

Podoplanin Expression Correlates with Disease Progression in Mycosis Fungoides

Alina JANKOWSKA-KONSUR¹, Christopher KOBIERZYCKI², Jędrzej GRZEGRZÓŁKA², Aleksandra PIOTROWSKA², Agnieszka GOMULKIEWICZ², Natalia GLATZEL-PLUCINSKA², Adam REICH¹, Marzenna PODHORSKA-OKOŁÓW², Piotr DZIĘGIEL³ and Jacek C. SZEPIETOWSKI¹

¹Department of Dermatology, Venereology and Allergology, ²Department of Histology and Embryology, Wrocław Medical University, and ³Department of Physiotherapy, University School of Physical Education, Wrocław, Poland

The aim of this study was to investigate the role of lymphangiogenesis in the clinical progression and outcome of mycosis fungoides. Immunohistochemistry and Western blot techniques were used to assess the expression of podoplanin and vascular endothelial growth factor C in mycosis fungoides. Expression of vascular endothelial growth factor C measured by immunohistochemistry was significantly higher in mycosis fungoides samples in comparison with control cases (chronic benign dermatoses) ($p=0.0012$). Increased expression of podoplanin was found in advanced vs. early mycosis fungoides ($p<0.0001$), and was positively correlated with cutaneous and nodal involvement ($p<0.001$, $p<0.0001$; respectively). Higher podoplanin expression was also significantly associated with shorter survival ($p<0.001$). Strong positive correlation was observed between expression of podoplanin analysed by immunohistochemistry and Western blot ($r=0.75$, $p<0.0001$). A similar association was shown regarding expression of vascular endothelial growth factor C ($r=0.68$, $p=0.0007$). In conclusion, these results suggest that increased expression of podoplanin is associated with poor clinical course, as well as shorter survival, of patients with mycosis fungoides.

Key words: mycosis fungoides; primary cutaneous T-cell lymphoma; lymphangiogenesis; podoplanin; VEGF-C.

Accepted Aug 17, 2016; Epub ahead of print Aug 22, 2016

Acta Derm Venereol 2017; 97: 235–241.

Corr: Jacek C. Szepietowski, Department of Dermatology, Venereology and Allergology, University of Medicine, Chalubinskiego 1, PL-50-368 Wrocław, Poland. E-mail: jacek.szepietowski@umed.wroc.pl

Mycosis fungoides (MF) is the most common form of primary cutaneous T-cell lymphoma, accounting for approximately 50% of cases (1). It arises in peripheral T lymphocytes and is characterized by a relatively benign remitting/relapsing course. Initially, MF develops solely in the skin, evolving through consecutive stages, from patches through plaques to tumours, often ulcerated. The disease can also occur in the erythrodermic form, in which inflammation of the skin covers more than 80% of the body surface area. In some cases, the disease manifests aggressive behaviour, disseminating to the lymph nodes and internal organs. The distinctive nature of MF

is reflected in the current staging system, in which special attention has been paid to the character and extent of the skin lesions (2). Despite numerous studies over the last decades, the aetiology of MF is not fully understood, but, similarly to solid tumours and other haematological malignancies, recent studies suggest the involvement of lymphangiogenesis in the development and progression of the disease.

Lymphangiogenesis is a multistep process of formation of new lymphatic vessels from the existing vascular network through extension and branching in response to local stimulation (3). It plays a significant role in physiological processes, mainly during embryogenesis, the ovarian cycle and gestation. However, it is also fundamental for metastasis of malignancies (4). The importance of this process in the dissemination of malignant neoplasms has been suggested due to the discovery of specific markers of lymphatic vessels. Among them, a highly O-glycosylated mucin-like transmembrane glycoprotein, podoplanin (PDPN), is commonly used in lymphatic vasculature assessment. PDPN is involved in the processes of adhesion and migration of endothelial cells, and its expression is observed on lymphatic endothelium. Moreover, physiologically its expression has been found in osteocytes, peritoneal mesothelial, glandular myoepithelial, and ependymal cells, as well as in stromal reticular and follicular dendritic cells of lymphoid organs. Expression in oncological lesions was shown in breast, oesophageal and testicular cancer, as well as in germ cell tumours (5–8).

Numerous lines of evidence indicate an important role of the family of vascular endothelial growth factors (VEGF) in lymphangiogenesis. The VEGF family consists of 6 proteins: VEGF-A, -B, -C, -D, -E, and placental growth factor (PGF), of which, the most potent stimulator of lymphangiogenesis is VEGF-C (9). VEGF-C promotes proliferation, migration and survival of lymphatic endothelial cells through a specific transmembrane receptor VEGFR-3/FLT4 (10, 11). Many recent studies have revealed a strong association between VEGF-C expression and clinicopathological data, i.e. grade of histological malignancy, progression, dissemination as well as prognosis of the disease (12–15). In breast, prostate and colon cancer increased expression of VEGF-C correlated with high lymphatic vessel density (LVD) and

poor survival (16–21). In haematological malignancies, increased lymphangiogenesis was found to be correlated with aggressiveness and poor clinical outcome of lymphoma (22, 23).

In studies on lymphangiogenesis in lymphoproliferative disorders relatively little attention was paid to primary cutaneous T-cell lymphomas (CTCL). Therefore, using immunohistochemical (IHC) and molecular methods (Western blot; WB), we evaluated the expression of VEGF-C and PDPN, important markers of lymphangiogenesis, in relation to clinicopathological data and their potential impact on patients' outcomes in MF.

