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ANALYSIS OF IMMUNOHISTOCHEMICAL EXPRESSION OF PECAM-1 AND ENDOGLIN IN NORMAL SKIN, BENIGN MELANOCYTIC NEVI, AND CUTANEOUS MALIGNANT MELANOMA

Marcos V. M. Oliveira*[1,2], Erivelton P. Santos[1], Camila S. Pereira[1,2], Valéria C. Quintão[1], Maria L. M. Pinheiro[1], Ludmilla R. Sousa[1], John R. Basile[3], Otávio C. Filho[1], Sérgio H. S. Santos[1,4], André L. S. Guimaraes[1,5] and Alfredo M. B. De-Paula[1,5]

[1]Health Science Post-graduate Programme, Health Research Laboratory, Nucleus of Epidemiological and Molecular Research Catrumano, Universidade Estadual de Montes Claros, 39401-001, Montes Claros, MG, Brazil; [2]PhD. Department of Medicine, Centro UniversitárioFIPMoc, 39408-007, Montes Claros, MG, Brazil; [3]DDS, PhD. Department of Oncology and Diagnostic Sciences, University of Maryland School of Dentistry, Baltimore, MD 21201, USA; [4]PhD. Department of Pharmacology, Universidade Federal de Minas Gerais, 31270901 Belo Horizonte, MG, Brazil; [5]DDS, PhD. Department of Dentistry, Universidade Estadual de Montes Claros, 39401-001, Montes Claros, MG, Brazil

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Corresponding author:
Marcos V. M. Oliveira

ABSTRACT

Objetive: We analyzed expression of PECAM-1 and endoglin for a correlation with clinicopathological behavior in CMM. **Methods**: Control (n = 12), CMN (n = 48), and CMM (n = 44) samples were submitted for immunohistochemistry. PECAM-1 and endoglin expression were counted in the stroma (*hot spots*) of all samples in order to calculate the MVD. Data analyses were performed using univariate statistical tests, with significance set at $p<0.05$. **Results**: Our findings showed that CMM exhibited higher MVD estimates for both PECAM-1 and endoglin compared to control and CMN samples ($p<0.001$, for all associations). Moreover, CMN samples exhibited higher MVD compared to control samples ($p<0.001$ for all associations). CMM from subjects with metastatic disease showed higher MVD by PECAM-1 ($p=0.036$) and endoglin ($p=0.015$) compared to non-metastatic CMM. **Conclusion:** Increasing MVD from normal skin to benign and malignant melanocytic tumors suggests the importance of a rich vascular network in the peritumoral stroma to support greater metabolic and energetic demands, which favors the dissemination of melanocytic tumor cells.

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INTRODUCTION

Cutaneous malignant melanoma (CMM) represents a relevant public health issue in many countries worldwide. Although CMM represent less than 10% of all skin cancers, it is responsible for over 90% of deaths associated to these types of cancer. Currently, CMM patients with metastatic disease exhibit a 5-year overall survival of about 5-10% (White, Stanley *et al*. 2002, Erdmann, Lortet-Tieulent *et al*. 2013). During early stages of CMM progression, endogenous factors, such as pale-skin, red or blonde hair, occurrence of many freckles on the upper back, use of oral contraceptives for ≥ 5 years, a previous history of blistering sunburns and actinic keratosis, a number of cutaneous melanocytic nevi, and family history of melanoma, and exogenous factors such as intermittent high intensity of ultraviolet radiation exposure, promote a series of genetic and epigenetic disturbances that affects cell differentiation, proliferation, and cell death control (Russak and Rigel 2012, Guarneri and Guarneri 2014). These disturbances might promote the development of benign cutaneous melanocytic nevi (CMN), the common or dysplastic subtypes, which might progress to cutaneous melanoma malignant (CMM) eventually. However, CMM also can directly arise from a normal melanocyte without necessarily going through the benign

