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Correlation between lymphatic vessel density and microvessel density in cutaneous malignant melanoma

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Abstract

Background: Malignant melanoma is an aggressive neoplasm, known for its propensity to early metastatic spread, *via* lymphatic as well as blood vessels. Tumor progression to an aggressive phenotype is associated with angiogenesis. Tumor lymphangiogenesis may represent a marker for assessing the risk of metastasis in the regional lymph nodes. *Materials and Methods*: We studied the lymphatic vessel density in peritumoral and intratumoral areas compared to overall microvessel density in 12 cases of malignant melanoma of the face. All cases were primary invasive melanomas, with a Clark level of invasion III and IV. Lymphatic vessels were marked with D2-40 murine monoclonal antibody and their density evaluated through hot-spot method by examination on optic microscopy (200×). Overall microvessel density was assessed using the same method, vascular endothelial cells being visualized using CD31 monoclonal antibody. Statistical analysis was made using SPSS 17.0 software package (Pearson correlation test and Student's *t*-test). *Results*: The disposition and aspect of the lymphatic vessels were different in peritumoral and intratumoral areas. Thus, in peritumoral areas lymphatics were generally regular, large, dilated vessels whereas intratumoral lymphatic vessels were smaller, with an irregular lumen. Lymphatic vessel density was generally higher in peritumoral areas. Intratumoral lymphatic vessel density was lower, but significantly correlated to overall microvessel density in these areas. Overall microvessels density was increased in thick cutaneous melanoma. Vessels in the peritumoral areas were larger and more numerous compared to those found in normal tissue. In cases with a dense peritumoral inflammatory infiltrate, we found the highest vascular density. Intratumoral angiogenesis was moderate in most cases, with irregular, smaller or collapsed vessels. *Conclusions*: Evaluation of the lymphatic vessel density may prove to be useful for the prognostic assessment in malignant melanoma, as it may predict the patients with a risk of developing lymph node metastasis.

Keywords: malignant melanoma, lymphatic vessel density, microvessel density, immunohistochemistry.

Introduction

Malignant melanoma is one of the most aggressive neoplasms, with a tendency to early metastasize in regional lymph nodes. The spread *via* lymphatic vessels is considered the preferred route of metastasizing. The hematogenous spread, although it can occur, appears to be less frequent than the lymphatic spread [1].

The evidence that the intensity of angiogenesis in a human tumor could predict the likelihood of metastasis was first reported in cutaneous melanoma [2]. Tumor angiogenesis requires a combination of angiogenic factors and stromal remodeling occurring because of the interaction between melanoma cells and their micro-environment. It was shown that melanoma cells release a range of soluble factors such as bFGF, MGSA/GRO, IL-8, IL-6, PDGF-A, IL-10 with autocrine action and PDGF, EGF, TGF-β, IL-1, GM-CSF, IGF-I, NGF, VEGF with a paracrine effect by modulating stromal formation, host immune response and angiogenesis [3–5]. Some of these factors have been demonstrated to also induce lymphangiogenesis (bFGF, VEGF) [1, 6, 7]. Up to date there are few published studies regarding lymphatic vessel density in malignant melanoma of the skin, with inconsistent results.

The aim of the study was to evaluate lymphatic vessel density in peritumoral and tumoral areas of cutaneous malignant melanoma, using a specific marker for lymphatic and assess the correlation with overall microvessel density as a hallmark of tumor angiogenesis.