METHODS

Specimens

The material for the IHC comprised 73 paraffin-embedded samples obtained from patients with MF diagnosed and treated in the Department of Dermatology, Venereology and Allergology (Wroclaw Medical University) in the period 1994–2015. The diagnosis was established and re-evaluated based on clinical, histopathological and IHC examinations, according to the World Health Organization (WHO) classification (2008) (1). The staging was assessed according to TNMB (Tumour, Nodes, Metastases, Blood) system (ISCL/EORTC revision) presented in our previous study (24). To examine potential differences between benign and malignant skin conditions chronic benign dermatitis paraffin samples (16 lichen planus, 3 disseminated eczema cases) were used as a control. Moreover, skin biopsies were collected from 21 patients with MF and 4 with benign dermatoses and stored at -80°C in order to perform WB analysis. The material for WB was obtained simultaneously from the same lesions as those for IHC study. The study was approved by the ethics committee of Wroclaw Medical University (approval number KB 574/2011). All patients' clinicopathological data are shown in **Table I**.

Immunohistochemistry

All reactions were performed on 4- μm -thick paraffin sections. In order to examine the expression of PDPN, a protocol using Dako Autostainer Link48 (Dako, Glostrup, Denmark) was introduced. Deparaffinization, rehydration and unmasking the antigens was conducted by boiling in EnVision FLEX Target Retrieval Solution (pH 9, 20 min, 97°C ; Dako) using Pre-Treatment Link platform (Dako). Activity of endogenous peroxidase was blocked by 5 min incubation in EnVision FLEX Peroxidase-Blocking Reagent (Dako). Sections were incubated with the primary monoclonal mouse anti-human PDPN antibody (clone D2-40, RTU, catalogue number: IR072; Dako) for 20 min at room temperature (RT). Following this EnVision FLEX /HRP – secondary antibodies were applied (20 min at RT; Dako). Diaminobenzidine (DAB, Dako) was utilized as the peroxidase substrate and the sections were incubated for 10 min at RT. In order to examine the expression of VEGF-C manual protocol was performed. The sections were deparaffinized in xylene, rehydrated and boiled in a citrate buffer, pH 6. Subsequently, endogenous peroxidase was blocked using 3% H_2O_2 (5 min, RT). The sections were incubated with primary mouse antibody anti-VEGF-C (1:100, catalogue number: 101-M90; ReliaTech GmbH, Wolfenbüttel, Germany) for 18 h at 4°C . The next reaction stages were conducted according to the manufacturer's instructions for the LSAB+ System-HRP visualization system (Dako). All slides were counterstained with EnVision FLEX Hematoxylin (Dako). Subsequently, the preparations were mounted in Mounting Medium (Dako).

Table I. Clinicopathological data

	Immunohistochemistry (<i>n</i> = 73)	Western blot (<i>n</i> = 21)
Age, years, mean \pm SD (range)	58.82 \pm 13.75 (19–81)	56.48 \pm 14.81 (31–77)
Sex, <i>n</i> (%)		
Male	46 (63.89)	12 (57.14)
Female	27 (37.50)	9 (42.86)
Tumour size, <i>n</i> (%)		
T1	20 (27.40)	6 (28.57)
T2	22 (30.14)	3 (14.29)
T3	15 (20.54)	6 (28.57)
T4	16 (21.92)	6 (28.57)
Lymph nodes, <i>n</i> (%)		
N0	42 (58.33)	9 (42.86)
N1, N2, N3	31 (43.06)	12 (57.14)
Metastasis, <i>n</i> (%)		
M0	72 (98.63)	21 (100.00)
M1	1 (1.37)	0 (0)
Blood involvement, <i>n</i> (%)		
B0	69 (94.52)	20 (95.24)
B1	4 (5.48)	1 (4.76)
Pathological tumour–node–metastasis, <i>n</i> (%)		
I A	19 (26.03)	6 (28.57)
I B	16 (21.92)	2 (9.52)
II A	6 (8.22)	1 (4.76)
II B	11 (15.07)	5 (23.81)
III	15 (20.55)	6 (28.57)
IV A	5 (6.85)	1 (4.76)
IV B	1 (1.36)	0 (0)
Clinical advancement, <i>n</i> (%)		
Early (stage IA–IIA)	46 (63.01)	12 (57.14)
Advanced (stage IIB–IVB)	27 (36.99)	9 (42.86)

Evaluation of immunohistochemistry reactions

All sections were evaluated using a BX-41 light microscope (Olympus, Tokyo, Japan) by 2 pathologists who were blinded to the patients' clinical data. In doubtful cases, a re-evaluation with a double-headed microscope was performed until a consensus was achieved. PDPN expression was observed in a cytoplasm of lymphatic vessels endothelial cells. Initially, whole slide was determined as follows: the sections were first scanned at low power ($\times 40$ and $\times 100$ magnification) to identify the hot-spots (areas of potentially highest lymphatic vessel density) as it is used in the microvessel density (LVD) count. Subsequently, these areas were examined under $\times 200$ magnification using a Chalkley Point Array graticule (Pyser Sgi., Edenbridge, UK). The Chalkley count was regarded as the number of grid points that hit stained vessels. A mean score was determined for 3 hot-spots. Expression of VEGF-C was conducted using the semiquantitative immunoreactive score (IRS) of Remmele and Stegner, which is based on the intensity of the colour reaction and the percentage of positive neoplastic cells in the whole slide (25). The scale takes into account the percentage of cells with positive reaction (0 points: absence of cells with positive reaction, 1 point: 1–10% cells, 2 points: 11–50%, 3 points: 51–80%, 4 points: over 80% cells with positive reaction) as well as intensity of the reaction (0 points: no reaction, 1 points: low, 2 points: moderate, and 3 points: strong intensity of colour reaction). The product of both these parameters comprises the final score, ranging from 0 to 12 points.

