidermis, where mean expression was assessed as 1.0±1.2. The podoplanin expression was significantly higher in SCC tumor cells in comparison to BCC, AK and healthy skin where mean expression was at similar level ($p<0.001$) (Figure 1).

The mean expression observed in stromal cells was of 1.5±2.3, 0.65±1.57 and 3.2±2.4 for BCC, AK and SCC, respectively. In the dermis of the controls no expression of podoplanin was found. We revealed that podoplanin expression was significantly higher in SCC stromal cells than in other analyzed groups ($p<0.001$). There were no significant differences between other studied groups.

The comparison of podoplanin-immunoreactivity within the particular groups revealed that in AK ($p=0.01$) and SCC ($p=0.001$) a significantly higher expression in tumour cells was found than in the stroma. In BCC no differences between mean podoplanin expression in tumour mass and stroma were observed (Table IV).

For all podoplanin expression samples no associations were found with regard to patients' age, gender, lesions diameter and duration of the disease. Moreover, we found that neither depth nor strength of inflammation have an influence on podoplanin expression in tumour and stromal cells. It is noteworthy that there was a significant strong positive correlation between expression of podoplanin and Clark's scale in SCC (R=0.31, $p=0.02$ and R=0.57; $p<0.0001$ for tumour mass and stroma, respectively)

Table III. *Scoring system according to Remmele and Stegner.*

Percentage of positive cells	Staining intensity
0 No positive cells	0 No detectable stain
1 <10 %	1 Weak nuclear stain
2 11-50 %	2 Moderate nuclear stain
3 51-80 %	3 Strong nuclear stain
4 >80%	

IRS total=Percentage of positive cells (0-4) × Intensity of stain (0-3).

Table IV. *Mean podoplanin expression in tumour mass and in stroma of the analyzed squamous cell cancer (SCC), basal cell cancer (BCC) and actinic keratosis (AK).*

	Mean podoplanin expression			
	Tumour mass/AK/ epidermis		Stroma/AK/ dermis	
SCC-total	5.6±3.9		3.2±2.4	
SCC akeratotic	3.3±4.4	$p=0.03$	5.4±2.5	$p=0.005$
SCC keratotic	6.2±3.7		2.7±2.2	
BCC-total	1.2±1.8		1.5±2.3	
BCC nodular	1.7±2.1		1.0±2.0	
BCC superficial	0.5±0.9	$p=0.04$	2.0±2.5	$p=0.04$
AK	1.45±1.14		0.65±1.57	
Healthy skin	1.0±1.2		0	

