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Tumor Lymphangiogenesis

A Novel Prognostic Indicator for Cutaneous Melanoma Metastasis and Survival

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Malignant melanomas of the skin are distinguished by their propensity for early metastatic spread via lymphatic vessels to regional lymph nodes, and lymph node metastasis is a major determinant for the staging and clinical management of melanoma. However, the importance of tumor-induced lymphangiogenesis for lymphatic melanoma spread has remained unclear. We investigated whether tumor lymphangiogenesis occurs in human malignant melanomas of the skin and whether the extent of tumor lymphangiogenesis may be related to the risk for lymph node metastasis and to patient survival, using double immunostains for the novel lymphatic endothelial marker LYVE-1 and for the panvascular marker CD31. Tumor samples were obtained from clinically and histologically closely matched cases of primary melanomas with early lymph node metastasis ($n = 18$) and from non-metastatic melanomas ($n = 19$). Hot spots of proliferating intratumoral and peritumoral lymphatic vessels were detected in a large number of melanomas. The incidence of intratumoral lymphatics was significantly higher in metastatic melanomas and correlated with poor disease-free survival. Metastatic melanomas had significantly more and larger tumor-associated lymphatic vessels, and a relative lymphatic vessel area of >1.5% was significantly associated with poor disease-free and overall survival. In contrast, no differences in the density of tumor-associated blood vessels were found. Vascular endothelial growth factor and vascular endothelial growth factor-C expression was equally detected in a minority of cases in both groups. Our results reveal tumor lymphangiogenesis as a novel prognostic indicator for the risk of lymph node metastasis in cutaneous melanoma. *(Am J Pathol 2003, 162:1951–1960)*

Malignant melanoma of the skin is a common and frequently lethal neoplasm with increasing worldwide incidence.[1] In 1998, 42,000 new cases of cutaneous malignant melanoma and 7300 related deaths were reported in the United States.[1] Complete surgical excision with wide margins represents the therapy of choice for primary melanomas. The vertical thickness[2] and the anatomical level of invasion (Clark level)[3] of the primary tumor are the most valuable prognostic indicators for the metastatic risk of cutaneous melanoma.[4–7] However, it is still difficult to predict the outcome after excision of the primary tumor, in particular in thin melanomas.[8,9] Hence novel indicators for the prognostic risk of metastatic melanoma spread are urgently needed.

Malignant melanomas metastasize via blood and lymphatic vessels, and the induction of tumor angiogenesis provides a possible explanation for how tumor cells escape their original site by invading the newly formed vascular bed.[10] Tumor vascularization has been observed in human melanoma both experimentally[11,12] and clinically.[13] The importance of tumor angiogenesis for the prognosis of primary malignant melanomas of the skin, however, has remained controversial.[14] Whereas several studies found an inverse correlation of tumor microvessel

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density with disease-free and overall survival,[15,16] other reports did not detect any significant differences of tumor microvessel density between metastasizing and nonmetastasizing melanomas.[17] Therefore, the potential prognostic value of tumor vascularization in malignant melanoma remains at present unclear.

Malignant melanomas of the skin are distinguished by their propensity for early metastatic spread via lymphatic vessels to regional lymph nodes, even at the early stages of tumor invasion. Hence lymph node metastasis, as determined by the analysis of sentinel lymph nodes, is a major determinant for the staging and clinical management of melanoma.[5,18] In contrast to the extensive studies on melanoma-associated angiogenesis, little is known about the mechanisms by which melanoma cells gain entry into the lymphatic system. Dilated tumor-associated lymphatic vessels, sometimes containing tumor cells, have been observed by routine histology and by electron microscopy in cutaneous melanoma.[19,20] However, the importance of tumor-induced lymphangiogenesis for lymphatic melanoma spread has remained unclear, and the very existence of melanoma-associated lymphangiogenesis has even been questioned.[21] Recent experimental evidence strongly suggests that tumors can actively induce lymphangiogenesis via production of lymphangiogenic factors such as vascular endothelial growth factor (VEGF)-C and VEGF-D, and that the extent of tumor lymphangiogenesis is directly correlated with the extent of experimental metastatic tumor spread to regional lymph nodes.[22,23] We have previously reported that VEGF-C is expressed in several human melanoma cell lines *in vitro*;[24] however, because of the lack of specific markers for the lymphatic endothelium in human cancers, the importance of tumor-associated lymphangiogenesis for melanoma progression has remained unclear. The recent discovery of the lymphatic endothelial hyaluronan receptor-1 (LYVE-1) as a specific marker for normal[25] and tumor-associated lymphatic vessels[26] has now provided the tool for a detailed analysis of tumor lymphangiogenesis in melanoma.

In the present study, we investigated whether tumor lymphangiogenesis occurs in human malignant melanomas of the skin and whether the extent of tumor lymphangiogenesis may be related to the risk for lymph node metastasis and to patient survival, using tumor samples obtained from clinically and histologically matched primary cutaneous malignant melanomas from patients who developed early lymph node metastasis and from patients who remained metastasis-free. Our results reveal, for the first time, a higher incidence of intra- and peritumoral lymphangiogenesis in metastatic melanoma, as compared with nonmetastatic melanomas. Moreover, multivariate proportional hazards analysis identified peritumoral lymphatic vascular density as a novel prognostic indicator for the risk of lymph node metastasis in cutaneous melanoma.