Western blot

Frozen samples were thawed in CellLytic MT Cell Lysis Solution (SigmaAldrich, Munich, Germany) with the addition of protease inhibitors, Benzonase – 50 U/ul (Merck; Millipore, Bedford, MA, USA) and 0.2 mM phenylmethanesulfonyl fluoride (PMSF; SigmaAldrich). Protein concentrations of whole-cell lysates were determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Equal amounts of total protein (30 μg) were mixed with

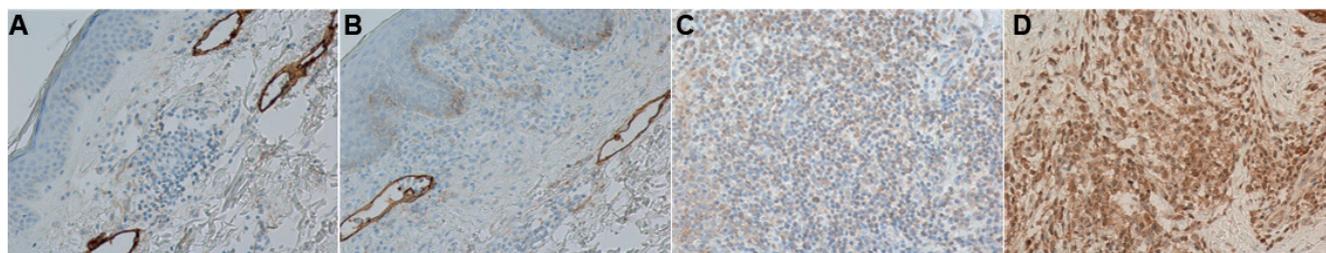


Fig. 1. Immunohistochemical expression of (A, B) podoplanin in cytoplasm of endothelial cells of lymphatic vessels, and (C, D) expression of vascular endothelial growth factor C, with (C) moderate and (D) strong cytoplasmic localization in neoplastic cells in patients with mycosis fungoides. Original magnification $\times 200$.

sample buffer and dithiothreitol (DTT) and resolved by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). After completion of the electrophoresis, the samples were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore) and incubated in a 4% bovine serum albumin (BSA) solution in Tris-buffered saline (TBS) with the addition of 0.1% Tween-20. Subsequently, the membranes were incubated with rabbit anti-human VEGF-C antibody – 1:500 (AP2042c; Abgent, San Diego, CA, USA) and rabbit anti-human PDPN antibody – 1:750 (11629-1-AP; Proteintech Europe, Manchester, UK) for a night at 4°C. At the end the membranes were treated with the peroxidase-conjugated donkey anti-rabbit secondary antibody – 1:3,000 (711-035-152; Jacksons Immunoresearch, Suffolk, UK) for 1 h, rinsed, and incubated with the Luminata Classico Western HRP Substrate (Merck) overnight. Protein quantifications were based on the total protein normalization with the use of the TGX Stain Free FastCast Acrylamide Kit (Bio-Rad, Hercules, CA, USA). Protein separation was visualized on the gels after electrophoresis and analysed by using Image Lab 5.0 Software (Bio-Rad) and ChemiDock MP System (Bio-Rad).

Statistical analysis

Shapiro-Wilk test was used to evaluate the normality assumption of examined groups. To compare the differences between the expression of examined markers in the all patients’ pairs of groups and clinicopathological data the unpaired *t*-test and Mann-Whitney test were used. To compare the differences between more than 2 groups the Kruskal-Wallis and Dunn’s multiple comparison test was performed. In addition, the Spearman’s correlation test was used to analyse the existing correlations. The Kaplan–Meier method was used to construct survival curves. To evaluate the analysis of survival Mantel–Cox test was performed. A Cox proportional hazards model with forward stepwise selection was used to calculate univariate and multivariate hazard ratio for the study

variables. All statistical analyses were performed using Prism 5.0 (GraphPad, La Jolla, CA, USA) and Statistica 10 (StatSoft Inc. Tulsa, OK, USA). The results were considered as statistically significant when $p < 0.05$.

RESULTS

Immunohistochemical expression of PDPN and VEGF-C was observed in 71 out of 73 cases of MF and in 18 out of 19 control cases. Expression of PDPN was observed in the cytoplasm of endothelial cells of lymphatic vessels (Fig. 1A, B), whereas VEGF-C expression was found in the cytoplasm of atypical T cells (Fig. 1C, D). The IHC expression of PDPN was higher in MF than in the control group; however, it did not reach statistical significance ($p = 0.07$; Mann-Whitney test, Fig. 2A). On the other hand, when comparing the chronic benign dermatoses and advanced MF group significantly higher expression was present in the latter ($p < 0.0001$; Mann-Whitney test; Fig. 2B). The expression of VEGF-C was significantly higher in MF samples compared with the control group ($p = 0.0012$, Mann-Whitney test; Fig. 2C). VEGFC was increased in both early and advanced cases compared with the control group (both $p < 0.01$, Mann-Whitney test; Fig. 2D).

