

# Induction of Vascular Endothelial Growth Factor Receptor-3 Expression on Tumor Microvasculature as a New Progression Marker in Human Cutaneous Melanoma<sup>1</sup>

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## ABSTRACT

The anatomical relation between a malignant tumor and its vascular and lymphatic bed is an important factor influencing metastasis. Lack of specific markers for the lymphatic endothelium has long hampered a reliable detection of lymphatics. Here, we demonstrate that lymphatic endothelium can reliably be identified in a panel of different normal tissues and of benign and malignant tumors. Application of the previously described PAL-E/CD31 double staining protocol differentiates between blood capillaries and veins on one hand and lymphatic vessels on the other. Blood vessel marker CD34, absent from lymphatics, was used additionally to classify arteries. We found that the lymphatic vascular endothelial growth factor receptor-3 (VEGFR-3, also known as Flt-4) was present on both lymphatic and blood vessels in 76 of 113 malignant tumors [adenocarcinoma of kidney ( $n = 3$ ), colon ( $n = 3$ ) and liver ( $n = 3$ ), breast ( $n = 9$ ) and squamous cell carcinoma ( $n = 5$ ), primary ( $n = 81$ ), and metastatic ( $n = 9$ ) melanoma]. No evident signs of tumor-induced lymphangiogenesis were observed. Evaluation of a series of 110 melanocytic skin lesions indicated that VEGFR-3 expression is confined to the lymphatic vasculature in benign lesions. However, its expression emerges on the blood neovasculature in malignant lesions as soon as metastatic potential develops. We conclude that induction of VEGFR-3 expression on tumor blood vessels may be a general phenomenon that would make VEGFR-3 a marker for tumor endothelium. In addition, we propose VEGFR-3 expression as a new microvascular progression marker in cutaneous melanoma.

## INTRODUCTION

The dependence of tumors on the presence of a vascular bed for outgrowth and metastasis has now been firmly established (1, 2). Although tumor cell invasion of lymphatics worsens prognosis significantly, (3, 4) it is still unknown why several tumors have a tendency to spread via the lymphatic bed. Although evaluation of the anatomical relation between tumor mass and lymphatic bed has not been studied in much detail because of the lack of specific lymphatic markers, (5) the possibility that the formation of new lymphatic vasculature (lymphangiogenesis) may occur in human malignant tumors is suggested by several animal studies demonstrating that xenografted tumors induced lymphangiogenesis as a result of overexpression of VEGF<sup>3</sup>-C or D (6–10).

More recently, several different specific lymphatic endothelial markers have been described that are not yet widely available however (11–14). Therefore, blood and lymphatic vessels have been differentiated often by exploiting histomorphological differences (15–17). In a previous study (18), we developed a staining protocol (using the pan-endothelial marker CD31 and the blood vessel endothelial marker PAL-E) that differentially highlights blood and lymphatic

vasculature in normal skin and primary melanoma lesions. Arteries are not decorated by this staining protocol, but this problem was overcome by supplementing the staining protocol with a mAb to the endothelial marker CD34 (19). Staining for CD34 of the endothelia of arteries, venules, and capillaries has been demonstrated in normal and tumor tissues (15–17). The issue whether CD34 is expressed on lymphatic vessels is not completely settled since its absence (12, 15) but also a variable, presumed lymphatic staining have been described (16, 17, 20).

Another direct way of identification of lymphatics was more recently reported using the 9D9 mAb (21). 9D9 specifically recognizes the VEGFR-3 (also known as Flt-4) that is expressed on lymphatic endothelium in normal adult tissues (22). However, the use of VEGFR-3 as a marker for lymphatic vessels in human disease is dubious because VEGFR-3 expression on blood vessels has been reported in different types of malignant tumors and granulation tissue (19, 23–25).

Recent data suggest that VEGFR-3 expression on blood vessels may be a general phenomenon in human cancer (19, 23, 24). Furthermore, if this expression does not occur in normal tissues, it may have biological and clinical implications. Theoretically, it may be related to the rate of malignancy and therapeutical interventions using VEGFR-3 as a target might selectively reduce tumor growth by inhibition of both hemangiogenesis and lymphangiogenesis with possibly little side effects. In human skin, proliferation of melanocytes can give rise to common acquired melanocytic nevus, atypical (dysplastic) nevus, melanoma *in situ*, primary melanoma, and melanoma metastasis (referred to as stages of melanoma progression). The probability of metastasis considerably rises once the stage of a thick (lesion thickness > 1.5 mm) primary melanoma lesion has been reached. In this study, this series of melanocytic lesions was taken as a model to evaluate whether VEGFR-3 expression on tumor blood vessels may serve as a tumor progression marker.