Materials and Methods

Patient Population and Histological Analyses

Patients were identified retrospectively through review of survival data from the German Melanoma Registry. Of a total of 1050 patients with primary cutaneous malignant melanoma, 19 patients with nonmetastatic primary melanoma (mean disease-free follow-up of 6.5 years; range, 34 to 119 months) were closely matched with a group of 18 patients who had documented early (<1 year) lymph node metastasis (Table 1). The two groups were matched for gender, age at diagnosis, tumor thickness, Clark's level of invasion, histological type, and presence of ulceration. Exclusion criteria were acrolentiginous melanoma and adjuvant therapy after the surgical excision of the primary tumor. The diagnosis of melanoma, the tumor thickness, and the level of tumor invasion were reconfirmed by two pathologists (SSD and MCM). Additional parameters such as the frequency of mitoses, tumor regression, vascular invasion, and microsatellites were also evaluated. Peritumoral inflammation was evaluated as

Table 1. Clinical and Pathological Characteristics of Patients with Melanoma

Category	Nonmetastatic	Metastatic
Number of patients	19	18
Male	11	10
Female	8	8
Time of lymph node metastasis (months)		
Mean	—	24.4
Range	—	5–69
Time of visceral organ metastasis (months)		
Mean	—	38.0
Range	—	14–76
Age at diagnosis (years)		
Mean	53.8	54.9
Range	21–79	23–76
Histologic type		
Superficial spreading	14	14
Nodular	4	4
Lentigo maligna	1	0
Breslow thickness (mm)		
<1.5	5	2
≥1.5	14	16
Mean	2.5	2.6
Clark level of invasion		
II	2	1
III	4	6
IV	12	10
V	1	2
Mean	3.63	3.47
Ulceration		
Present	4	4
Absent	15	14
Site		
Upper Extremity	5	2
Lower Extremity	7	4
Head and neck	3	3
Trunk	4	9
Mitoses (mm^2)		
>6.0	4	4
1–6	11	13
0	3	0
Regression		
Present	4	2
Absent	15	15
Peritumoral inflammation		
Absent	6	7
Present	9	9
Brisk	4	2

absent, present (nonbrisk), and brisk.[27] The degree of inflammation was further graded as low (≤50% of tumor area) or high (>50% of tumor area).

Immunostains and in Situ *Hybridization*

Paraffin sections (6 μm thickness) were dewaxed, hydrated, and treated with 0.01% protease XXIV (Sigma, St. Louis, MO) in phosphate-buffered saline for 20 minutes at 37°C. Sections were double-stained using a rabbit polyclonal antibody against human LYVE-1 (1:600)[25] and a mouse monoclonal anti-human CD31 antibody (1:40; DAKO, Carpinteria, CA), followed by incubation with the respective secondary antibodies that were labeled with either Texas Red (1:50) or with fluorescein isothiocyanate (1:50) (Jackson ImmunoResearch, West Grove, PA) as previously described.[24] Cell nuclei were counterstained with Hoechst bisbenzimide (Sigma) at 20 μg/ml. Additional immunohistochemical stains were performed using affinity-purified rabbit polyclonal antibodies against human VEGF-C (C-terminus; Zymed, San Francisco, CA) or against human LYVE-1, followed by incubation with conjugated rabbit anti-human immunoglobulin (1:200), using the 3-amino-9-ethylcabazole peroxidase substrate kit (Vector Laboratories, Burlingame, CA). To detect proliferating cells, the Zymed proliferating cell nuclear antigen (PCNA) 3,3′-diaminobenzidine staining kit was used as previously described.[28] For specificity controls, either the secondary antibody was omitted or the primary anti-VEGF-C antibody was preincubated with a 40-fold molar excess of recombinant human VEGF-C (a generous gift from Dr. K. Alitalo, University of Helsinki, Finland). *In situ* hybridization was performed as previously described,[29] using a riboprobe for human VEGF that detects all known VEGF splice variants[29] or a 808-bp human VEGF-C riboprobe described previously.[30] Transcription reactions were performed using the Riboprobe Gemini II kit (Promega, Madison, WI) in the presence of [α-^{35}S]UTP. For autoradiography, slides were coated with NTB2 film emulsion and exposed for 4 weeks. Sections were examined using a Nikon E-600 microscope (Nikon, Melville, NY) and digital images were captured using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Computer-Assisted Morphometric Vessel Analysis

To analyze the lymphatic and blood vessel density and size within and surrounding the 37 primary melanomas, we performed double-immunofluorescence stains for CD31 and LYVE-1. Sections were examined using a Nikon E-600 microscope and digital images were captured using a SPOT digital camera. For each tumor section, three fields with the highest lymphatic vascular density (hot spots) were evaluated at ×100 magnification. Digital images of tumor-associated lymphatic vessels and blood vessels were captured in the same field. Peritumoral lymphatic vessels were defined as LYVE-1-positive vessels within an area of 100 μm from the tumor border. Intratumoral lymphatic vessels were defined as LYVE-1-positive vessels located within the tumor mass and not confined by invagination of normal tissue. Tumor borders were determined on serial sections using Hoechst nuclear stains and hematoxylin and eosin stains. Morphometric analyses of lymphatic vessels and of blood vessels were performed using the IP-Lab software (Scanalytics, Fairfax, VA) to determine the vessel number per mm^2, the average vessel size, and the relative tumor area occupied by vessels as described.[31] The relative lymphatic vascular area was determined in the peritumoral area and was calculated as the area covered by lymphatic vessels divided by the total area examined times 100, expressed in percent.

Statistical Analyses

The unpaired Student's *t*-test was used to determine the statistical significance (*P* value) of the mean for all vascular parameters. The chi-square square test was used to evaluate differences in the frequency of intratumoral lymphatic vessels. Disease-free survival and overall survival intervals were determined as the time period from initial diagnosis to the time of first metastasis or the time of death. Patients with no events (ie, no lymph node metastasis) were censored and the disease-free interval for these patients was the same as their overall follow-up time. Disease-free survival and overall survival analyses were performed using the Kaplan-Meier method. The comparison between survival functions for different strata was assessed with the log-rank statistic. Univariate and multivariate analyses of prognostic factors were based on the Cox proportional hazards model with model selection based on the backwards elimination process.