Regarding advancement of the disease, increased PDPN expression was found in advanced vs. early MF ($p < 0.0001$; Mann-Whitney test; Fig. 2B). PDPN expression increased with more infiltrated cutaneous lesions (tumours, stage T3) and with more extensive skin

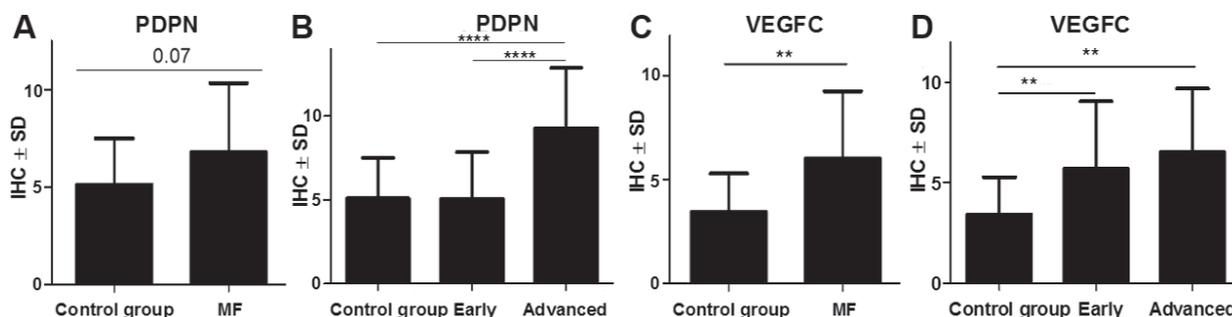


Fig. 2. Comparison of immunohistochemical expression of podoplanin (PDPN) with regard: to (A) disease presence and (B) clinical advancement. Comparison of immunohistochemical expression of vascular endothelial growth factor-C (VEGF-C) with regard to (C) disease presence and (D) clinical advancement. MF: mycosis fungoides; IHC: immunohistochemistry, early MF – stage IA–IIA; advanced MF – stage IIB–IVB. $**p < 0.01$, $****p < 0.0001$, Mann-Whitney test.

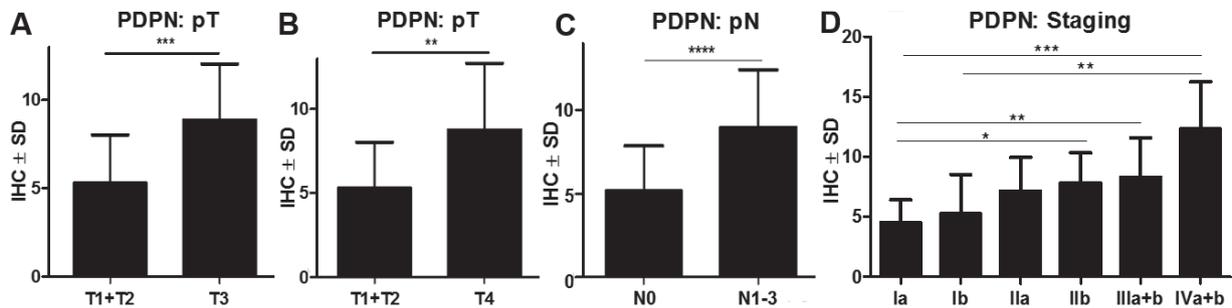


Fig. 3. Comparison of immunohistochemical expression of podoplanin (PDPN) with regard to: (A, B) skin involvement, (C) nodal involvement, and (D) staging. MF: mycosis fungoides; IHC: immunohistochemistry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; Mann-Whitney test.

involvement (erythroderma, stage T4, compared with less infiltrated skin changes with the limited involvement of the body surface (patches and plaques, stages T1, T2) ($p = 0.0004$, $p = 0.0014$, respectively, Mann-Whitney test; **Fig. 3A, B**). Moreover, higher PDPN expression was associated with nodal involvement, N1-3 vs. N0 ($p < 0.0001$; Mann-Whitney test; **Fig. 3C**). Evident trend of increased PDPN expression was found in higher stages of MF clinical advancement (Mann-Whitney test; **Fig. 3D**). In contrast, no association between VEGF-C expression and disease advancement, extracutaneous involvement as well as staging in MF was disclosed.

To confirm the results obtained by IHC, expression of PDPN and VEGF-C in frozen samples of MF and control cases (21 and 4 samples, respectively) was assessed using the WB technique (**Fig. 4**). WB expression of PDPN and VEGFC proteins was observed in all MF and control cases. Slightly higher expression of both studied markers was found in the MF group, albeit not reaching statistical significance (**Fig. 5**). In addition, no associations were found between expression of both examined proteins and clinicopathological data of MF (data not shown). Nevertheless, strong positive correlation between PDPN expression analysed by IHC and WB was observed ($r = 0.75$, $p < 0.0001$, Spearman's rank correlation test; **Fig. 6A**). Similar association regarding VEGF-C expression was shown ($r = 0.68$, $p = 0.0007$, Spearman's rank correlation test; **Fig. 6B**). Analysing mutual correlations between the IHC expression of studied markers, a strong positive correlation between PDPN and VEGF-C was found ($r = 0.70$, $p < 0.0001$; Spearman's rank correlation test; **Fig. 6C**). Correlation between PDPN and VEGF-C

analysed by WB did not reach statistical significance ($r = 0.38$, $p = 0.083$, Spearman's rank correlation test; **Fig. 6D**).