neoplasm phase (Clark, Elder *et al*. 1984, Tucker, Fraser *et al*. 2002). Typically, CMM cells exhibit a highly efficient, precocious metastatic capacity to invade local and distant organs. In early stages, CMM is morphologically characterized by initial radial growth phase, with a typical spreading of melanoma cells in the superficial layers of the skin. Later, disseminating melanoma cells promote the development of vertical growth phase of the malignancy, morphologically characterized as invasion of disseminating melanoma cells into the peritumoral stroma. Invading CMM cells use both lymphatic and haematogenous vascular networks to disseminate to local or distant organs during the metastatic process (Massi, Puig *et al*. 2006, Leong, Gershenwald *et al*. 2011). Tumor angiogenesis involves a series of molecular disturbances that promote extracellular matrix degradation, recruitment of circulating endothelial progenitor cells, survival, migration and proliferation of endothelial cells (ECs), and ultimately, the formation of neovasculature from pre-existing blood vessels. This complex process ensures the supply of nutrients and oxygen to metabolically active cancer cells, removal of catabolic wastes from cancer cells and the tumor microenvironment, and provides a hematogenous route for disseminating cancer cells (Folkman 1971, Hanahan and Folkman 1996, Gensicka, Glowacka *et al*. 2015). Tumor angiogenesis results in increasing activity of ECs induced by a spectrum of angiogenic signaling factors. Endoglin (CD105) is a proliferation-associated and hypoxia-inducible transmembrane phosphorylated glycoprotein component of the receptor complex of transforming growth factor-beta (TGF-β). Notably, endoglin is preferentially expressed in active, proliferative ECs and has a pivotal role for vascular development (Li, Sorensen *et al*. 1999, Newman and Newman 2003, Fonsatti and Maio 2004). Endoglin promotes migration and endothelial cell turnover by stimulating the TGF-β/ALK-1/Smad5 phosphorylation pathway while inhibiting the TGF-β/ALK-5/Smad2-3 signaling pathway (She, Matsuno *et al*. 2004). Platelet endothelial cell adhesion molecule (PECAM-1, also known as CD31), is a vascular-associated adhesion and signaling membrane glycoprotein expressed on filopodia of leukocytes, platelets, and ECs. PECAM-1 plays important roles in regulation of leukocyte trans-endothelial migration and motility and adhesion mechanisms of ECs (Gratzinger, Canosa *et al*. 2003, Fujiwara 2006, DeLisser 2011). The quantification of tumor microvessel density (MVD) is a good indicator of tumor angiogenesis and the total vascular network of a malignancy. As a result, MVD plays a pivotal role intumor progression, as well as acting as a reliable prognostic indicator for human solid cancers (Weidner 1995, Sharma, Sharma *et al*. 2005, Raica, Cimpean *et al*. 2009). However, the significance of such vascular alterations in the progression of cutaneous melanocytic neoplasms (Barnhill, Fandrey *et al*. 1992, Vacca, Ribatti *et al*. 1993, Michaylira and Nakagawa 2006) as well as their influence on metastasis of melanoma cells remains inconclusive (Kashani-Sabet, Sagebiel *et al*. 2002, Depasquale and Thompson 2005, Ribatti, Annese *et al*. 2010, Helfrich and Schadendorf 2011). In this study, we aimed to analyze the MVD, as determined by both endoglin and PECAM-1 expression, in the stroma of normal skin, CMN, and CMM human samples. Moreover, we investigated if MVD is associated with sociodemographic and clinicopathological factors related to CMM patients.

METHODS

Ethical aspects: Ethical approval for this study was obtained from a relevant local ethic committee (Committee on ethic in research - Unimontes: protocol no: 691.408/2014).

Samples: This retrospective, cross-sectional study was performed on archived tissue blocks of normal human skin (n = 12 women, mean age: 37.2 ± 9.4 years old) and resected primary CMN (n = 48, mean age: 33.8 ± 11.8 years old, male:female ratio: 1:2.7, white skin color: 53.8%), and CMM (n = 44, mean age: 55 ± 14.9 years old, male:female ratio: 1:1.93, white skin color: 87.5%), with confirmed histopathological diagnosis. Sociodemographic and clinicopathological data were obtained from clinical charts from patients attended at public health centers for Oncology treatment at Montes Claros city, Minas Gerais state, Brazil.

