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Lymphangioinvasion in routine H&E staining is strongly associated with poor clinical outcome in lymph node-negative cutaneous melanoma patients

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Abstract

Introduction. Lymphatic invasion (LYI) and lymphangiogenesis in the primary tumor are important processes related to the dissemination of neoplasms. The aim of the study was to examine the relationship of LYI status in cutaneous melanoma with patient survival and clinicopathological data.

Material and methods. LYI status was assessed in 104 hematoxylin-eosin (H&E) stained melanoma primary tumor samples and analyzed in relation to patient survival and other clinicopathological and histopathological characteristics.

Results. LYI was found in 30 (28.8%) patients. It was observed more frequently in ulcerated, proliferating, and thicker tumors. It correlated with the presence of sentinel lymph node, regional and distant metastases. The presence of LYI significantly correlated with shorter overall survival, cancer specific overall survival and disease-free survival in Kaplan-Meier analysis (all $P < 0.001$). Positive LYI status was a factor of unfavorable prognosis also in patients without regional lymph node metastases.

Conclusions. Our results support earlier observations that LYI is a powerful prognostic factor. We recommend an assessment of LYI status during routine review of cutaneous melanoma slides stained with H&E as a standard, potentially informative, yet economically beneficial procedure. *(Folia Histochemica et Cytobiologica 2016, Vol. 54, No. 3, 126–133)*

Key words: lymphatic invasion; prognosis; disease-free survival; malignant melanoma

Introduction

Lymphatic dissemination is a critical step in the natural history of melanoma. The presence of nodal metastases is one of the most crucial determinants of patient's prognosis that greatly influences the therapeutic approach [1]. Although the patients with no evidence of nodal involvement generally have a better prognosis than those in metastatic stage of the disease, the N0 group remains very heterogeneous with respect to survival [2]. It motivates researchers to continue to seek novel prognostic factors which would further stratify these patients and help delineate a group that could benefit from more aggressive treatment and intensified follow-up.

One of the parameters potentially useful for risk stratification is lymphatic invasion (LYI). Its pro-

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gnostic significance was postulated, although not always unanimously, in melanoma [3–7] and multiple other cancer types, *e.g.* lung [8], breast [9, 10], cervical [11, 12], ovarian [13], gastric [14], colorectal [15, 16], prostate [17] or bladder [18] cancers. Invasion of lymphatic vasculature within the primary tumor occurs ahead of nodal involvement in the course of cancer dissemination [19]. Therefore, morphological determination of LYI offers the possibility to spot aggressive, but relatively early-stage melanomas in which no nodal foci of cancer are yet apparent, but invasive melanocytes had already entered lymphatic vessels — a situation placing these tumors somewhat on the line between localized and metastatic phase. Interestingly, several studies demonstrated the relationship between angiolymphatic invasion and positive sentinel lymph node biopsy (SLNB) status, an observation that may be helpful in the selection of patients for this procedure [6, 7, 20, 21].

Despite presumable prognostic significance of LYI status and a recommendation for its assessment in cutaneous melanoma by the American College of Pathologists [22], it is frequently omitted in the final melanoma pathology report. Unfortunately, comprehensive and large-scale studies on LYI are still insufficient, therefore the actual value of this potentially powerful parameter in American Joint Committee on Cancer (AJCC) melanoma staging remains marginal [2].

The aim of the present study was to examine the relationship of LYI status in hematoxylin and eosin (H&E) stained melanoma primary tumor samples with patient survival and other clinicopathological and histopathological characteristics. Special attention was placed on the prognostic significance of LYI in lymph node-negative patients.

Material and methods

Patients. The study group consisted of 104 patients with cutaneous malignant melanoma diagnosed between 2005 and 2010 and treated in the Lower Silesian Oncology Center in Wroclaw, Poland. The group was selected on the basis of the availability of tissue material (paraffin blocks and H&E-stained slides) and medical documentation. Comprehensive clinical data were obtained from archival medical records. The diagnostic and therapeutic procedures were determined on the basis of medical records of the Oncology Outpatient Clinic of the Lower Silesian Oncology Center and data provided by the Lower Silesian Cancer Registry and Civil Register Office (Wroclaw, Poland). The study was approved by the Ethical Committee of the Wroclaw Medical University, Poland.