Univariate analysis using the Mantel-Cox test revealed that, in 73 patients tested, higher PDPN expression was significantly associated with shorter overall survival (OS; **Fig. 7A**). Regarding VEGF-C, increased expression of this marker was also associated with shorter survival, albeit not reaching statistical significance (**Fig. 7B**). Univariate and multivariate Cox regression model analysis is shown in **Table II**.

DISCUSSION

So far, only a few studies have analysed the process of lymphangiogenesis in CTCL. The current study demonstrated that higher PDPN expression is significantly correlated with disease progression and shorter survival in MF. First, we noticed the increased PDPN expression in MF compared with the control group, albeit not reaching statistical significance. However, the significantly higher PDPN expression was found in advanced MF while comparing with chronic benign dermatitis. Numerous data indicate the close association between lymphangiogenesis and chronic inflammation. In the skin, the increased expression of VEGF-C and enhanced LVD measured utilizing antibodies against PDPN was observed in cutaneous lichen planus, psoriasis and atopic eczema (26, 27). MF lesions, especially in the early stages, are characterized by mixed infiltration consisting of malignant lymphocytes mingled with reactive cells. Our data suggest, however, more pronounced process

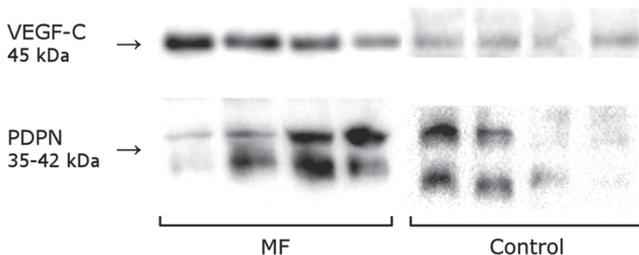


Fig. 4. Sample results of Western blot analysis of vascular endothelial growth factor C (VEGF-C) and podoplanin (PDPN) expression in mycosis fungoides (MF) and control cases.

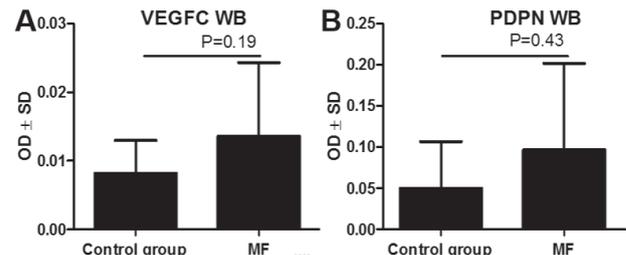


Fig. 5. Comparison of vascular endothelial growth factor C (VEGF-C) and podoplanin (PDPN) expression in Western blot (WB) analysis with regard to disease presence. MF: mycosis fungoides.

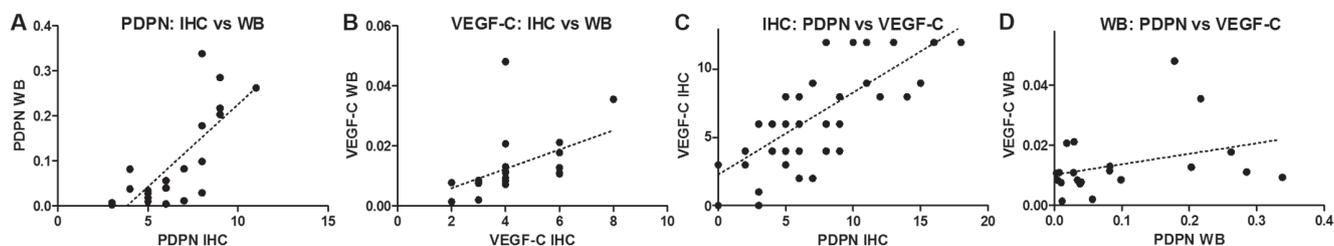


Fig. 6. Spearman's rank correlation plots presenting associations between expressions of podoplanin (PDPN) and vascular endothelial growth factor C (VEGF-C) analysed by immunohistochemistry (IHC) and Western blot (WB) technique: (A) PDPN WB vs. PDPN IHC ($r=0.75$, $p<0.0001$), (B) VEGF-C WB vs. VEGF-C IHC ($r=0.68$, $p=0.0007$), (C) VEGF-C IHC vs. PDPN IHC ($r=0.70$, $p<0.0001$) and (D) VEGF-C WB vs. PDPN WB ($r=0.38$, $p=0.083$) in patients with mycosis fungoides.

of lymphangiogenesis in the course of malignancy than in inflammation.

Secondly, we found an association between disease progression and PDPN expression. Advanced cases of MF were characterized by increased marker expression compared with early cases. Expression of PDPN also correlated with skin and nodal involvement. Increased lymphatic vascularization has been found in more infiltrated as well as in more extensive cutaneous lesions (tumours vs. patches and plaques, erythroderma vs. patches and plaques). Our results are consistent with accumulated lines of evidence that strongly suggest the role of tumour-induced lymphangiogenesis in progression and metastasis of various cancers, i.e. prostate adenocarcinoma, oesophageal squamous cell carcinoma, gastric, breast and lung cancer (28–32). Considering haematological malignancies, positive correlation between increased number of PDPN-positive vessels and disease progression was reported in Sézary syndrome, the most common, aggressive CTCL (33). Increased LVD was also associated with aggressive histology in non-Hodgkin's lymphomas (NHL) (23).