Results

Detection of Intratumoral and Peritumoral Lymphatic Vessels in Malignant Melanomas

We closely matched two cohorts of patients with metastatic (n = 18) or with nonmetastatic (n = 19) primary cutaneous malignant melanoma for age, gender, tumor type, thickness, invasion level, and presence of ulceration (Table 1). Histological analysis of hematoxylin and eosin-stained paraffin sections revealed that additional prognostic parameters such as mitotic activity, peritumoral inflammation, or regression also showed comparable distribution in both groups (Table 1). Next, melanoma-associated lymphatic and blood vessels were simultaneously visualized in all primary melanomas, using immunofluorescence double stains for the lymphatic vessel marker LYVE-1 and for the panvascular marker CD31. Whereas CD31-positive/LYVE-1-negative blood vessels were homogeneously distributed throughout the tumors, CD31-positive/LYVE-1-positive lymphatic vessels were found in prominent hotspots both within and around primary cutaneous melanomas (Figure 1; A to F). Such hotspots were seen in thick melanomas (Figure 1; A to C) as well as in thin melanomas (Figure 1; D to F). Foci of intratumoral lymphatic vessels were preferentially local-

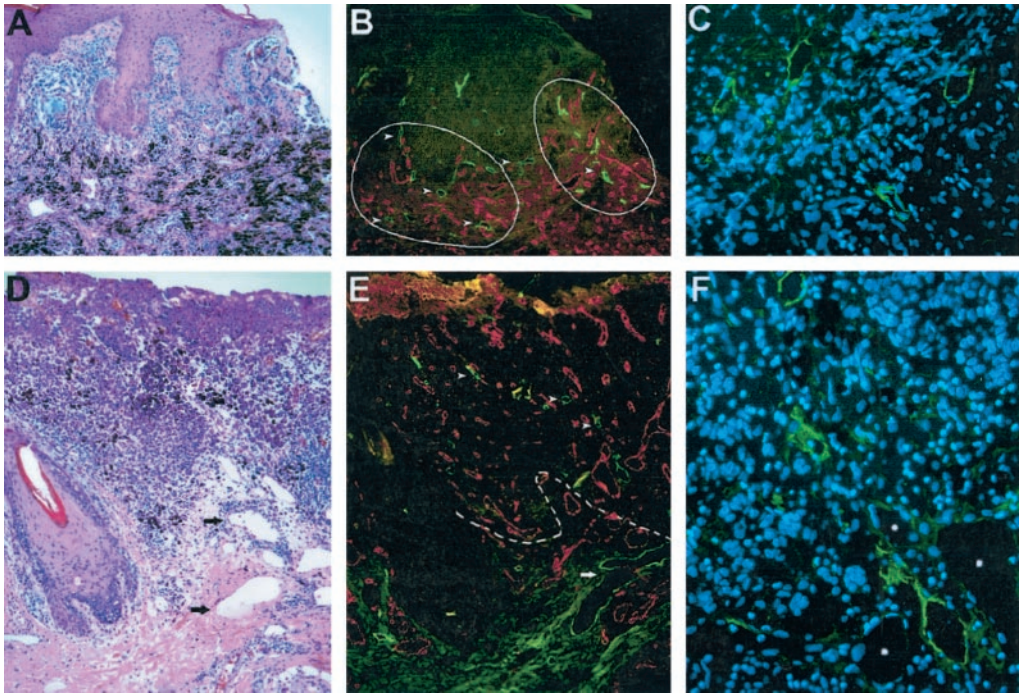

Figure 1. Detection of intratumoral and peritumoral lymphatic vessels in cutaneous malignant melanomas. **A:** Histology of a thick melanoma (4.5 mm) shows compact masses of frequently pigmented tumor cells. **B:** Immunofluorescent stain of a serial section of the same tumor for the lymphatic marker LYVE-1 (green) and the panvascular marker CD31 (red) reveals prominent hotspots of high lymphatic vessel density (hotspots are **circled** by **solid line**). In contrast, blood vessels are homogeneously distributed throughout the tumor. **C:** Thin-walled, LYVE-1-positive intratumoral lymphatic vessels with open lumina. **D:** Histology of a thin melanoma (≤1.5 mm) with dilated peritumoral lymphatics (**arrows**). **E:** Immunofluorescent stain of a serial section for LYVE-1 (green) and CD31 (red) reveals lymphatic vessels within (**arrowheads**) and surrounding (**arrows**) the tumor border (**dotted line**). **F:** LYVE-1-positive intratumoral lymphatic vessels with open lumina. The adjacent blood vessels (**asterisks**) are LYVE-1-negative. Cell nuclei are counterstained (blue) with Hoechst (**C** and **F**). Original magnifications: ×100 (**A**, **B**, **D**, and **E**); ×400 (**C** and **F**).

ized near the tumor border in metastatic melanomas (Figures 1E and 2A). In all cases, peritumoral lymphatic vessels with frequently open lumina were found within a distance of 100 μm from the tumor border (Figures 1E and 2A). Intratumoral lymphatic vessels frequently exhibited a thin-walled, basket-like morphology (Figure 2B) resembling that seen in blood vessel networks during VEGF-induced angiogenesis.[32] Importantly, intratumoral lymphatics were found more frequently in metastatic melanomas (77.8% of all cases) than in nonmetastatic melanomas (36.8%, $P = 0.01$, chi-square test; Figure 2D). Pigmented tumor cells within LYVE-1-positive intratumoral lymphatics were found in 2 of 18 (11%) metastatic melanomas (Figure 2, E and F), whereas no intralymphatic tumor cells were detected in nonmetastatic melanomas. Differential immunostains for LYVE-1 and for PCNA revealed PCNA-positive nuclei in the majority of melanoma cells and in several LYVE-1-positive lymphatic endothelial cells (Figure 2C), confirming the occurrence of active intratumoral lymphangiogenesis in primary human melanomas.