Thus, we evaluated blood and lymphatic vessel staining by the PAL-E/CD31 staining protocol and by the QBEnd/10 (CD34) and 9D9 (anti-VEGFR-3; Refs. 21, 22) mAbs in a panel of different human normal and tumor tissues. The analysis focused on the following questions: (a) can blood and lymphatics be identified using serial sections stained by PAL-E/CD31 and CD34 in these tissues; (b) is VEGFR-3 expressed on blood vessels in the included tumor types; (c) are intratumoral lymphatics present in the various tumor types and can signs of lymphangiogenesis be observed; and (d) is VEGFR-3 expression on blood vessels a tumor progression marker?

## MATERIALS AND METHODS

**Patient Material.** Frozen specimens of 20 normal and 52 tumor human tissues (Table 1) were obtained from the pathology archives of the University Hospital Nijmegen where they were stored at  $-130^{\circ}\text{C}$ . Additional primary melanoma lesions ( $n = 10$ ) were kindly provided by Dr. Uta Hofmann (Universitäts-Hautklinik, Würzburg, Germany). Sixty-eight paraffin-embedded primary cutaneous melanoma lesions (36 tumors with a Breslow diameter of <0.75 mm; 17 tumors between 0.76–1.50 mm; 7 tumors between 1.50–3.00 mm; and 8 tumors of >3.00 mm) and three paraffin-embedded squamous cell carcinomas of the larynx were obtained from our pathology archives. All specimens had been diagnosed by a pathologist.

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<sup>3</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; mAb, monoclonal antibody; PAb, polyclonal antibody; MVD, microvascular density; HGP, horizontal growth phase; VGP, vertical growth phase.

Table 1 *Tissues included in this study*<sup>a</sup>

Normal tissues	Tumor tissues
Breast ( <i>n</i> = 3)	Breast cancer (invasive intraductal carcinoma, <i>n</i> = 8; invasive lobular carcinoma, <i>n</i> = 1)
Colon ( <i>n</i> = 3)	Colon (adenocarcinoma, <i>n</i> = 3)
Kidney ( <i>n</i> = 3)	Kidney (adenocarcinoma, <i>n</i> = 3)
Liver ( <i>n</i> = 3)	Liver (hepatocellular carcinoma, <i>n</i> = 3)
Preputial skin (5)	Larynx (squamous cell carcinoma, <i>n</i> = 5)
Skin ( <i>n</i> = 3)	Skin (common acquired melanocytic nevus, <i>n</i> = 10) <sup>b</sup>
	Skin (atypical (dysplastic) nevus, <i>n</i> = 10) <sup>b</sup>
	Skin (melanoma <i>in situ</i> , <i>n</i> = 7) <sup>b</sup>
	Skin (superficial spreading melanoma lesions, <i>n</i> = 60) <sup>b</sup>
	Skin (nodular melanoma lesions, <i>n</i> = 11) <sup>b</sup>
	Skin (acrolentiginous melanoma lesions, <i>n</i> = 3) <sup>b</sup>
	Skin (melanoma metastasis lesions, <i>n</i> = 9) <sup>b</sup>

<sup>a</sup> In total, 20 normal and 133 tumor tissues were included.

<sup>b</sup> Stages of melanoma progression.

**Antibodies.** The mAbs used for immunohistochemistry were the endothelial markers CD34 (QEnd/10; Dako, Glostrup, Denmark) and VEGFR-3 (9D9; Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, Helsinki, Finland), the pan-endothelial marker CD31 (PECAM-1; British Biotechnology, ITK Diagnostics, Uithoorn, the Netherlands), and the blood vessel endothelial marker mAb PAL-E (University Medical Centre, Nijmegen, the Netherlands). PABs to VEGFR-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Immunohistochemistry.** By H&E staining, representative sections of the included normal or tumor tissues (Table 1) were identified. Areas in the normal tissue sections corresponding to tumor sites were used as controls. On three successive sections of each specimen, the distribution of 9D9, QEnd/10, and PAL-E mAbs binding was evaluated using a standard three-step avidin-biotin complex method (Vectastain, Vector Laboratories) as described previously (18). In part of the tumor tissues, a similar staining procedure was conducted with the PABs to VEGFR-3. All sections were developed in 3-amino-9-ethyl-carbazole solution (Aldrich, Steinheim, Germany). Sections pre-stained with PAL-E were incubated with the anti-CD31 mAb and developed with a mixture of Fast Blue, naphthol phosphate and levamisole (Sigma-Aldrich, Bornem, Belgium). In paraffin-embedded serial sections, 9D9 and QEnd/10 mAbs were detected as described above.