Our study has also revealed increased expression of the key lymphangiogenesis factor, VEGF-C, in MF compared with the control group. VEGF-C overexpression was also observed in Sézary syndrome in the IHC study of Karpova et al. (33). In further analysis, we observed comparable levels of VEGF-C expression in early as well as in advanced stages, suggesting that VEGF-C could play a role on the subsequent steps of disease initiation and progression. Analogously, comparable expression level of another VEGF family member, VEGF-A, was observed in early and advanced disease in the skin biopsy

tissue obtained from patients with MF (34). According to the authors, VEGF-A overexpression observed in all disease stages should be considered as an inner phenomenon associated with MF development. This hypothesis could be also applicable to the role of VEGF-C in MF.

A number of studies has documented VEGF-C expression in tumour cells, inflammatory cells and tumour-associated fibroblasts in primary human tumours and their stroma. IHC analysis allowed us to locate the VEGF-C in the cytoplasm of the tumour cells. VEGF-C expression has been also observed in malignant T cells in the course of tumour progression in a xenograft mouse model of MF, and in the cells of suprabasal layers of the epidermis in another IHC study on the MF skin samples (33). Interestingly, *in vitro* study failed to detect VEGF-C in CTCL cells, while co-culture with malignant lymphocytes induced increased VEGF-C expression in fibroblasts, which implies the modulating effect of the tumour microenvironment on VEGF-C expression level in different cell types (35).

In the current study we observed a positive correlation between PDPN and VEGF-C expression. Our results are consistent with the report of Karpova et al., who found the same association in Sézary syndrome and erythrodermic MF (33). A positive correlation between VEGF-C, and PDPN expression was also reported in other malignancies, i.e. gastric and bladder cancer (17, 36).

Taking into account observed PDPN and VEGF-C expression in MF, the potential impact of lymphangiogenesis on the patients' outcome was analysed. Increased expression of PDPN significantly correlated with shorter survival. Moreover, association between increased expression of VEGF-C and poor outcome was found,

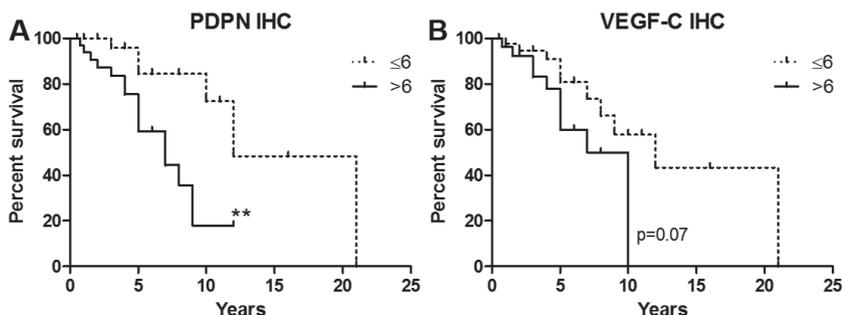


Fig. 7. Analysis of survival in the mycosis fungoides group according to (A) immunohistochemical expression of podoplanin (PDPN) and (B) vascular endothelial growth factor C (VEGF-C). $**p<0.001$, Mantel-Cox test.

Table II. Univariate and multivariate Cox regression model analysis of survival in mycosis fungoides

Characteristics	Overall survival		
	Hazard ratio	95% CI	p-value
<i>Univariate Cox regression model analysis</i>			
VEGF-C (≤ 6 vs. > 6)	0.7512	0.4378–1.2891	0.2993
Podoplanin (≤ 6 vs. > 6)	0.6658	0.3953–1.1215	0.1263
Stage (I–IIa vs. IIb–IVb)	0.3928	0.2139–0.7211	0.0025
pT (T1–2 vs. T3–4)	0.3948	0.2160–0.7215	0.0025
pN (N0 vs. N1–3)	0.3900	0.2106–0.7223	0.0027
pM (M0 vs. M1)	0.2222	0.0225–2.1891	0.1975
pB (B0 vs. B1)	0.3661	0.0503–2.6614	0.3208
Age (≤ 62 vs. > 62 years)	0.9489	0.7771–1.1585	0.6066
Sex (male vs. female)	0.7321	0.4177–1.2831	0.2762
<i>Multivariate Cox regression model analysis</i>			
Stage (I–IIa vs. IIb–IVb)	1.2501	0.0679–23.010	0.8805
pT (T1–2 vs. T3–4)	0.4648	0.0288–7.4917	0.5891
pN (N0 vs. N1–3)	0.5533	0.2404–1.2734	0.1640

Statistically significant values are given in bold.
VEGF-C: vascular endothelial growth factor C; CI: confidence interval.

although it did not reach statistical significance. However, this phenomenon may be due to the relatively small number of patients included in the study. Accumulated lines of evidence indicate the association between tumour lymphangiogenesis and poor survival in solid tumours, i.e. increased VEGF-C expression correlated with poor outcome in breast cancer, and enhanced LVD was associated with shorter survival in colon and gastric cancer (29, 37–39). Considering haematological malignancies, in a heterogeneous group of NHL, mainly diffuse large B-cell lymphoma (DLBCL) and follicular B-cell lymphoma as well as Hodgkin's lymphomas, the LVD correlated with poor outcome (22). Another study revealed that increased expression of VEGF-C in blood and tumour tissue correlated with mortality and high International Prognostic Index score in patients with DLBCL (40). In Hodgkin's lymphomas, Rueda et al. (41) found that higher tissue expression of VEGF-C was a negative prognostic factor and correlated with shorter progression-free survival.