The clinicopathological profile of patients included the following parameters: age and gender, primary tumor location, tumor stratification according to AJCC (pT), presence or absence of nodal (pN) and distant (pM) metastases, information on disease recurrence and SLNB procedures (Table 1).

Tumor samples and histopathological evaluation. Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin. All H&E stained sections were examined by two pathologists. The parameters of the primary tumor recorded in pathology reports were Breslow thickness, Clark level, growth phase, histologic type, mitotic rate (number of mitotic figures per 1 mm^2), presence of ulceration, lymphangioinvasion, microsatellitosis, intensity of lymphocytic inflammatory infiltrate (TILs, tumor-infiltrating lymphocytes) and microscopic evidence of regression (Table 2).

Statistical analysis. Statistical analysis was performed using the Statistica 10.0 (Statsoft, Tulsa, OK, USA) and IBM SPSS 21 (IBM, Armonk, NY, USA) software packages. Overall survival (OS) was defined as the time between the primary surgical treatment and death, and OS was censored at the last follow-up of patients who were still alive. Disease-free survival (DFS) was defined as the time between the primary surgical treatment and the date of relapse. DFS was censored at the last follow-up of patients who survived without disease recurrence or at the date of non-cancer associated death. Cancer-specific overall survival (CSOS) was defined as the time between the primary surgical treatment and cancer-associated death, and was censored at the last follow-up of surviving patients.

A χ^2 test, exact Fisher test in the case of 2×2 tables, and Spearman rank correlation were used to analyze associations between mitotic rate and the presence of ulceration and clinicopathological parameters. Differences between the means were tested with a nonparametric test (Mann-Whitney U test and Kruskal-Wallis test). The log-rank test was used to compare survival in two groups, and the overall survival rate was estimated by the Kaplan-Meier method. The influence of explanatory variables on death risk was analyzed by means of the Cox proportional hazard regression. P values < 0.05 were considered statistically significant.

Results

The frequency of lymphatic invasion in 104 melanoma patients

The presence of LYI was observed in 30 (28.8%) patients (Figure 1). With regard to regional lymph node status, LYI was detected in 15 out of 86 pN-negative patients, whereas 15 out of 18 patients in the pN-positive group had observable LYI (Table 1).

Correlations between lymphatic invasion and clinicopathological parameters

The presence of lymphatic invasion significantly correlated with higher regional advancement of the primary tumor (pT; $P < 0.001$). Moreover, it was

Table 1. Clinicopathological characteristics of the patients with or without lymphangioinvasion (LYI)

Clinicopathological characteristics	No *(%)*	Absence of LYI	Presence of LYI	*P* value
All patients	104 (*100.0*)	74 (*71.2*)	30 (*28.8*)	
Age in years (range 21–79; median 58.5 y)	56.5 ± 15.4[a]			0.411[b]
Gender[c]				
Female	60 (*57.7*)	43	17	0.532
Male	44 (*42.3*)	31	13	
Primary tumor location[d]				
Head/neck	15 (*14.4*)	10	5	
Extremities	45 (*43.3*)	34	11	0.777
Hand/foot	2 (*1.9*)	1	1	
Trunk	42 (*40.4*)	29	13	
Primary tumor (pT)[b]				
pT1	34 (*32.7*)	33	1	
pT2	21 (*20.2*)	18	3	**< 0.0001**
pT3	26 (*25.0*)	12	14	
pT4	23 (*22.1*)	11	12	
Sentinel lymph node biopsy status (SNLB)[c]	60 (*57.7*)			
No metastases (SNLB–)	48 (*80.0*)	44	4	**< 0.0001**
Metastases present (SNLB+)	12 (*20.0*)	3	9	
Regional lymph nodes status (pN)[c]				
No metastases (pN–)	86 (*82.7*)	71	15	**< 0.0001**
Metastases present (pN+)	18 (*17.3*)	3	15	
Recurrence[c]				
No	87 (*83.7*)	69	18	**< 0.0001**
Yes	17 (*16.3*)	5	12	
Distant metastases[c]				
No	99 (*95.2*)	74	25	**0.002**
Yes	5 (*4.8*)	0	5	