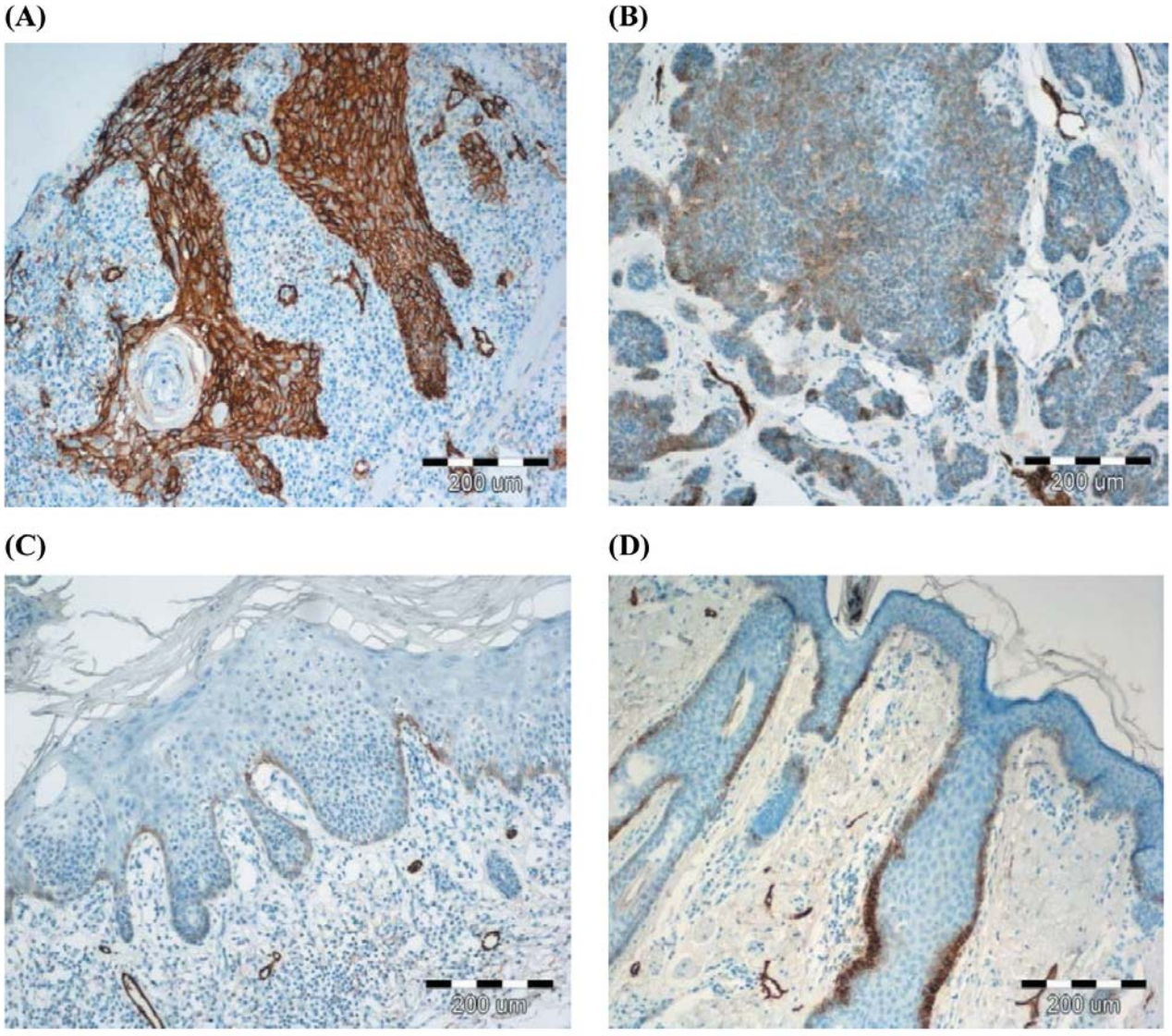

Figure 1. *Podoplanin expression in sections of squamous cell cancer (A), basal cell cancer (B), actinic keratosis (C) and healthy skin (D).*

(Figure 2). Stronger podoplanin expression was linked to the deeper neoplastic infiltration to the skin.

The mean lymphatic vessel density in the lesion mass was counted as 4.4±4.2, 0.5±1.8 and 8.5±7.3 for BCC, AK and SCC respectively. The number of lymphatics in AK was significantly lower than in BCC and SCC ($p<0.001$ for both comparisons), whereas the mean LVD in BCC and SCC was at a similar level. The results of mean LVD in stromal cells were of 13.6±8.0, 10.9±3.3 and 13.4±6.5 for BCC, AK and SCC, respectively. They did not differ statistically from each other. It is noteworthy that we revealed that in tumour mass of SCC, the stronger intensity of podoplanin expression was correlated with higher mean LVD (R=0.28; $p=0.03$) (Figure 3). For the rest of assessments no such correlations were found for both tumour and stromal cells.