In our study, the expression of VEGF-C and PDPN observed in the IHC study was confirmed utilizing WB technique. A strong correlation was found between the results of the IHC and WB analyses regarding both studied factors. However, in-depth analysis of the WB results did not show significant associations. Given the small sample size these findings are not surprising for us, but are a limitation of the study.

In summary, this study provides new data indicating the role of lymphangiogenesis in the progression, skin and nodal involvement of MF, as well providing evidence for potential future use of increased expression of PDPN as a predictor of shorter survival in patients with MF.

ACKNOWLEDGEMENTS

This work was financially supported by the National Science Center (decision no. DEC-2011/01/B/NZ4/01052).

The authors declare no conflict of interest.

REFERENCES

- Rakfkiaer E, Cerroni L, Sander CA, Smoller BR, Willemze R. Mycosis fungoides. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. In: WHO classification of tumours of hematopoietic and lymphoid tissues. Lyon: International Agency for Research on Cancer (IARC), 2008: p. 269–317.
- Olsen E, Vonderheid E, Pimpinelli N, Willemze R, Kim Y, Knobler R, et al. Revisions to the staging and classification of mycosis fungoides and Sezary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC). *Blood* 2007; 110: 1713–1722.
- Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* 2002; 21: 1505–1513.
- Rutkowski JM, Ihm JE, Lee ST, Kilarski WW, Greenwood VI, Pasquier MC, et al. VEGFR-3 neutralization inhibits ovarian lymphangiogenesis, follicle maturation, and murine pregnancy. *Am J Pathol* 2013; 183: 1596–1607.
- Niemiec J, Sas-Korczynska B, Harazin-Lechowska A, Martynow D, Adamczyk A. Lymphatic and blood vessels in male breast cancer. *Anticancer Res* 2015; 35: 1041–1048.
- Li JC, Li Y, Ai JY, Chen K, Zhu YH, Fu L, et al. Podoplanin-positive cancer cells at the edge of esophageal squamous cell carcinomas are involved in invasion. *Mol Med Rep* 2014; 10: 1513–1518.
- Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, Detmar M. Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumours. *Am J Pathol* 2005; 166: 913–921.
- Sonne SB, Herlihy AS, Hoei-Hansen CE, Nielsen JE, Almstrup K, Skakkebaek NE, et al. Identity of M2A (D2-40) antigen and gp36 (Aggrus, T1A-2, podoplanin) in human developing testis, testicular carcinoma in situ and germ-cell tumours. *Virchows Arch* 2006; 449: 200–206.
- Takahashi M, Yoshimoto T, Kubo H. Molecular mechanisms of lymphangiogenesis. *Int J Hematol* 2004; 80: 29–34.
- Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, et al. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J* 2001; 20: 4762–4773.
- Salameh A, Galvagni F, Bardelli M, Bussolino F, Oliviero S. Direct recruitment of CRK and GRB2 to VEGFR-3 induces proliferation, migration, and survival of endothelial cells through the activation of ERK, AKT, and JNK pathways. *Blood* 2005; 106: 3423–3431.
- Decio A, Tarabozetti G, Patton V, Alzani R, Perego P, Fruscio R, et al. Vascular endothelial growth factor c promotes ovarian carcinoma progression through paracrine and autocrine mechanisms. *Am J Pathol* 2014; 184: 1050–1061.
- Kimura H, Kato H, Tanaka N, Inose T, Faried A, Sohda M, et al. Preoperative serum vascular endothelial growth factor (VEGF-C) levels predict recurrence in patients with esophageal cancer. *Anticancer Res* 2008; 28: 165–169.
- Kinoshita J, Kitamura K, Kabashima A, Saeki H, Tanaka S, Sugimachi K. Clinical significance of vascular endothelial growth factor-C (VEGF-C) in breast cancer. *Breast Cancer Res Treat* 2001; 66: 159–164.
- Juchniewicz A, Niklińska W, Kowalczyk O, Laudański W, Sułewska A, Dziegielewska P, et al. Prognostic value of vascular endothelial growth factor-C and podoplanin mRNA expression in esophageal cancer. *Oncol Lett* 2015; 10: 3668–3674.
- Mohammed RA, Green A, El-Shikh S, Paish EC, Ellis IO, Martin SG. Prognostic significance of vascular endothelial cell growth factors-A, -C and -D in breast cancer and their relationship with angio- and lymphangiogenesis. *Br J Cancer* 2007; 96: 1092–1100.
- Kigure W, Fujii T, Sutoh T, Morita H, Katoh T, Yajima RN, et al. The association of VEGF-C expression with tumor lymphatic