[a]Mean ± SD; [b]*P* value of Mann-Whitney's U test; [c]*P* value of Fisher's exact test; [d]*P* value of χ^2 test. Statistically significant differences are shown in bold font ($P < 0.05$).

observed more often in patients with positive SLNB status ($P < 0.001$). Furthermore, the presence of LYI was associated with the presence of regional lymph node (pN; $P < 0.001$) and distant metastases (pM; $P = 0.002$). Importantly, the disease recurred more frequently in patients with detected LYI ($P < 0.001$). No statistically significant relationships between LYI and age at the moment of diagnosis, gender or primary tumor location were observed (Table 1).

Correlations between lymphatic invasion and histopathological parameters

Higher tumor advancement according to Breslow thickness and Clark level was associated with the presence of LYI ($P < 0.001$ in both cases). Moreover, LYI-positive tumors were more frequently ulcerated ($P < 0.001$) and presented higher levels of mitotic rate ($P < 0.001$). Interestingly, LYI was observed more often in melanomas of nodular and acral-lentiginous histologic types ($P = 0.037$). No significant correlations were observed between LYI and other histopathological characteristics: TIL grade, growth phase, microsatellitosis and tumor regression (Table 2).

Impact of lymphatic invasion status on 5-year survival of melanoma patients

Kaplan-Meier analysis revealed that the presence of lymphatic invasion significantly correlated with

Table 2. Correlations between lymphangioinvasion and histopathological characteristics of primary tumors

Histopathological parameters	No *(%)*	Lack of LYI	Presence of LYI	*P* value
Breslow thickness[a]				
≤ 1 mm	34 (*32.7*)	33	1	
1.01–2.00 mm	20 (*19.2*)	17	3	**< 0.001**
2.01–4.00 mm	27 (*26.0*)	13	14	
> 4 mm	23 (*22.1*)	11	12	
Clark thickness[a]				
I	0 (*0.0*)			
II	18 (*17.3*)	18	0	**< 0.001**
III	49 (*47.1*)	37	12	
IV	26 (*25.0*)	16	10	
V	11 (*10.6*)	3	8	
Histologic type[b]				
Superficial spreading melanoma (SSM)	68 (*65.4*)	54	14	**0.037**
Nodular malignant melanoma (NMM)	32 (*30.8*)	18	14	
Acral-lentiginous melanoma (ALM)	4 (*3.8*)	2	2	
Mitotic rate[a]				
0	45 (*43.3*)	43	2	**< 0.001**
1–2	26 (*25.0*)	20	6	
≥ 3	33 (*31.7*)	11	22	
Ulceration[c]				
No	55 (*52.9*)	49	6	**< 0.001**
Yes	49 (*47.1*)	25	24	
TILs[c]				
No and non-brisk	52 (*50.0*)	33	19	0.065
Brisk	52 (*50.0*)	41	11	
Growth phase[c]				
Radial	3 (*2.9*)	3	0	0.356
Vertical	101 (*97.1*)	71	30	
Microsatellitosis[c]				
No	98 (*94.2*)	72	26	0.056
Yes	6 (*5.8*)	2	4	
Tumor regression[c]				
No	96 (*92.3*)	68	28	0.581
Yes	8 (*7.7*)	6	2	

[a]*P* value of Mann-Whitney's U test; [b]*P* value of χ^2 test; [c]*P* value of Fisher's exact test. Statistically significant results ($P < 0.05$) are shown in bold font.

shorter overall survival (OS, Figure 2A, $P < 0.001$), cancer-specific overall survival (CSOS, Figure 2B, $P < 0.001$) and disease-free survival (DFS, Figure 2C, $P < 0.001$). Another survival analysis was performed in a subgroup of patients without regional lymph-node metastases. Positive LYI status was found to be a factor of unfavorable prognosis defined as shorter OS ($P = 0.001$, Figure 2D), CSOS ($P < 0.001$, Figure 2E) and DFS ($P < 0.001$, Figure 2F).