Increased Tumor Lymphangiogenesis in Metastatic Melanomas

Metastatic melanomas exhibited more hotspots of intratumoral and peritumoral LYVE-1-positive lymphatic vessels than nonmetastatic tumors (Figure 1B), whereas the extent of blood vascularization was uniform throughout both metastatic and nonmetastatic tumors. The density of both lymphatic and blood vessels was higher in melanomas than in neighboring normal human skin (data not shown). Computer-assisted morphometric analysis confirmed a comparable blood vascular density, average blood vessel size, and tumor area covered by blood vessels in metastatic and nonmetastatic melanomas (Figure 3; A to C). In contrast, the number of peritumoral lymphatic vessels was significantly increased in metastatic melanomas (16.5 ± 1.62 vessels/mm^2) as compared with nonmetastatic melanomas (9.1 ± 0.76 vessels/mm^2, $P = 0.0002$; Figure 3D). Moreover, the average lymphatic vessel size was significantly larger in metastatic melanomas (1773.0 ± 280.9 μm^2) than in nonmetastatic melanomas (908.6 ± 86.2 μm^2, $P = 0.0048$; Figure 3E). The relative peritumoral area covered by lymphatic vessels was threefold higher in metastatic melanomas (2.25 ± 0.27%) than in nonmetastatic melanomas (0.75 ± 0.07%, $P < 0.0001$; Figure 3F).

VEGF-C Expression in Malignant Melanomas

Because VEGF-C expression has been recently linked to tumor lymphangiogenesis in experimental models, we next analyzed VEGF-C protein expression in all 37 primary melanomas by *in situ* hybridization and by immunohistochemistry. By *in situ* hybridization, we detected focal, low-level tumor cell expression of VEGF-C mRNA (Figure 4; A to C) in 44% of the metastatic melanomas and in 44%

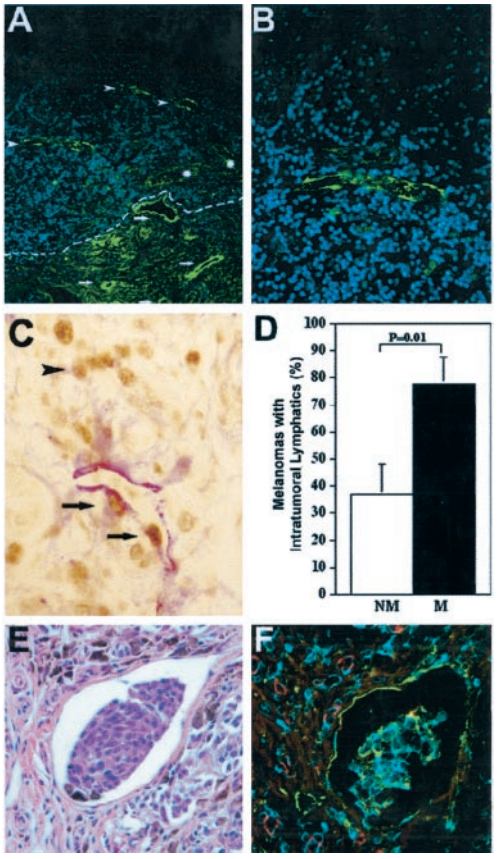

Figure 2. Higher frequency of intratumoral lymphangiogenesis in metastatic melanomas. **A:** Immunofluorescent stain for LYVE-1 (green) depicts thick-walled peritumoral (**arrows**) and thin-walled intratumoral lymphatics (**arrowheads**) in a thin melanoma (1.05 mm). The tumor border is indicated by a **dotted line**. Blood vessels (**asterisks**) are negative for LYVE-1. **B:** Higher magnification of intratumoral lymphatics reveals thin-walled, basket-like morphology. **C:** Double immunostain for LYVE-1 (red) and PCNA (brown) reveals an intratumoral lymphatic vessel with proliferating lymphatic endothelial cells (**arrows**) and adjacent melanoma cells (**arrowhead**). **D:** Significantly increased frequency of detectable intratumoral lymphatic vessels in metastatic melanomas (M; $n = 18$), as compared with nonmetastatic (NM; $n = 19$) tumors (mean ± SEM, chi-square test, $P = 0.01$). **E:** Detection of melanin-containing tumor cells within an intratumoral lymphatic vessel. H&E stain. **F:** Immunofluorescent stain of a serial section for LYVE-1 (green) and CD31 (red) confirms that tumor cells are located within a lymphatic vessel. Cell nuclei are counterstained blue with Hoechst (**B** and **F**). Original magnifications: ×200 (**A**); ×400 (**B**, **C**, **E**, **F**).

of the nonmetastatic melanomas. VEGF-C mRNA expression was frequently also detected in epidermal keratinocytes overlying the tumors and in peritumoral stromal cells (Figure 4D). Moreover, mRNA expression of the angiogenesis factor VEGF was detected in 33.3% of the metastatic and in 30% of the nonmetastatic melanomas (data not shown). Focal cytoplasmic VEGF-C protein expression (Figure 5, A and B) was found more frequently in tumor cells of metastatic melanomas (50.0%) than in nonmetastatic melanomas (31.6%); however, these differences did not reach statistically significant levels ($P > 0.05$). VEGF-C expression was also detected in peritumoral dermal fibroblasts near the invasive edge of metastatic (55.6%) (Figure 5C) and of nonmetastatic melanomas (31.6%, $P = 0.14$).