In control sections, primary antibodies were omitted. The following tissues were included as positive controls: normal skin, melanoma and breast carcinoma lesions in case of PECAM-1, PAL-E, and 9D9 stainings (18, 23, 24, 26); and hepatocellular carcinoma in case of QEnd/10 (27–29). The 9D9 and QEnd/10 stainings were counterstained for 45 s with Harris' hematoxylin (Merck, Darmstadt, Germany) at room temperature. All sections were mounted in Insol-mount medium (Klinipath B.V., Duiven, the Netherlands).

**Determination of Blood Vessel Density.** In case of the primary melanoma lesions, we selected the superficial spreading melanoma lesions to evaluate the MVD as a parameter of the extent of hemangiogenesis, which is a measure for tumor progression. The nodular and acrolentiginous melanoma lesions were excluded to avoid biasing data obtained. By visual examination of the PAL-E/CD31 and CD34 immunostainings at  $\times 63$  magnification, blood vessels in 10 nonoverlapping fields/tumor lesion were counted and averaged. Vascular counts included complete cross sections, partial cross sections, and small groups of positive cells.

## RESULTS

**Evaluation of the PAL-E/CD31 and CD34 Staining Protocols in Frozen Sections.** In all normal and tumor tissues (Table 1), both immunostainings decorated vascular structures, which could be identified on the basis of their architecture and characteristic staining pattern, as described earlier (Refs. 15–18; Table 2, Fig. 1A–D).

As previously shown, (18, 19) the PAL-E/CD31 double staining differentially highlighted blood and lymphatic capillary beds in all normal (Fig. 1A) and tumor tissues (Fig. 1B). Because PAL-E staining on arterial vessels was absent (30), PAL-E-negative and CD31-positive (CD31<sup>+</sup>/PAL-E<sup>-</sup>) ones were classified as lymphatic or arterial vessels, whereas PAL-E and CD31-positive (PAL-E<sup>+</sup>) ones were

classified as blood vessels (18, 19). Staining of infiltrating leukocytes by CD31 was prominent as well (Fig. 1B).

Compared with the PAL-E/CD31 double staining, the anti-CD34 mAb stained PAL-E<sup>+</sup> blood vessels and CD31<sup>+</sup>/PAL-E<sup>-</sup> vessels in all included tissues. Using the differences in morphological characteristics of arterial and lymphatic vessels, the CD34<sup>+</sup> and CD31<sup>+</sup>/PAL-E<sup>-</sup> vessels could be classified as arteries. No staining of lymph vessels by the anti-CD34 mAb was observed in any of the normal tissues and tumor tissues (Fig. 1, C and D). Also, stromal areas in sections of colon, colon carcinoma, and (adnexal structures in) skin were positive using CD34 immunohistochemistry.

**Vascular VEGFR-3 Expression in Normal and Tumor Tissue on Frozen Sections.** Despite extensive testing, immunostaining by the anti-VEGFR-3 PABs in our hands resulted in a high background staining of especially stromal areas and unreliable, irreproducible staining of the vasculature. Therefore, we excluded these PABs from additional analysis.

Using serial PAL-E/CD31 and CD34 stainings, VEGFR-3 expression detected by the anti-VEGFR-3 mAb could be localized on lymphatic vessels in all normal tissues (Table 2, Fig. 1E). No or very weak expression of VEGFR-3 on blood vessels was observed. However, we observed expression of VEGFR-3 on both blood and lymphatic vessels in 33 of 42 investigated malignant tumors (Tables 2 and 3, Fig. 1, B and F). In most malignant tumors, blood vessels surrounding nests of tumor cells were stained by the anti-VEGFR-3 mAb. In kidney and hepatocellular carcinoma, a more intense staining of the intratumoral blood vessels occurred compared with the other tumor types. In several malignant tumors within one section, both negative and positive VEGFR-3 staining of blood vessels was observed. The VEGFR-3-positive blood vessels were present in areas directly surrounding the tumor cells, whereas VEGFR-3-negative ones were localized at further distance (Fig. 1F).

**Lymphatics and Lymphangiogenesis in Tumor Tissue.** Presence of lymph vessels of kidney and liver carcinoma lesions were confined to the peritumoral areas. In common acquired melanocytic nevi, atypical (dysplastic) nevi, primary and metastatic melanoma, breast, colon, and larynx carcinoma lesions, lymph vessels were also observed in the stroma between nests of melanocytes or tumor cells. These vessels may represent preexisting lymphatics or may be newly formed (Fig. 1B). However, we were unable to observe evident signs of lymphangiogenesis such as locally increased numbers of lymphatics or lymphatic sprouting. In addition, no major differences in localization or lymphatic density could be observed in the different stages of melanoma progression.