Discussion

Lymphatic spread is a crucial mechanism of melanoma dissemination, and LYI along with lymphangiogenesis

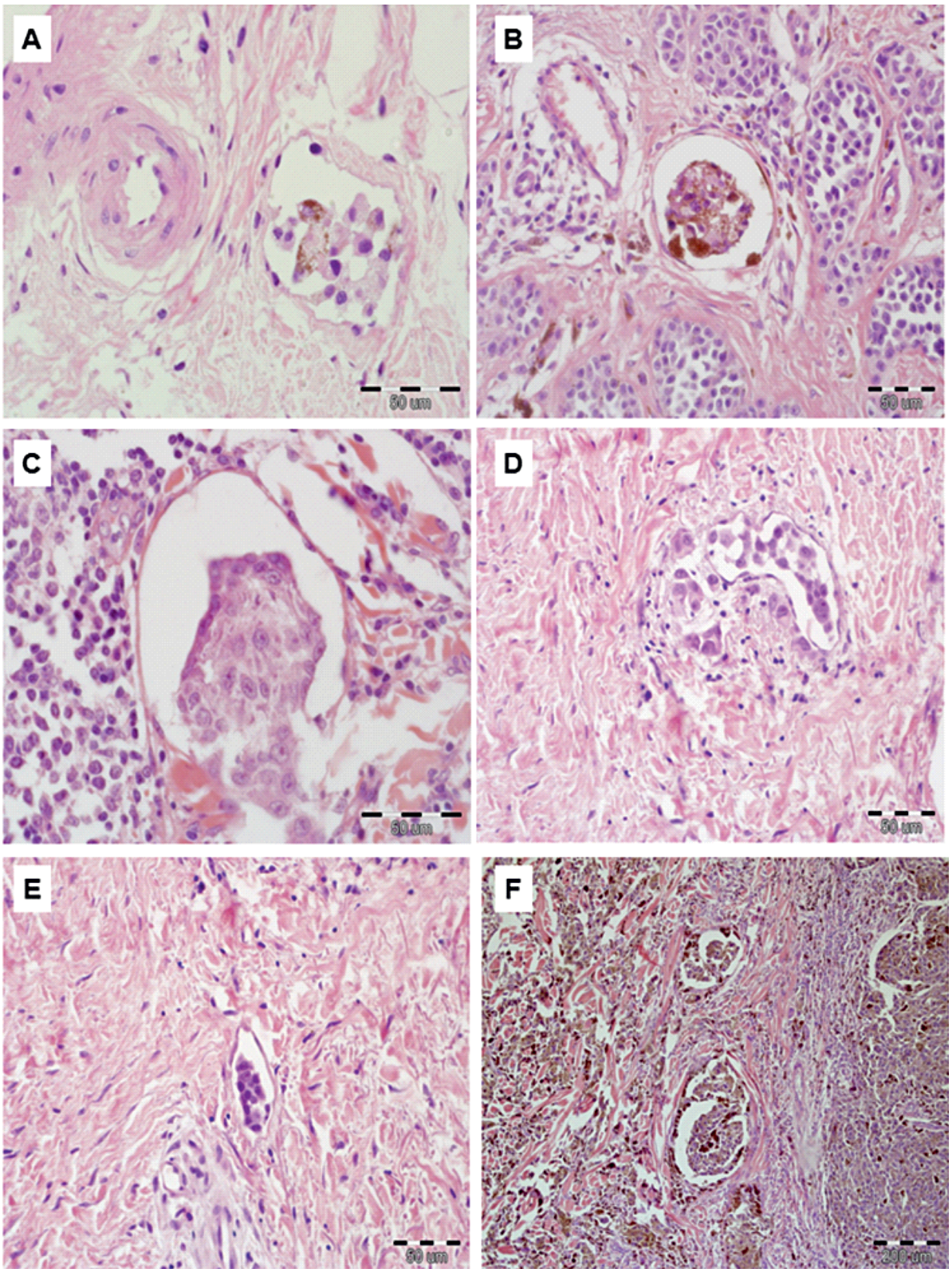

Figure 1. Lymphatic invasion in hematoxylin-eosin stained sections of melanoma patients. **A.** Tumor thrombus in a thin--walled lymphatic vessel (×600); **B.** Nest of melanoma cells in the lymphatic vessel with abundant melanin in the cancer cell cytoplasm (×400); **C.** Massive invasion in the lymphatic vessel with vast destruction of the wall and breakdown of lymphatic endothelium (×600); **D and E.** Tumor thrombi within deep lymphatic vessels in the dermis (×400); **F.** Advanced cutaneous melanoma (Breslow thickness 4.2 mm, Clark V) with distinct lymphangioinvasion. Abundant melanin in the tumor infiltration and melanoma cells located in the thin-walled lymphatic vessels (×200).