- vessel density and lymph node metastasis in patients with gastric cancer and gastrointestinal stromal tumor. *Hepato-gastroenterology* 2013; 60: 277–280.
18. Zhao YC, Ni XJ, Wang MH, Zha XM, Zhao Y, Wang S. Tumor-derived VEGF-C, but not VEGF-D, promotes sentinel lymph node lymphangiogenesis prior to metastasis in breast cancer patients. *Med Oncol* 2012; 29: 2594–2600.
 19. Tanaka T, Ishiguro H, Kuwabara Y, Kimura M, Mitsui A, Katada T, et al. Vascular endothelial growth factor C (VEGF-C) in esophageal cancer correlates with lymph node metastasis and poor patient prognosis. *J Exp Clin Cancer Res* 2010; 29: 83.
 20. Jennbacken K, Vallbo C, Wang W, Damber JE. Expression of vascular endothelial growth factor C (VEGF-C) and VEGF receptor-3 in human prostate cancer is associated with regional lymph node metastasis. *Prostate* 2005; 65: 110–116.
 21. Soumaoro LT, Uetake H, Takagi Y, Iida S, Higuchi T, Yasuno M, et al. Coexpression of VEGF-C and Cox-2 in human colorectal cancer and its association with lymph node metastasis. *Dis Colon Rectum* 2006; 49: 392–398.
 22. Kadowaki I, Ichinohasama R, Harigae H, Ishizawa K, Okitsu Y, Kameoka J, et al. Accelerated lymphangiogenesis in malignant lymphoma: possible role of VEGF-A and VEGF-C. *Br J Haematol* 2005; 130: 869–877.
 23. Ma SP, Lin M, Liu HN, Yu JX. Lymphangiogenesis in non-Hodgkin's lymphoma and its correlation with cyclooxygenase-2 and vascular endothelial growth factor-C. *Oncol Lett* 2012; 4: 695–700.
 24. Jankowska-Konsur A, Kobierzycki C, Reich A, Grzegorzolka J, Bieniek A, Dziegiel P. Expression of SATB1, MTI/II and Ki-67 in mycosis fungoides. *Anticancer Res* 2016; 36: 189–197.
 25. Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologie* 1987; 8: 138–140.
 26. Výboňová D, Mellová Y, Adamicová K, Adamkov M, Hešková G. Quantitative comparison of angiogenesis and lymphangiogenesis in cutaneous lichen planus and psoriasis: immunohistochemical assessment. *Acta Histochem* 2015; 117: 20–28.
 27. Shi VY, Bao L, Chan LS. Inflammation-driven dermal lymphangiogenesis in atopic dermatitis is associated with CD11b+ macrophage recruitment and VEGF-C up-regulation in the IL-4-transgenic mouse model. *Microcirculation* 2012; 19: 567–579.
 28. Ambrosio MR, Rocca BJ, Barone A, Ginori A, Crivelli F, Pirtoli L, et al. Lymphatic vascularization in prostate adenocarcinoma: correlation with tumor grade, androgen withdrawal and prognosis. *Anticancer Res* 2015; 35: 5595–5600.
 29. Pak KH, Jo A, Choi HJ, Choi Y, Kim H, Cheong JH. The different role of intratumoral and peritumoral lymphangiogenesis in gastric cancer progression and prognosis. *BMC Cancer* 2015; 15: 498.
 30. Ma W, Wang K, Yang S, Wang J, Tan B, Bai B, et al. Clinicopathology significance of podoplanin immunoreactivity in esophageal squamous cell carcinoma. *Int J Clin Exp Pathol* 2014; 7: 2361–2371.
 31. El-Gendi S, Abdel-Hadi M. Lymphatic vessel density as prognostic factor in breast carcinoma: relation to clinicopathologic parameters. *J Egypt Natl Canc Inst* 2009; 21: 139–149.
 32. Sun JG, Wang Y, Chen ZT, Zhuo WL, Zhu B, Liao RX, et al. Detection of lymphangiogenesis in non-small cell lung cancer and its prognostic value. *J Exp Clin Cancer Res* 2009; 28: 21.
 33. Karpova MB, Fujii K, Jenni D, Dummer R, Urosevic-Maiwald M. Evaluation of lymphangiogenic markers in Sézary syndrome. *Leuk Lymphoma* 2011; 52: 491–501.
 34. Pileri A, Agostinelli C, Righi S, Fuligni F, Bacchi F, Sabattini E, et al. Vascular endothelial growth factor A (VEGFA) expression in mycosis fungoides. *Histopathology* 2015; 66: 173–181.
 35. Pedersen IH, Willerslev-Olsen A, Vetter-Kauczok C, Krejsgaard T, Lauenborg B, Kopp KL, et al. Vascular endothelial growth factor receptor-3 expression in mycosis fungoides. *Leuk Lymphoma* 2013; 54: 819–826.
 36. Zhou M, He L, Zu X, Zhang H, Zeng H, Qi L. Lymphatic vessel density as a predictor of lymph node metastasis and its relationship with prognosis in urothelial carcinoma of the bladder. *BJU Int* 2011; 107: 1930–1935.
 37. Lin Y, Liu F, Fan Y, Qian X, Lang R, Gu F, et al. Both high expression of pyruvate kinase M2 and vascular endothelial growth factor-C predicts poorer prognosis in human breast cancer. *Int J Clin Exp Pathol* 2015; 8: 8028–8037.
 38. Jiang H, Shao W, Zhao W. VEGF-C in non-small cell lung cancer: meta-analysis. *Clin Chim Acta* 2014; 427: 94–99.
 39. Sundov Z, Tomic S, Alfirevic S, Sundov A, Capkun V, Nincevic Z, et al. Prognostic value of MVD, LVD and vascular invasion in lymph node-negative colon cancer. *Hepatogastroenterology* 2013; 60: 432–438.
 40. Pazgal I, Boycov O, Shpilberg O, Okon E, Bairey O. Expression of VEGF-C, VEGF-D and their receptor VEGFR-3 in diffuse large B-cell lymphomas. *Leuk Lymphoma* 2007; 48: 2213–2220.
 41. Rueda A, Olmos D, Vicioso L, Quero C, Gallego E, Pajares-Hachero BI, et al. Role of vascular endothelial growth factor C in classical Hodgkin lymphoma. *Leuk Lymphoma* 2015; 56: 1286–1294.