**VEGFR-3 Expression in Different Stages of Melanoma Progression.** The stages of melanoma progression are represented by the benign lesions common acquired melanocytic nevus, the premalignant lesions atypical (dysplastic) nevus and melanoma *in situ* and the malignant lesions primary cutaneous melanoma (composed of superficial spreading, nodular, and acrolentiginous melanoma) and mela-

Table 2 *Summary of the endothelial staining patterns in frozen sections*

Antigen	mAb	Normal tissues		Tumor tissues	
		Blood vessel	Lymphatics	Blood vessel	Lymphatics
CD31	PECAM-1	<sup>a</sup>	<sup>+</sup> <sup>b</sup>	<sup>a</sup>	<sup>+</sup>
Unknown	PAL-E	<sup>+</sup>	<sup>-</sup>	<sup>+</sup>	<sup>-</sup>
CD34	QEnd/10	<sup>+</sup>	<sup>-</sup>	<sup>+</sup>	<sup>-</sup>
VEGFR-3	9D9	<sup>-</sup>	<sup>+</sup>	<sup>+</sup> <sup>c</sup>	<sup>+</sup>

<sup>a</sup> Because PAL-E and anti-CD31 antibodies were used in a double staining protocol, it is not possible to identify CD31 staining of the capillary and venous blood vessels. Capillary and venous CD31 staining is overruled by the PAL-E staining (Figs. 1–3; Ref. 18).

<sup>c</sup> The occurrence of blood vessel staining in the various tumors by the anti-VEGFR-3 mAb is shown in more detail in Table 3.