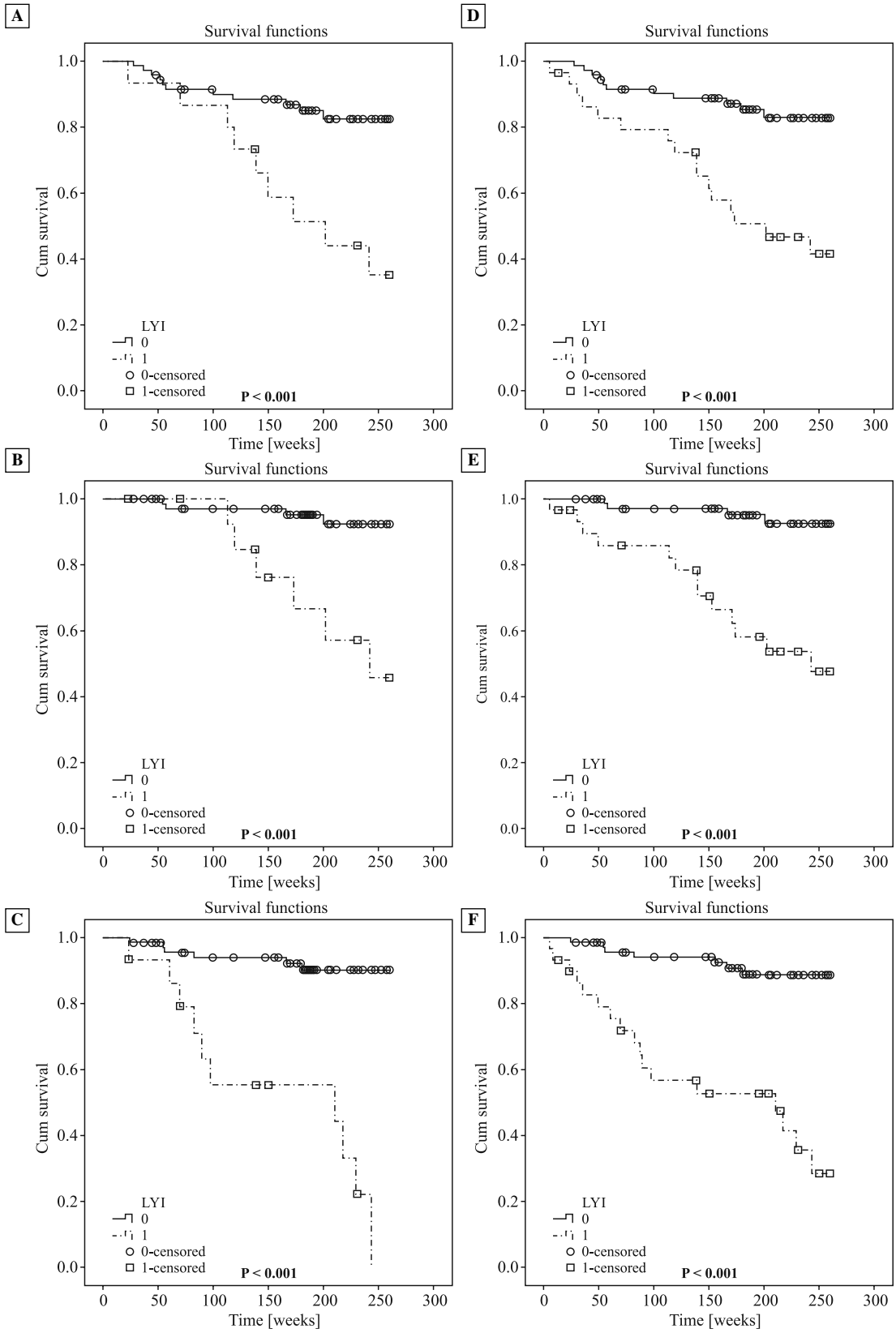

Figure 2. Prognostic significance of lymphatic invasion (LYI) status for 5-year survival of melanoma patients. Presence of LYI was strongly related to unfavorable prognosis defined as shorter overall survival (**A**), cancer-specific overall survival (**B**) and disease free survival (**C**) in the entire study group. Moreover, also in lymph node-negative patients positive LYI status was a factor indicating poor outcome; **D —** overall survival; **E** — cancer-specific overall survival; **F** — disease free survival (DFS).