Correlation of Lymphangiogenesis with Peritumoral Inflammation

We next studied whether the degree of lymphangiogenesis in metastatic melanomas was correlated with the degree of peritumoral inflammation, as assessed by routine histology. We found that the lymphatic vascular area was significantly increased ($P = 0.005$) in melanomas with high-grade nonbrisk inflammation (3.15 ± 0.10%), as compared with melanomas with low-grade inflammation (1.18 ± 0.10%). LYVE-1-positive macrophages near dilated, LYVE-1-positive lymphatic vessels were frequently found in metastatic melanomas with high-grade nonbrisk inflammation (Figure 5E). VEGF-C-expressing inflammatory cells were detected in 3 of 18 (16.7%) metastatic melanomas, but not in nonmetastatic tumors. The peritumoral mononuclear inflammatory cells, mostly located near the invasive tumor edge, expressed VEGF-C in a cytoplasmic granular pattern (Figure 5F).

Lymphangiogenesis Is a Novel Prognostic Parameter for Melanoma Metastasis and Survival

Because metastatic melanomas were characterized by a significant increase of lymphatic vessel density and lymphatic vessel size, we next investigated whether the degree of tumor lymphangiogenesis might serve as a new prognostic parameter for the risk of melanoma metastasis. A univariate proportional hazard analysis revealed that the presence of intratumoral lymphatic vessels and a higher level of peritumoral lymphatic vascular area were significantly associated with a more rapid development of lymph node metastasis (Table 2). A multivariate proportional hazard analysis, using backward elimination of all other variables, revealed that only the level of peritumoral lymphatic area was an independent predictor of the time to lymph node metastasis. An additional univariate proportional hazard analysis for the effect of different risk factors on overall survival identified the presence of intratumoral lymphatics and the extent of peritumoral lymphatic vascularization as the only two parameters that were significantly associated with reduced overall survival (Table 3). Because both groups of metastatic and nonmetastatic melanomas were exactly matched for tumor thickness, no prognostic significance could be assigned to this parameter by the hazard analyses.

We next used Kaplan-Meier analyses to calculate the overall survival and the disease-free survival for patients with or without the detection of intratumoral lymphatics, and for patients with low (≤1.0%), medium (1.0% to ≤1.5%), or high (>1.5%) peritumoral lymphatic vascular area (Figure 6; B to D). This classification was based on the distribution of lymphatic vascular areas in our patient population (Figure 6A). We found that a high level of tumor lymphangiogenesis was a significant prognostic factor for reduced overall survival (Figure 6B; $P < 0.0001$) and for reduced disease-free survival (Figure 6C; $P < 0.0001$). The estimates for 3, 5, and 10 years for each stratum were calculated with pair-wise comparisons,

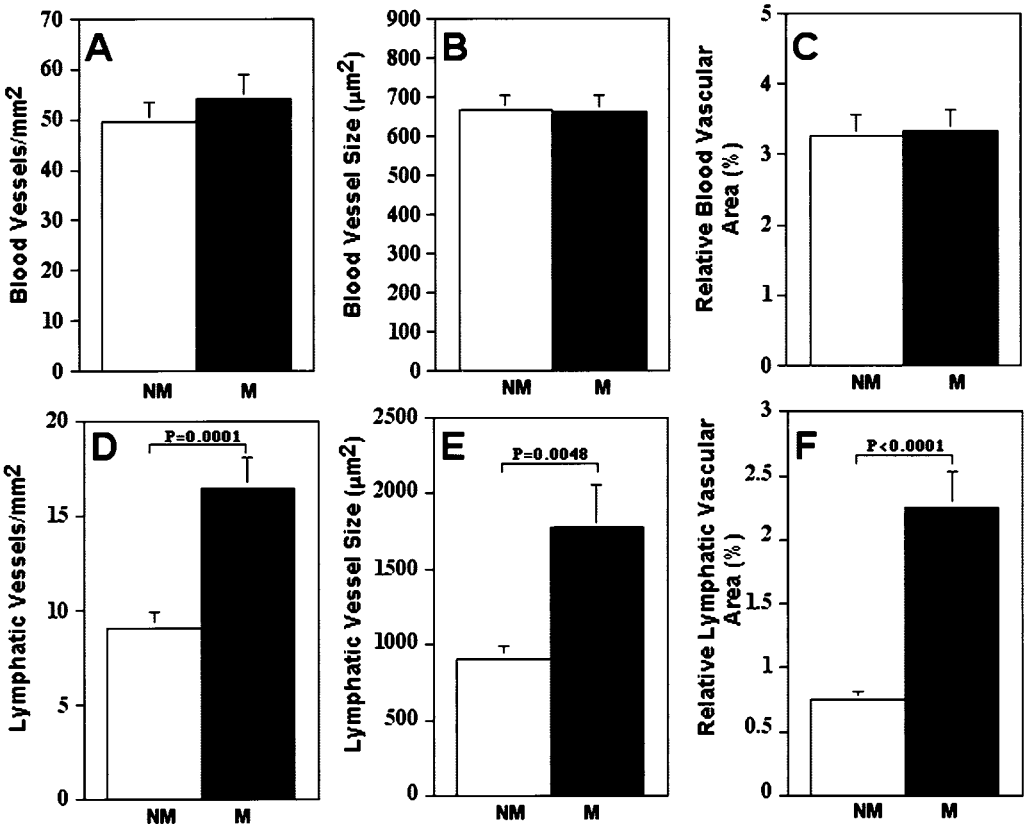

Figure 3. Computer-assisted image analysis of tumor-associated blood vessels and peritumoral lymphatic vessels in nonmetastatic *versus* metastatic melanomas. **A–C:** Comparable blood vascular density, average blood vessel size, and relative area occupied by blood vessels in metastatic (M; $n = 18$) and nonmetastatic (NM; $n = 19$) malignant melanomas. **D–F:** Significant increase of lymphatic vascular density, average lymphatic vessel size, and relative area occupied by lymphatics in metastatic malignant melanomas, as compared with nonmetastatic melanomas. Mean ± SEM.

which were all significant ($P < 0.05$) for both overall and disease-free survival. Importantly, the detection of intratumoral lymphatic vessels was significantly associated with a poorer disease-free survival, as compared with melanomas without intratumoral lymphatics (Figure 6D; $P = 0.0177$).