Clinicopathological analyses: Samples of sunlight-exposed (n = 1) and non-exposed (n = 11) normal skin from 12 healthy individuals were used as controls. These samples were obtained from patients who underwent to esthetic or corrective surgical procedures. Clinically, CMN were acquired melanocytic nevi exclusively and were found in both sunlight-non-exposed (n = 17) and sunlight-exposed (n = 31) cutaneous sites. All CMM were classified according to the *American Joint Committee on Cancer* (AJCC) melanoma staging (Balch, Gershenwald *et al*. 2009). TNM clinical staging IA-IB and IIA-IIB-IIC (localized melanoma) was observed in 9 CMM samples (20.5%), while stages III and IV (regional and distant metastatic disease, respectively) were noted in 35 (79.5%) of CMM patients. CMM presented the clinical forms as follow: superficial spreading (n = 18, 40.9%), nodular (n = 7, 15.9%), lentigo malignant (n = 10, 22.7%), and acral lentiginous melanomas (n = 9, 20.5%). Primary tumor ulceration was detected in 6 cases (13.6%) and recurrence was noted in 10 (14.3%) primary CMM. The risk o death in CMM individuals has a significant association with prognostic factors from different cutaneous anatomical sites where melanoma occurred. CMM samples were classified as *low-risk* (lower trunk, thigh, lower leg, foot, lower arms, hands, and face) and *high-risk sites* (back and breast/thorax, upper arm, neck, and scalp) for death caused by CMM (Garbe, Buttner *et al*. 1995, Masback, Olsson *et al*. 2001, Homsi, Kashani-Sabet *et al*. 2005). According to primary tumor thickness, CMM patients were categorized as T1 (≤ 1 mm thickness, n = 13), T2 (1.01 to 2 mm thickness, n = 10), T3 (2.01 to 4 thickness, n = 13), and T4 (> 4 mm thickness, n = 8). Both distant and locoregional metastatic diseases were diagnosed in 29 (66%) of CMM patients. Formalin fixed and paraffin embedded normal skin, CMN, and CMM tissues sample were submitted to histopathological analysis. Tissue sections were cut at a thickness of 3–5μm and stained with hematoxylin and eosin (H&E). CMM samples were subjected to analysis of tissue invasion of melanoma cells by Breslow's thickness (Breslow 1970) and Clark's level (Clark, From *et al*. 1969) criteria. According to Breslow's thickness grade, CMM samples were categorized as follows: TI (up to 0.75mm, n = 3, 6.8%), TII (from 0.75 to 1.5mm, n = 7, 15.9%), TIII (1.5 to 3mm, n = 27, 61.4%), and TIV (3 to 4mm, n = 7, 15.9%). According to Clark's level (degree of invasion), CMM samples were categorized as follows: level I (intraepidermal and epithelium adnexal lesion, n = 3, 6.8%), level II (invasion up to the papillary dermis, n = 7, 15.9%), level III (invasion fills the entire reticular dermis, though without invading it, n = 18, 40.9%), level IV (invasion of the reticular dermis, n = 13, 29.5%), and level V (invasion of the hypodermis, n = 3, 6.8%).

Immunohistochemical reactions: PECAM-1 and endoglin expression were performed using immunohistochemical (IHC) method with streptavidin–biotin-peroxidase detection system. 4μm-thick CMN and CMM sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Sections were submitted to antigen retrieval combined with pressure cooking. Afterwards, endogenous peroxidase, biotin, and streptavidin were blocked by using specific reagents prior to incubation with each primary antibody (mouse monoclonal anti-PECAM-1, clone 1A10, 1:100; Novocastra, Newcastle, United Kingdom; and rabbit polyclonal anti-endoglin, 1:100; Abcam, Cambridge, United Kingdom), overnight at 4°C. The sections were thereafter incubated with LSAB™-Kit Plus Peroxidase® (DakoCytomation, Glostrup, Denmark) for 1h. Tissues were stained with a chromogen (3,3′-diaminobenzidine tetrahydrochloride, DAB), counterstained with Mayer's hematoxylin, cover slipped, and visualized under an optical microscope. Positive and negative controls were applied according to the manufacturer's instructions (DakoCytomation, Glostrup, Denmark).