in the primary tumor are important phenomena in the course of events leading to metastasis [23, 24]. In our study, we demonstrated several correlations between LYI status and other histopathological and clinicopathological features in cutaneous melanoma patients. LYI was observed more frequently in tumors with deeper infiltration according to Breslow and Clark, and in tumors with ulceration and high MR. Moreover, LYI correlated with the presence of SLN, regional and distant metastases as well as the disease recurrence.

Although definitive collective conclusions have yet not been drawn and considerable differences exist between various studies, a number of groups presented statistically significant associations between LYI status and key melanoma characteristics such as tumor thickness [3, 25, 26], ulceration [3, 25, 26], MR [25, 26], or SLN [6, 7, 20, 27]. The observed correlations give good reasons for further exploring LYI as a parameter of potential practical application. Furthermore, our results indicate that positive LYI status is related to poor outcome defined as shorter CSOS, DFS, as well as OS. Prognostic significance of LYI in the context of patient survival was demonstrated by several laboratories [3, 6, 28], while other groups did not confirm this observation [7, 26, 29].

Importantly, positive LYI status was strongly correlated with shorter survival also in N0 subgroup of patients. This association was evident with regard to all considered endpoints: CSOS, DFS and OS. It is likely that the presence of neoplastic cells within lymphatic vasculature of the primary tumor is a manifestation of their aggressive phenotype and ability to metastasize. However, it has to be considered that some patients develop metastasis without evidence of LYI in the primary tumor, and conversely — LYI may be present with no indication of concurrent metastatic spread. Considering this inconsistency, in lymph node-negative patients, LYI-positivity may shortly precede the metastatic spread, or even be associated with already existent submicroscopic micrometastases, detectable solely by reverse transcriptase-polymerase chain reaction assay [30, 31]. What might result from this pitfall are more aggressive therapies which should be performed in LYI(+) patients. In our study, the recurrence rate within LYI-positive N0 patients was extraordinarily high (11 of 15; 73.3%; data not shown). The study of Borgstein *et al.* revealed that evidence of LYI in the primary tumor significantly correlated with locoregional cutaneous recurrence [32]. Among clinical stage I patients, 93% of LYI-positive subjects developed in-transit metastases compared with 1.6% in LYI-negative group [32]. Vuylsteke *et al.* demonstrated in a group of 209 clinical stage I/II patients after SLN dissection, that LYI was a strong predictor of DFS and OS both in univariate and multivariate analysis [33]. Interestingly, LYI was the strongest predictor in a multivariable model of DFS analysis, followed by ulceration, SLN status and Breslow thickness [33]. In the case of OS, LYI was superior to SLN status and Breslow thickness, whereas ulceration was found non-significant [33].

Many of the above cited studies used immunohistochemical markers for blood and/or lymphatic vasculature like D2-40, LYVE-1, CD31 or CD34. With the advent of modern biotechnology, such methods have become increasingly popular for scientific and diagnostic purposes. Nevertheless, classical H&E staining remains the standard diagnostic technique available for every pathologist. An important outcome of our results is that LYI assessment may be beneficial even with this simple tool.

In conclusion, we demonstrated that positive LYI status was related to poor outcome defined as shorter CSOS, DFS and OS in the whole study population and in lymph node-negative patients. We recommend an assessment of LYI status to be done during routine review of H&E slides of cutaneous melanoma as a standard, potentially informative, yet economically beneficial procedure. Comprehensive and large-scale studies should follow this preliminary report to establish LYI status as a stratification criterion in future editions of AJCC staging system.

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Conflict of interest

The authors declare that there is no conflict of interest.

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