Discussion

Basal cell carcinoma and squamous cell carcinoma constitute about 30% of malignant neoplasms in human. The incidence of these cancers rises during the last decades, increase of NMSC is about 3-8% per year (18). NMSC are characterized by a very low lethality, slow growth and low incidence of metastases, nevertheless they can destroy surrounding tissues

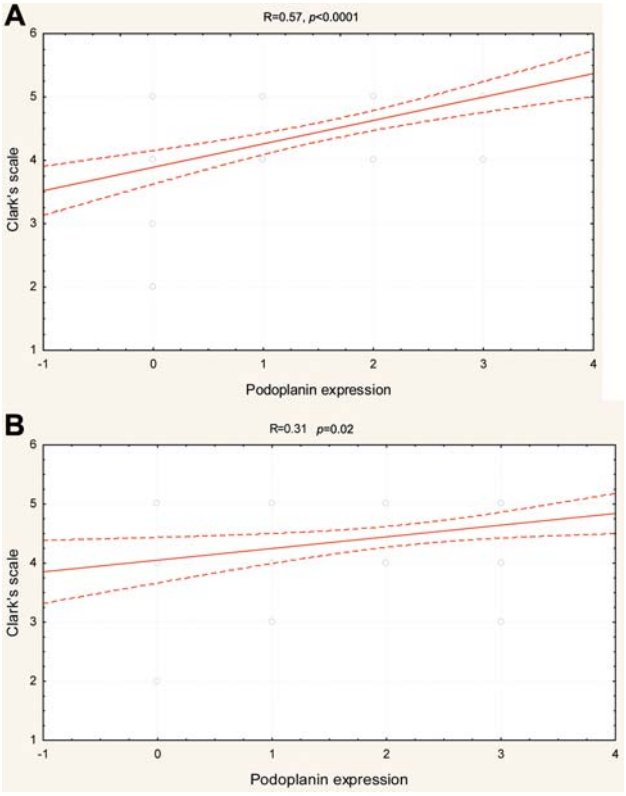

Figure 2. *Correlation between Clark's scale and podoplanin expression in SCC stromal (A) and tumour cells (B). Spearman correlation test. (A) Correlation between Clark's scale and podoplanin expression in SCC stromal cells. (B) Correlation between Clark's scale and podoplanin expression in SCC tumour cells.*

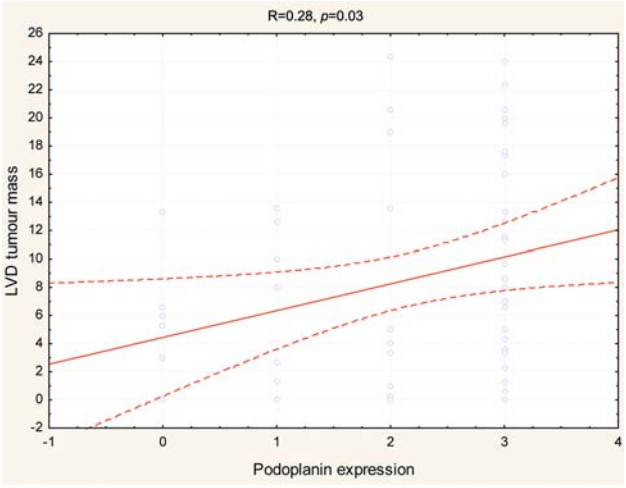

Figure 3. *Correlation between mean lymphatic vessel density (LVD) and podoplanin expression in tumor cells of squamous cell cancer. Spearman correlation test.*

or lead to their deformation (16, 20). Podoplanin expression has been found in a number of normal cells and also in many neoplasms. Overexpression of this protein in cancer cells was mainly connected with decreased survival and worse prognosis (1, 4, 21, 22). In the work of Vinicius *et al*. (23) expression of podoplanin was assessed in cutaneous squamous cell carcinoma. Interestingly podoplanin positivity was described in 41.8% of primary tumors and in 41.7% of metastatic lesions. Podoplanin expression in cutaneous squamous cell carcinoma was not connected with the presence of lymph node metastases but was associated with reduced survival. According to the authors these results could indicate influence of podoplanin in invasiveness and proliferative activity (23). In our study expression of podoplanin in cancerous stroma was found in 79% of SCC and in 44.7% of BCC. In cancer cells expression was described in 87.7% in SCC and 32.1% of BCC. It is noteworthy that in stroma and in neoplastic cells mean podoplanin expression was significantly higher in SCC in comparison to BCC and AK. In BCC, AK and healthy skin mean podoplanin expression was at similar levels. In SCC and AK expression of podoplanin was higher in tumour mass than in cancer stroma. This result may indicate that podoplanin may play a role in skin carcinogenesis. Furthermore, lesions connected with worse prognosis as SCC may present higher podoplanin expression.