Discussion

The prognosis of cutaneous malignant melanoma is determined by its ability to metastasize, and the evaluation of the metastatic risk of a primary melanoma poses a challenge to clinicians and pathologists alike. Although a number of clinical, histological,[2,3,27,33] and molecular[34] prognostic indicators have been described, the current method of cutaneous melanoma prognostication is predominantly based on the tumor thickness.[5] However, the prognostic value of tumor thickness is limited because a considerable number of patients with thin melanomas die of metastatic disease whereas many of those with thick tumors experience long-term survival. This clinical observation was confirmed in our matched cohort study of 19 patients with nonmetastatic primary melanoma and of 18 patients with early (<1 year) lymph node metastasis that were selected from a pool of 1050 melanoma patients. Thus, novel prognostic indicators of metastasis for malignant melanoma are urgently needed. The discovery of the lymphatic endothelial hyaluronan receptor-1 (LYVE-1) as a specific marker for both normal[25] and tumor-associated lymphatics[26] has now paved the road to study tumor lymphangiogenesis not only in experimental tumor models, but also in spontaneously arising human tumors. The specificity of LYVE-1 as a lymphatic marker has been recently challenged because of its presence on hepatic sinusoidal endothelial cells that are involved in hyaluronan uptake.[35] However, recent studies, using differential immunostains for LYVE-1 and for the lymphatic-specific transcription factor Prox1 have confirmed that LYVE-1 is selectively expressed by lymphatic vessels, but not by blood vessels, in murine and in human tumors.[36–38]

In our present study, using double immunostains for CD31 and LYVE-1, we detected both peri- and intratumoral lymphatic vessels in a large number of primary cutaneous melanomas. Although intratumoral lymphatics have been proposed to be nonfunctional in experimental mouse tumor models,[39,40] univariate proportional hazard analysis revealed that the presence of intratumoral lymphatics was a significant risk factor for the development of lymph node metastasis in patients with cutaneous melanoma. Not only did intratumoral lymphatics occur at a significantly higher incidence in metastatic melanoma, but their presence was also related to a significantly increased risk of lymph node metastasis. These results suggest that intratumoral lymphatics play a clinically sig-

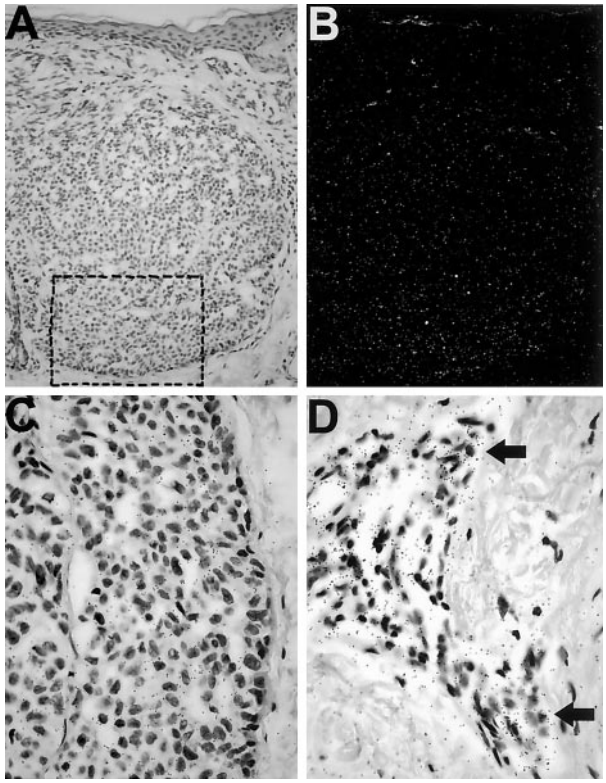

Figure 4. VEGF-C mRNA expression in cutaneous melanomas. **A:** Bright-field microscopy shows a dermal melanoma nodule. **B:** Dark-field microscopy reveals increased signal (white grains) in the tumor area but not the dermis. **C:** Bright-field microscopy shows increased black grains under a higher magnification from the **rectangle** in **A**. **D:** VEGF-C mRNA expression by peritumoral stromal cells. **Arrows** indicate areas of strong hybridization signals. Original magnifications: ×200 (**A** and **B**); ×600 (**C** and **D**).

Table 2. Univariate Analysis for the Effect of Different Risk Factors on the Development of Lymph Node Metastasis

Covariate	Hazard ratio	*P* value
Intratumoral lymphatics*	3.559	0.0263
Lymphatic Vascular† Area (%)	1.602	0.0002
Vascular Invasion*	1.971	0.372
Ulceration*	1.515	0.4344
Microsatellites*	1.165	0.882
Peritumoral inflammation†	0.835	0.5942
Site†	0.698	0.3115
Regression*	0.624	0.5292
Mitoses (>6/mm^2)*	0.531	0.2305

* Dichotomous variable.
† Continuous variable.

nificant role as conduits for the metastasis of cutaneous melanoma, proposing an active role of lymphangiogenesis as evidenced by hot spots of lymphatic vessels within and surrounding metastatic melanomas. In contrast, a traditional view asserts that lymphatic endothelium plays a passive role during tumor metastasis and that lymphatic invasion only occurs via infiltrating tumor cells invading pre-existing lymphatic vessels.[41] However, several studies in animal tumor models provide direct experimental evidence that increased tumor lymphangiogenesis promotes lymphatic tumor spread to regional lymph nodes.[22,23,42–44] Taken together, the results obtained in animal tumor models and in human tumors[26] support the recently proposed concept of an active lymphangiogenesis model for tumor metastasis.[45]