Counting of immunostained cells and MVD estimates: All control, CMN, and CMM samples were morphologically evaluated by one independent observer without knowledge of the clinical factors (EP, Santos). Photomicrographs were taken at 100X and 400X magnification using an optical Olympus® BH2 microscope (model: CX31; RTSF, Miami, USA).

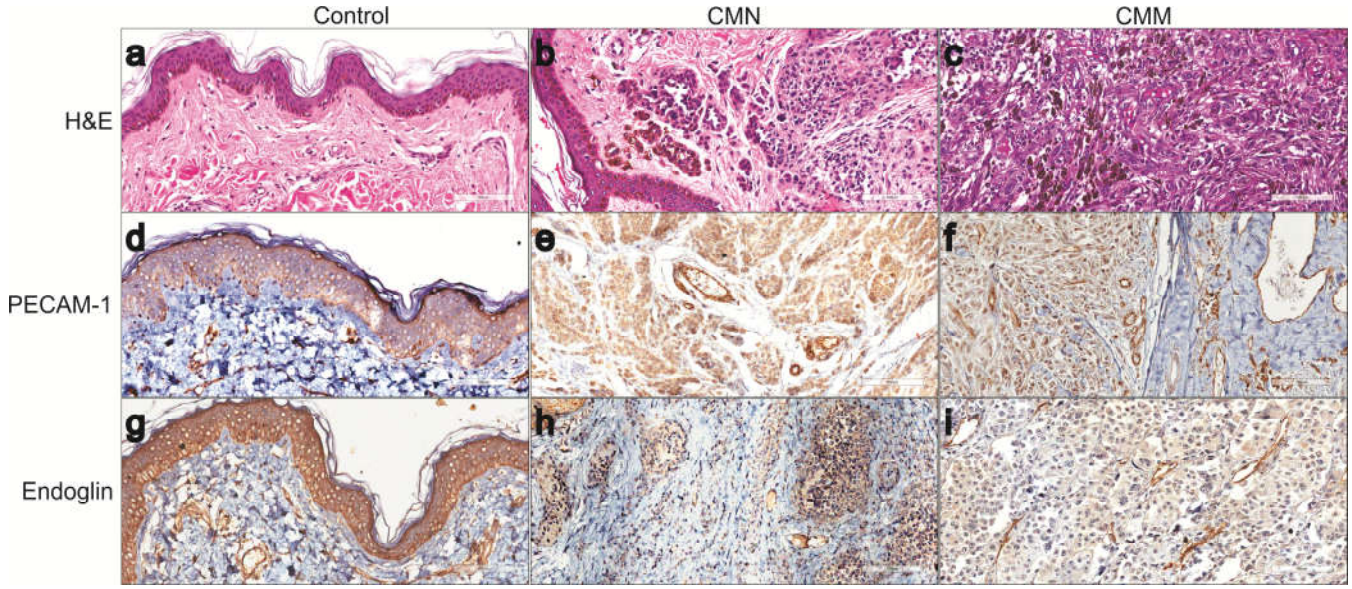

Figure 1. Morphological aspects of normal skin (control), cutaneous benign melanocytic nevi (CMN), and malignant melanoma (CMM) samples (Figures A, B, and C, respectively. H&E staining; higher magnification of 400x). Immunohistochemical expression of PECAM-1 (Figures D, E, and F) and endoglin (Figures G, H, and I) proteins in samples of control, CMN, and CMM (immunostaining: DAB; counterstaining: Mayer's hematoxylin; higher magnification of 400X)