Materials and Methods

We retrospectively studied 12 excision biopsy tissue samples (from Clinical Recovery Hospital, Iassy and "St. Spiridon" Clinical Emergency County Hospital, Iassy, Romania) from patients with cutaneous malignant melanoma of the face. The cohort included seven males and five females, age ranging between 54 and 85 years, with a median of 73.5 years. All cases were advanced primary melanomas, classified according to Clark's level of invasion (five cases Clark III and seven cases Clark IV). Specimens of each tissue were fixed in 10% neutral buffered formalin and embedded in paraffin. Serial 3-µm step sections were obtained and routine Hematoxylin–Eosin stain method was applied for morphologic study. Three µm thick histological sections were prepared for immunostaining. In order to visualize lymphatic vessels we used D2-40 monoclonal antibody (Dako, Glostrup,

Denmark). Prior to immunostaining, the sections were deparaffinized using successive benzene and graded ethanol baths, immersed in 3% hydrogen peroxide in distilled water for five minutes at room temperature to block endogenous peroxidase activity and pre-treated by microwave heating in citrate buffer, pH 6 for 30 minutes for antigen retrieval. After incubation for 30 minutes at room temperature, we applied LSAB+ working system and bound antibodies were visualized with 3,3'-diaminobenzidine. Separate sections were prepared for immunostaining with monoclonal murine anti-CD31 antibody (clone JC/70A, prediluted, Dako, Glostrup, Denmark) for highlighting endothelial cells. The same treatment was applied and after incubation with the antibody for 30 minutes, we applied LSAB+ working system and bound antibodies were visualized with 3-aminoethyl-carbazole.

All slides were automatically processed using Dako Cytomation Autostainer and then examined in optic microscopy using Nikon Eclipse E600 Microscope (200×).

Lymphatic vessel density (LVD) – lymphatics positive for D2-40 stained in brown and overall microvessel density (OMVD) (endothelial cells positive for CD31 – red) were evaluated using hot spot method in peritumoral and intratumoral areas. Three different microscopic fields for each area were examined for every case and the arithmetic means for LVD and OMVD were noted. For statistical analysis, we used Pearson correlation test and Student's *t*-test.

Results

The quantitative results of intra- and peritumoral LVD and OMVD are shown in Table 1.

LVD in peritumoral areas (mean of 8.5083) was twice as high as LVD in tumoral areas (mean of 3.9417) in correlation to OMVD that showed a mean of 25.3917 in peritumoral areas compared to 17.1917 in tumoral areas. In the peritumoral areas, we observed an increase in overall microvascular density. These vessels were large, regular, with thin walls limited by endothelial cells positive for CD31 (Figure 1). In areas with intense inflammatory infiltrate, there was an increased vessel density.

Table 1 – *LVD and OMVD in malignant melanoma of the skin: quantitative values*

		Peritumoral area	Intratumoral area
LVD	Minimum	7.41	3.08
	Maximum	9.83	4.83
	Mean	8.5083	3.9417
OMVD	Minimum	21.92	11.92
	Maximum	32.23	19.78
	Mean	25.3917	17.1917

LVD – Lymphatic vessel density; OMVD – Overall microvessel density.

Peritumoral lymphatics were large vessels, with a dilated lumen and regular disposition (Figure 2). Intratumoral lymphatics were smaller vessels, with an irregular lumen and variable disposition. We found sinuous lymphatic vessels with very small or collapsed lumen inside connective septa penetrating the tumor (Figure 3).

In three cases, there were tumoral emboli in the lymphatic vessels (Figure 4). All three cases developed regional lymph node metastasis within eight months from the excision of the primary tumor.

Intratumoral angiogenesis evaluated by microvessel count was moderate compared to that found in peritumoral area. Vessels inside the tumor areas were heterogeneous; they may be blood vessels with a sinuous, irregular lumen or very small neoformation vessels with irregular or collapsed lumen and tortuous disposition (Figure 5). We also found neovessels with sinuous orientation, with an inconstant lumen disposed in the connective tissue penetrating the tumor.

In peritumoral areas, there was a moderate correlation between the number of lymphatics and blood vessels, but this correlation did not reach statistical significance (p=0.147) (Table 2).

In intratumoral areas, we found a moderate, statistically significant correlation between LVD and OMVD (correlation coefficient 0.585, p=0.046) (Table 3).