We have previously reported that the lymphangiogenic factor VEGF-C is expressed by several melanoma cell lines *in vitro* and that overexpression of recombinant VEGF-C in human melanoma cell lines promoted tumor lymphangiogenesis after xenotransplantation in nude

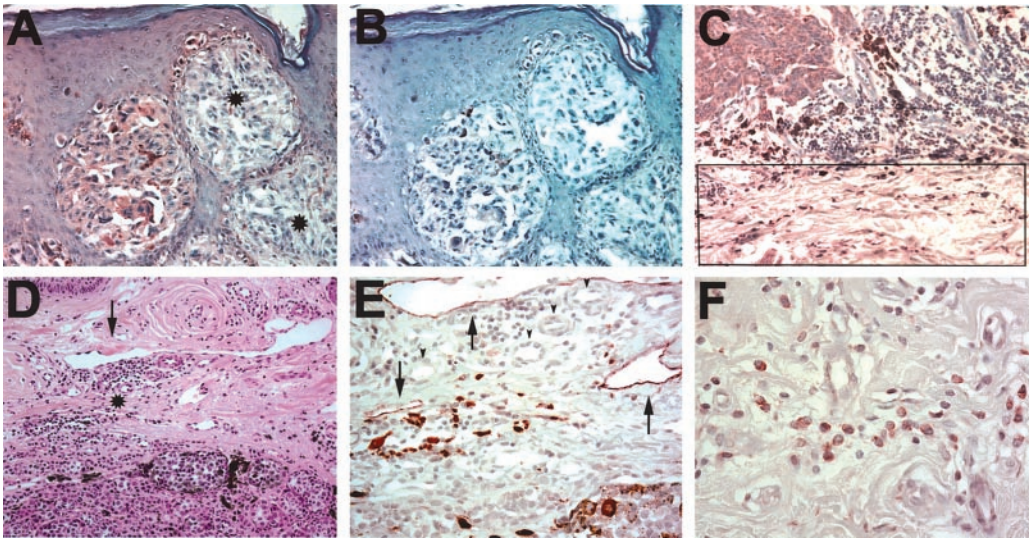

Figure 5. VEGF-C protein expression in cutaneous melanomas. **A:** Immunoperoxidase staining for VEGF-C (red) demonstrates focal expression in the cytoplasm of melanoma cells in one tumor nest but not in the adjacent nests (**asterisks**). **B:** Serial section negative control with omission of secondary antibody. **C:** Immunoperoxidase staining for VEGF-C (red) demonstrates VEGF-C expression by peritumoral fibroblasts (**rectangle**) at the invasive edge of a metastatic melanoma (**top left**) in the reticular dermis. **D:** H&E stain of the invasive tumor edge of a metastasizing cutaneous melanoma reveals mononuclear inflammation (**asterisks**) near dilated lymphatic vessel (**arrow**). **E:** Immunoperoxidase staining of a serial section for LYVE-1 (red) decorates dilated peritumoral lymphatics (**arrow**) and adjacent mononuclear infiltrate. Blood vessels are LYVE-1-negative (**arrowheads**). **F:** Immunoperoxidase staining for VEGF-C (red) stains the cytoplasm of peritumoral mononuclear cells in a granular pattern. Original magnifications: ×200 (**A–D**); ×400 (**E**); and ×600 (**F**).

Table 3. Univariate Analysis for the Effect of Different Risk Factors on Overall Survival

Covariate	Hazard ratio	*P* value
Intratumoral lymphatics*	6.133	0.0180
Lymphatic Vascular† Area (%)	1.555	0.0028
Vascular Invasion*	4.511	0.0619
Ulceration*	1.986	0.2607
Microsatellites*	NA	NS
Peritumoral inflammation†	0.570	0.1763
Site*	0.935	0.8697
Regression*	0.994	0.9933
Mitoses (>6/mm^2)*	0.696	0.5414

NA, not applicable; NS, not significant.
* Dichotomous variable.
† Continuous variable.

mice.[24] In the human melanomas studied in the present investigation, however, we only detected low-level and heterogeneous expression of VEGF-C by tumor cells that did not significantly correlate with the metastatic potential of the primary tumors. Similarly, only weak VEGF-C protein has been recently found in metastatic human squamous cell carcinomas of the head and neck,[26] whereas other studies found a correlation between the occurrence of tumor metastasis and tumor expression of VEGF-C mRNA in a number of epithelial cancers.[46] We also found detectable VEGF expression in only a minority of all melanomas examined, confirming previous studies reporting that VEGF was only expressed in some cases of primary melanomas.[47,48] Taken together, these results indicate that tumor-derived VEGF and VEGF-C likely do not represent the major source of angiogenic or lymphangiogenic activity in cutaneous melanomas. Therefore, it is tempting to speculate that the relative low levels of VEGF-C expression by melanoma cells are complimented by nontumoral, stromal sources such as dermal fibroblasts and peritumoral macrophages, as demonstrated in our study. Moreover, recent evidence indicates that peritumoral inflammation and VEGF-C production by inflammatory cells might also contribute to the induction of lymphangiogenesis.[24,49] Although we were unable to detect any expression of VEGF-D,[50] another known lymphangiogenic factor, in the 37 cases studied (data not shown), lymphatic vessel growth might be stimulated by other, yet unknown growth factors. The recent establishment of specific human lymphatic endothelial cell cultures[51–53] and the development of *in vivo* lymphangiogenesis assays[54] now provide the experimental tools for future *in vitro* and *in vivo* studies for the identification and characterization of novel lymphangiogenesis factors that might include members of the fibroblast growth factor family.[54]