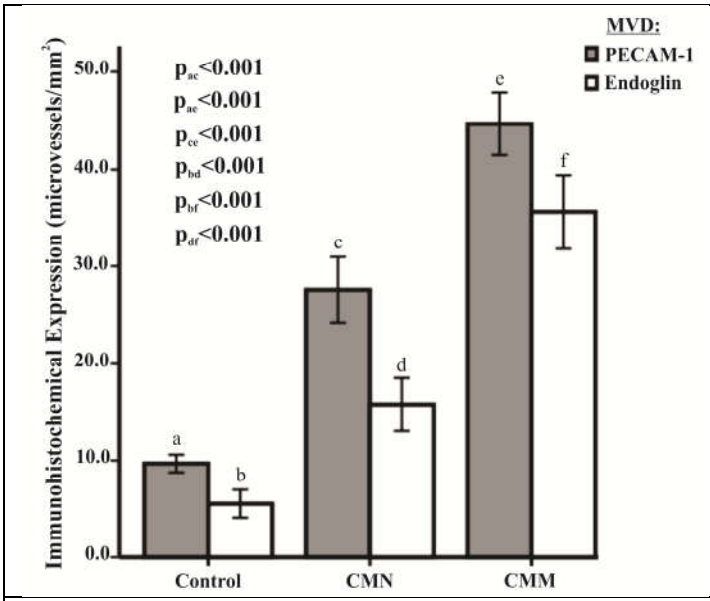

Figure 2. Expression of PECAM-1 and endoglin proteins for MVD estimative in normal skin (control), cutaneous benign melanocytic nevi (CMN), and malignant melanoma (CMM) samples. Our findings showed a significant increase of both protein expressions from control to CMN and CMM samples. Statistical analyses were performed by using Student´s t test and Anova. Level of significance was set at α = 5% (p < 0.05)

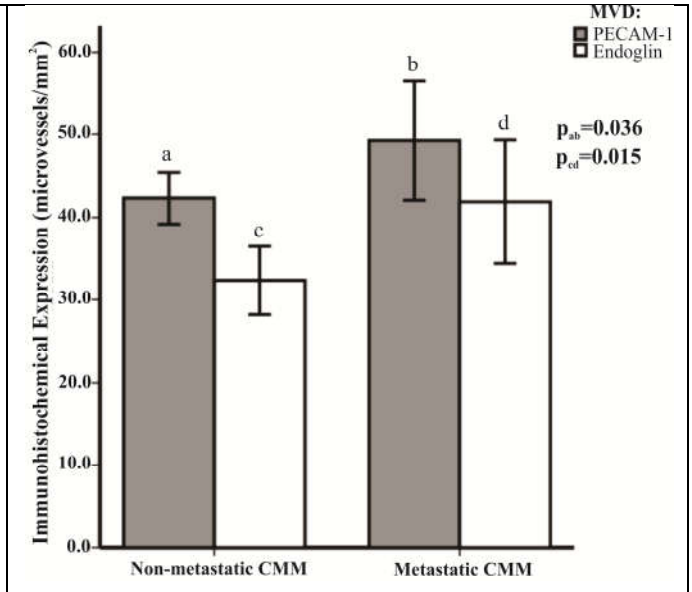

Figure 3. Expression of PECAM-1 and endoglin proteins for MVD estimative in primary metastatic and non-metastatic CMM samples. Our findings showed a significant increase of both protein expression in CMM lesions of individuals with metastatic disease (both local and distant). The statistical analyses were performed by using Student´s t test and ANOVA. Level of significance was set at α = 5% (p < 0.05)

The counts of immunostained cells for PECAM-1 and endoglin were performed using ImageJ® software, version 1.44 for Windows®. For MVD analyses, all samples of each group investigated were initially inspected at low magnification (40X) in order identify microscopic fields containing the greatest number of distinctly immunostained microvessels in normal or peritumoral stroma (referred to as *hot spots*). Microvessels were noted as isolated stained endothelial cells or transversally sectioned vascular tubes with a single layer of endothelial cells, either with or without a thin basement membrane. If two or more positive foci appeared to belong to a single continuous vessel, this was counted as only one microvessel. MVD estimated by PECAM-1 and endoglin expression were performed in each sample in three hot spots in which five microscopic areas were randomly selected for counting.