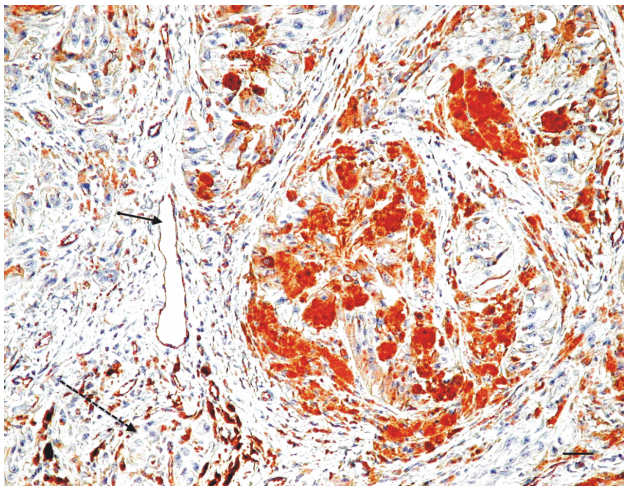

Figure 1 – *CD31, ob. ×10. Continuous arrow: peritumoral large, irregular microvessel with endothelial cells positive for CD31 (red). Dotted arrow: higher vascular density in areas with intense inflammatory infiltrate. Scale bar 100 µm.*

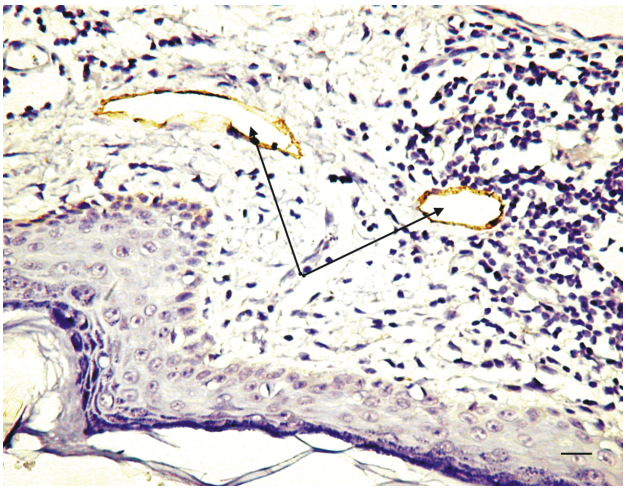

Figure 2 – *D2-40, ob. ×20. Peritumoral lymphatic vessels with large, regular lumen. Scale bar 50 µm.*

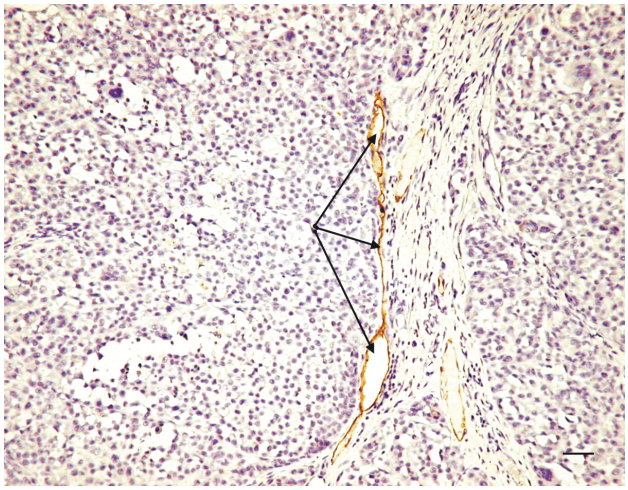

Figure 3 – ***D2-40, ob. ×10. Sinuous lymphatic vessels at the limit of tumoral area and a connective septum. Scale bar 100 µm.***