Surprisingly, we did not detect any significant differences in tumor angiogenesis between the metastatic and nonmetastatic matched cutaneous melanomas. The similarity in tumor angiogenesis may be because of the

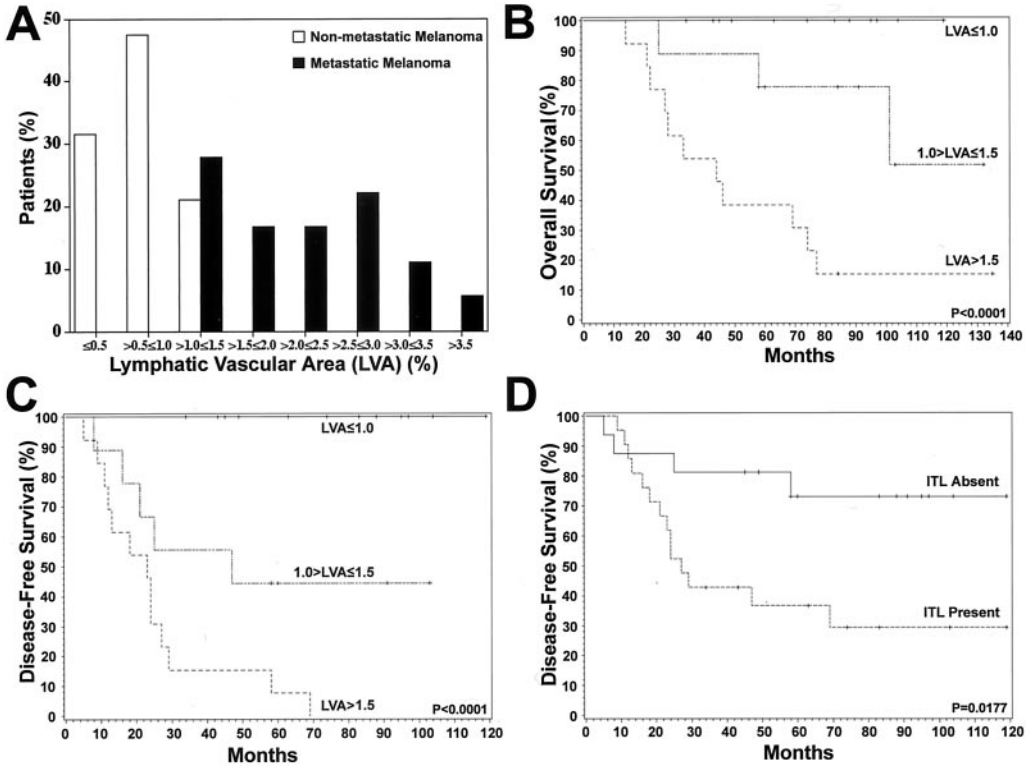

Figure 6. Kaplan-Meier analyses of overall and disease-free survival as a function of tumor lymphangiogenesis. **A:** The distribution of lymphatic vascular area (LVA) in the patient population indicates an overlap in the >1.0 to ≤1.5 range between the nonmetastatic and metastatic melanoma groups. **B**, **C:** Overall and disease-free survival curves were stratified by low (≤1.0%), medium (1.0 to ≤1.5%), or high (>1.5%) level of tumor lymphangiogenesis. Statistically significant correlation between the extent of tumor-associated lymphangiogenesis, expressed as percentage of area covered by lymphatic vessels, and reduced disease-free survival and overall survival are noted. **D:** The disease-free survival analysis revealed that the detection of intratumoral lymphatics was significantly associated with a poorer disease-free survival.

comparably weak levels of VEGF mRNA expression in both metastatic and nonmetastatic melanomas in our study that did not include cases with early organ metastasis. Moreover, both groups were exactly matched for tumor thickness, thereby excluding differences of local tumor progression that has been previously correlated with increased vascularity.[13,16,55,56] A recently published prospective study in 417 cutaneous melanoma patients found that tumor vascularity, as assessed on routine histological stains, was the most important determinant of overall survival.[20] In contrast, other investigators failed to detect any correlation between melanoma vascularization and prognosis. Using morphometric analysis of *Ulex europaeus* type I lectin-labeled sections obtained from 86 melanomas with no evidence of recurrence after a minimum follow-up period of 5 years and of 21 cases with locoregional recurrence and/or metastasis, one study found that tumor recurrence could not be predicted by any of the derived vascular parameters (vascular length, surface, and volume density) either independently or together with other histological and clinical features.[57] In another study of 60 cases of metastasizing and nonmetastasizing cutaneous melanomas that were matched for tumor thickness, age, sex, and anatomical site, there was no significant difference in the number of microvessels or in the pattern of vascular microarchitecture between metastasizing and nonmetastasizing tumors.[17] The reported increase of VEGF protein expression during the transition from horizontal growth to the vertical melanoma growth phase[55,56] suggests that angiogenesis may be important for promoting primary tumor growth whereas it seems to play a minor role if any in promoting lymphatic melanoma metastasis.

In summary, our study demonstrates, for the first time, the presence of intra- and peritumoral lymphangiogenesis in primary human cutaneous melanomas. It also provides the first evidence that the extent of tumor lymphangiogenesis may be related to the risk of lymph node metastasis as well as patient survival, and that peritumoral lymphatic vascular density might serve as a novel prognostic indicator for the risk of lymph node metastasis of human cutaneous melanomas. The feasibility of CD31/LYVE-1 double immunostains on routine paraffin sections eliminates the need for fresh-frozen tumor samples and will greatly facilitate larger, prospective, multi-institutional clinical trials that are needed to further validate the prognostic value of tumor lymphangiogenesis for metastasis and patient survival of primary malignant melanomas of the skin and, potentially, of other human malignancies.

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