A total of 15 microscopic fields (total area of 2.69 mm^2) were considered to calculate MVD, expressed as microvessels/mm^2 (Weidner 1995, Vermeulen, Gasparini *et al*. 1996).

Statistical analyses: All data was transferred and statistically tested using SPSS® 18.0 software (SPSS Inc., Illinois, USA). Statistical analyses between clinicopathological variables related to CMM group and MVD were performed using Student's t test. Association between PECAM-1 and endoglin expression and control, CMN, and CMM groups were tested using analysis of variance (Anova), after Bonferroni correction. Median follow-up time for CMM individuals was calculated using the reverse Kaplan-Meier method. Differences between groups were considered as statistically significant when $p < 0.05$.

RESULTS

PECAM-1 and endoglin immunohistochemical expression showed selective reactivity for isolated endothelial cells or immature/mature vascular structures located in normal or peritumoral stroma areas of skin, benign CMN, and CMM samples, respectively (Figure 1). PECAM-1 and endoglin expression was detected in controls (9.7 ± 1.4 and 5.5 ± 2.3, respectively), CMN (27.6 ± 15.8 and 15.8 ± 9.5, respectively), and CMM (44.7 ± 10.5 and 35.6 ± 12.6, respectively). Our findings showed CMM samples with higher PECAM-1 and endoglin expression compared to controls ($p < 0.001$ and $p < 0.001$, respectively) and CMN ($p < 0.001$ and $p < 0.001$, respectively) (Figure 2). Table 1 shows the association between clinicopathological variables related to CMM and MVD analyses. A significant association was noted between the occurrence of metastatic disease (both local and distant) and higher MVD performed for PECAM-1 ($p = 0.036$) and endoglin ($p = 0.015$) (Figure 3).

Table 1. Analysis between the microvessel density (MVD) determined by both PECAM-1 and endoglin expression and clinicopathological factors related to CMM

Variables	MVD			
	PECAM-1 Expression	p	Endoglin Expression	p
Anatomical site				
Low risk (n=28)	45.4 (± 11.9)	0.586	34.8 (± 14.4)	0.594
High risk (n=16)	43.5 (± 7.8)		37.0 (± 8.7)	
Clinical Size				
Small (n = 9)	42.8 (± 9.0)	0.516	31.1 (± 13.3)	0.197
Large (n = 35)	45.3 (± 11.0)		36.9 (± 12.2)	
Ulceration in Primary Tumor				
Absent (n = 38)	45.2 (± 11.1)	0.477	35.4 (± 13.3)	0.775
Present (n = 6)	41.8 (± 5.5)		37.0 (± 6.4)	
Recurrence				
Absent (n = 10)	40.1 (± 7.1)	0.113	32.2 (± 9.5)	0.341
Present (n = 34)	46.1 (± 11.0)		36.6 (± 13.3)	
Level of Invasion				
I/II/III (n = 28)	45.2 (± 10.5)	0.687	35.6 (± 13.4)	0.986
IV-V (n = 16)	43.8 (± 10.2)		35.6 (± 11.3)	
Tumor Thickness				
<2 mm (n = 23)	42.7 (± 8.1)	0.183	33.0 (± 11.2)	0.147
≥ 2 mm (n = 21)	46.9 (± 12.5)		38.5 (± 13.5)	

* Values bearing asterisks show significant association using Student's t test and Anova tests.