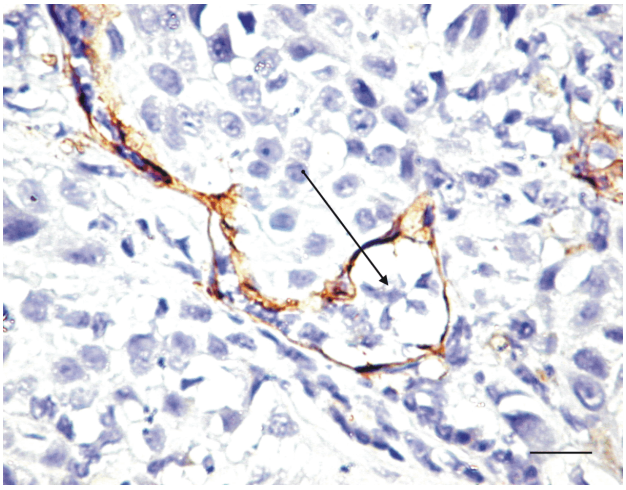

Figure 4 – ***D2-40, ob. ×20. Tumoral emboli in a lymphatic vessel. Scale bar 50 µm.***

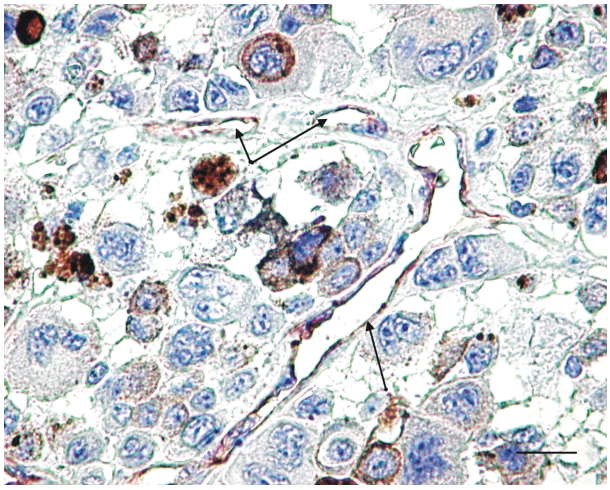

Figure 5 – ***CD31, ob. ×40. Intratumoral microvessels with sinuous, irregular lumen. Scale bar 20 µm.***

Table 2 – ***Pearson correlation test between LVD and OMVD in peritumoral areas***

Correlations			
		OMVD	***LVD***
OMVD	Pearson correlation	1	.445
	Sig. (two-tailed)		.147
	N	12	12
LVD	Pearson correlation	.445	1
	Sig. (two-tailed)	.147	
	N	12	12

OMVD – Overall microvessel density; LVD – Lymphatic vessel density.

Table 3 – ***Pearson correlation test between LVD and OMVD in intratumoral areas***

Correlations			
		OMVD	***LVD***
OMVD	Pearson correlation	1	.585*
	Sig. (two-tailed)		.046
	N	12	12
LVD	Pearson correlation	.585*	1
	Sig. (two-tailed)	.046	
	N	12	12

OMVD – Overall microvessel density; LVD – Lymphatic vessel density.
*Correlation is significant at the 0.05 level (two-tailed).

Discussion

One of the earliest signs of malignant tumor metastasis is the spread into the regional lymph nodes, and it occurs more frequently *via* lymphatic than hematogenous route in malignant melanoma [8].

Angiogenesis has been established to play an important role in the development and progression of many tumors. The prognostic importance of the degree of melanoma vascularization, however, has remained controversial. Some authors found a significant correlation between tumor vascularity and other histological prognostic factors for melanomas of the skin [9–11]. Thus, Depasquale and Thompson found that microvessel density is a reliable prognostic factor in cutaneous melanomas thicker than 2 mm [11]. Other authors also found a significant correlation between angiogenesis and prognosis in melanoma [2, 12–14], while Hillen *et al.* consider proliferating endothelial cells a better marker for tumor prognosis than microvessel density [15]. There have been numerous researches regarding the vascular invasion in relation to the risk of melanoma relapse, lymph node involvement and distant metastasis, results suggesting that vascular invasion may have an impact on melanoma prognosis similar to that of ulceration. Other research focused on the perivascular infiltration and found that melanoma cells migrate along the external surface of the vessels and disseminate even without intravascular invasion [16].