Recent studies have regarded podoplanin as a marker of carcinogenesis. Expression of this protein was mostly connected with patient's poor outcome and decreased survival. In the work of Kawase *et al*. (10) expression of podoplanin was assessed in a group of 177 patients with diagnosis of lung adenocarcinoma in both cancer and stromal cells. Podoplanin expression was found in 5.1% of cancer and in 30.5% of cancer-associated fibroblasts (CAFs). Expression was observed only in the invasive adenocarcinomas and none were found in non-cancerous lesions. Interestingly podoplanin positive cases were connected with shorter survival time. Evidence that podoplanin is possibly connected with tumour progression provided also Kreppel *et al*. (14). In a group of patients with cutaneous head and neck squamous cell carcinoma podoplanin expression was assessed. Authors found a connection between increased podoplanin expression and number of tumorous lymph nodes (14). In a group characterized by high podoplanin expression patients' survival was significantly decreased. These data suggest that podoplanin may impact tumourigenesis and high level of its expression may be connected with worse patient prognosis. In this studywe demonstrated that deeper infiltration of neoplastic cells due to Clark's scale was connected with higher podoplanin expression in SCC stroma and tumour

mass. We also found that stromal cells of akeratotic type were characterized by higher podoplanin expression in comparison to keratotic SCC. These results may confirm the role of podoplanin in skin carcinogenesis and its association with cancer aggressiveness. Interestingly in the work of other authors it was proposed that podoplanin plays an emerging role in early stages of tumour growth. Podoplanin expression was also present in dysplastic lesions of the larynx that was connected with higher incidence of laryngeal squamous cell carcinoma in these lesions (11). Nevertheless in laryngeal squamous cell carcinomas podoplanin expression was diminished during tumour progression (11). In our work in stroma of nodular BCC mean podoplanin expression was lower in comparison to the superficial type. Conversely to BCC stroma in neoplastic cells mean podoplanin expression was higher in the nodular type in comparison to superficial type. As basal cell carcinoma is characterized by a different course these results are in accordance with previous considerations. Slow growth and low metastatic potential are characteristic features of BCC.

It has also been shown that high podoplanin expression was connected with better prognosis. In the work of Yamanashi *et al*. expression of podoplanin in the tumor stroma of colorectal cancer was associated with good outcome of patients (24). In the study of Carvalho *et al*. podoplanin was also shown to be a favorable prognostic factor for squamous cervical carcinomas (22). The authors suggested that podoplanin might be a favorable prognostic factor for squamous cervical carcinomas (22). Good prognosis connected with lower incidence of nodal metastases was observed also in patients with lung squamous cell carcinoma. Suzuki *et al*. demonstrated that in lung squamous cell carcinoma low podoplanin expression was regarded as a predictor for poor prognosis of patients (25). Furthermore lesions characterized by high podoplanin expression showed a significantly longer overall survival and disease-free survival time (25).

In this study mean lymphatic vessel density did not vary in the stroma and tumour area in BCC, SCC and healthy skin. Only in tumour mass of AK mean LVD was significantly lower than of other lesions. These results may suggest that lymphatic vascularization plays small role in progression of NMSC. However in the tumour mass of SCC, expression was connected with LVD, intense podoplanin expression was associated with increased number of lymphatics. Higher mean podoplanin an expression in stroma of keratotic SCC and positive correlation between expression of podoplanin and mean lymphatic vessel density in SCC cells may support the notionthat this protein may serve as prognosticator of clinical course in skin cancers. Without any doubts podoplanin may play a role in development and progression of skin cancer however its exact role needs to be clarified.

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