DISCUSSION

Dynamic and sequential transformations occur from potentially malignant melanocytic cutaneous lesion to melanoma in susceptible individuals. Among these transformations, the stroma acquires an active and emergent vascular network that plays pivotal roles for both CMM tumorigenesis and progression (Barnhill, Fandrey *et al*. 1992, Streit and Detmar 2003, Ribatti, Annese *et al*. 2010). PECAM-1 is considered a pan-endothelial marker that identifies mature blood vessels, with minimal reactivity for lymphatic and macrophage (Horak, Leek *et al*. 1992, Zhou, Christofidou-Solomidou *et al*. 1999). On the other hand, endoglin has been considered a reliable marker of proliferating ECs, and therefore, angiogenesis (Li, Sorensen *et al*. 1999). In this study, we noted a significant, gradual increase of MVD from control to CMN to CMM samples. Additionally, we showed that CMM samples of individuals with metastatic disease exhibited a higher MVD. It has been shown that melanoma cells with high catabolic/metabolic and energetic demands need to induce a rich vascular network in the peritumoral stroma to sustain tumor progression (Michaylira and Nakagawa 2006, Helfrich and Schadendorf 2011). Notably, CMM exhibits a rapid progression towards widespread metastatic dissemination even when the primary tumor presents with small clinical size or microscopic thickness (Breslow 1970, Bedrosian, Faries *et al*. 2000, Corsetti, Allen *et al*. 2000). Premature metastatic behavior from melanoma cells has been associated with the developmental origins of melanocytes (from neural crest cells that have remarkable migratory behavior) (Gupta, Kuperwasser *et al*. 2005) and the presence of lymphatic and blood vascular networks in the stroma, which gradually increases with CMM progression (Barnhill, Fandrey *et al*. 1992). Malignant melanoma cells release various angiogenic growth factors that promote tumor angiogenesis from early stages of CMM progression (Vacca, Ribatti *et al*. 1993, Wanebo, Argiris *et al*. 2006, Elias, Hasskamp *et al*. 2010). A higher MVD identified by endoglin expression in our CMM samples suggests that melanoma cells might contribute to stimulation of EC proliferative activity. In turn, infiltrative melanoma cells that engage in a vascular metastatic route are capable of disseminating and establishing metastatic niches in target organs. The association between high MVD expression and metastatic CMM has been noted in some studies (Kashani-Sabet, Sagebiel *et al*. 2002, Valencak, Heere-Ress *et al*. 2004, Depasquale and Thompson 2005, Demirkesen, Buyukpinarbasili *et al*. 2006) but not in others (Busam, Berwick *et al*. 1995, Hillen, van de Winkel *et al*. 2006). These controversial findings are related to some factors such as the diversity of angiogenic antigens (e.g. factor VIII-related antigen, vascular endothelial growth factors, kinase insert domain receptor, ulex europaeus lectin-1, PECAM-1, CD34, endoglin, collagens types IV and XVIII, laminin, and neuropilin-1) that have been used to detect blood vessels and quantify the MVD of various human solid malignancies, including CMM. Moreover, the vascular density analysis performed with non-standardized microscopic assessment might contribute to the conflicting findings between MVD neoangiogenesis and metastasis, along with other clinicopathological factors (Vermeulen, Gasparini *et al*. 1996). Recent studies have investigated the presence of other vascular abnormalities that also result in a gain of vascularity for CMM, such as augmentation of the angiogenic response by recruitment of circulating progenitor ECs (Furuhashi, Sjoblom *et al*. 2004), vessel cooption (Dome, Paku *et al*. 2002), and vasculogenic mimicry (Maniotis, Folberg *et al*. 1999). Our study has some limitations that should be highlighted. First of all, the sample size is not large. Moreover, its cross-sectional design results in difficulty in establishing causal inferences. On the other hand, our findings add more evidence that MVD identified by immunostaining of PECAM-1 and endoglin might constitute a potential target in the selection of high risk CMM patients for complementary anti-angiogenic therapeutic strategies. However, further studies are necessary to clarify these possibilities. In conclusion, our findings suggest that higher MVD from normal skin to benign and malignant melanocytic tumors play important roles in supporting the growth of melanocytic neoplastic cells and favoring the dissemination of infiltrating melanoma cells.

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