The importance of tumor lymphangiogenesis in metastasis has been subject of few studies and whether this process is important for spread *via* the lymphatics is still not clear. During the last few years, the discovery of the key lymphatic growth factors VEGF-C and -D and their corresponding receptor VEGFR-3 allowed a better understanding of the molecular mechanisms underlying the development of lymphatic vessels and their role in health and disease [5, 6]. Recent studies led to the identification of several molecular markers specific for the lymphatic endothelium, allowing a clear discrimination between lymphatic and blood vessels [7]. D2-40 is one of these new markers, represented by a monoclonal murine

antibody against a 40 000 kD glycoprotein that specifically interacts with an epitope on the lymphatic endothelium [8]. There have been studies showing that neoplastic cells, including melanoma cells produce lymphangiopoietic vascular endothelial growth factors VEGF-C and -D [7, 8, 17, 18].

The role of lymphangiogenesis and its prognostic significance in cutaneous malignant melanoma has been studied lately, but with conflicting data reports in the literature. Thus, Skobe *et al.* demonstrated that over-expressing VEGF-C human melanoma transplanted onto nude mice led to intratumoral lymphangiogenesis [19]. De Waal *et al.*, in contrast, reported the absence of lymphangiogenesis in human primary melanomas, using double immunostaining techniques [20]. Straume *et al.* found decreased LVD in thick melanomas. The authors suggested that aggressive, thick melanomas might destroy the lymphatics rendering them undetectable by immuno-histochemical means. However, they found that an increased intratumoral LVD was an independent prognostic factor in vertical growth phase melanoma [21]. Our study on invasive, thick melanomas showed that lymphatic vessels were predominantly present in the peritumoral areas with an almost double LVD in these areas compared to LVD in intratumoral areas, supporting Straume *et al.* findings. In intratumoral areas, we found a statistically significant correlation between the number of lymphatics and the total number of neovessels, suggesting a correlation between lymphangiogenesis and angiogenesis (evaluated by OMVD) in malignant melanoma. Knowing the importance of angiogenesis in tumor growth and progression, we can assume that lymphangiogenesis in malignant melanoma may play a role in tumor spread.

Giorgadze *et al.* [22] compared intratumoral LVD in cutaneous malignant melanomas to LVD in other benign melanocytic lesions and found a significantly higher LVD in melanomas. The authors also found an increased LVD in the papillary dermis underneath *in situ* melanomas, suggesting that transformed melanocytes from the epidermis may secrete factors that influence the surrounding microenvironment. The significant difference between LVD in melanomas and LVD in benign melanocytic lesions lead to the importance of assessing intratumoral LVD as a method of differential diagnosis. Dadras *et al.* concluded that increased peri- and intratumoral lymph-angiogenesis was associated with a higher incidence of metastasis, showing that the incidence of intratumoral lymphatics was higher in patients with metastatic disease [23, 24]. Studies regarding lymphangiogenesis and lymphatic spread in malignant melanoma yielded conflicting results [25–27]. Thus, Padera *et al.* found lymphatic metastasis in the absence of functional intratumor lymphatics [27].

In our study, intratumoral LVD was approximately the same in all cases. We found the presence of tumoral emboli in the lymphatics in three cases and these patients developed regional lymph node metastasis more quickly than those with lymphatic vessels present in tumoral area, but with no tumoral emboli. This finding suggests that lymphangiogenesis may be a first step in tumor spread, followed by intralymphatic invasion and regional lymph node metastasis.

Conclusions

Increased overall microvessel density in thick cutaneous melanoma suggests a possible relation between tumor thickness – known as an independent prognostic factor in melanoma – and the number of new blood vessels found in peritumoral and intratumoral areas. The positive correlation between angiogenesis and lymphangiogenesis in intratumoral areas of thick cutaneous melanoma suggests that lymphatic vessel density may prove to be useful for the prognostic assessment in malignant melanoma, as it may predict the patients with a risk of developing lymph node metastasis.

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