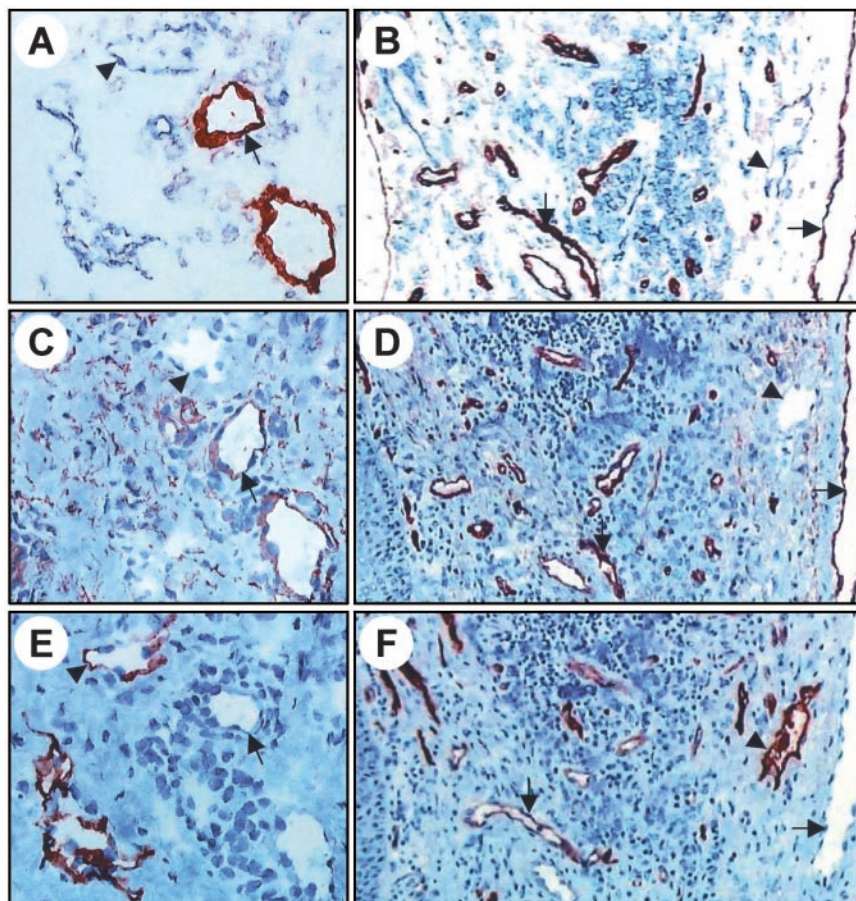


Fig. 1. Immunohistochemical analysis of the vasculature in serial sections of preputial skin (A, C, E) and of squamous cell carcinoma of the larynx (B, D, F); magnification:  $\times 200$ . A and B, differential staining of capillary and venous blood vessels and lymph vessels. Blood vessels (arrows) are stained red by mAb PAL-E, lymph vessels (arrowheads) and arteries are stained blue by anti-CD31 [note that the blue blood vessel (except arteries) endothelial staining by anti-CD31 is overruled by the red staining by PAL-E<sup>18</sup>]. C and D, adjacent sections stained with the mAb QBEnd/10 (CD34). Numerous stromal cells are CD34 positive. E and F, adjacent sections stained with the mAb 9D9 (VEGFR-3). No staining is observed of the blood vessel (arrow), whereas lymphatics are positive (arrowhead) in the preputial skin (E). Both VEGFR-3-positive (left arrow), near the tumor cells (T), and -negative blood vessels (right arrow) at a greater distance from the tumor were observed (F). The arrowheads in A, C, E and B, D, F mark identical lymph vessels and the arrows identical blood vessels. In C-F, the QBEnd/10 and 9D9 stainings were counterstained with Harris' hematoxylin.

noma metastasis (Table 1). In these lesions, we analyzed vascular VEGFR-3 expression to assess its value as a progression marker. Sections of all 42 frozen melanoma lesions listed above were evaluated. In addition, of the primary melanoma lesions, all 68 paraffin-embedded tumors were used. Lymphatic staining by the VEGFR-3 mAb was comparable between frozen and paraffin-embedded sections facilitating comparison of expression of VEGFR-3 by blood vessel endothelial cells in all specimens.

By comparison to a parallel PAL-E/CD31 double staining (frozen sections) or CD34 staining (paraffin-embedded sections), it became evident that VEGFR-3 expression in benign melanocytic lesions was strictly confined to lymphatics, whereas in 55 of 83 malignant melanoma lesions, it was present on both lymphatic and blood vessels (level of significance:  $P < 0.0001$ , two-sided Fisher's exact test; Table 3, Figs. 2-4). In two benign lesions, VEGFR-3 was also found on dendritic Langerhans cells in the epidermis (Fig. 2A). In two superficial spreading melanoma lesions, extensive positivity of the epidermis, tumor cells, and the epidermal area (*i.e.*, blood and lymphatic endothelial cells, fibroblasts) was observed. Occasionally, dull VEGFR-3 staining of melanoma-associated macrophages was observed.

In case of primary melanoma lesions, we analyzed our data with respect to tumor thickness according to Breslow (31), which is the most prominent prognostic marker in cutaneous melanoma. As shown in Table 3, none of the seven primary melanoma *in situ* lesions showed VEGFR-3 expression on dermal blood vessels, whereas all three acrolentiginous melanoma (thickness between 0.7 and 3.2 mm; median, 0.9 mm) expressed VEGFR-3 on their blood vessels. In case of 60 superficial spreading melanoma lesions, 28 lesions expressing no VEGFR-3 (thickness between 0.15 and 1.8 mm; median, 0.60 mm)

appeared to be thinner compared with 32 remaining lesions showing VEGFR-3 blood vessel expression (thickness between 0.3 and 7 mm; median, 1.2 mm;  $P = 0.0005$ , Mann-Whitney *t* test). In all 11 nodular melanoma (thickness between 2.1 and 11 mm; median, 4.2 mm), VEGFR-3 was present on the blood vasculature.

In 40 of 46 VEGFR-3-positive primary melanoma lesions, expression was present on a subset of blood vessels in restricted areas (often located at the invasive front of the tumor), whereas in the remaining 6 primary and 8 metastatic lesions, VEGFR-3 expression was ubiquitous (Fig. 3, A-D). No significant difference in thickness between the primary melanoma lesions with localized or ubiquitous VEGFR-3 expression was present.

Using routine H&E staining, all 60 superficial spreading melanomas were classified as HGP or VGP according to Elder *et al.* (32) and modified by Cook *et al.* (33). A higher number of VGP melanomas

Table 3 Incidence of VEGFR-3 expression on tumor blood vessels

Tumor type	Positive cases <sup>a</sup>
Breast (invasive intraductal and lobular carcinoma)	9/9
Colon (adenocarcinoma)	2/3
Larynx (squamous cell carcinoma)	4/5
Kidney (adenocarcinoma)	3/3
Liver (hepatocellular carcinoma)	3/3
Skin (common acquired melanocytic nevus)	0/10
Skin (atypical (dysplastic) nevus)	1/10
Skin (melanoma <i>in situ</i> )	0/7
Skin (superficial spreading melanoma lesions)	32/60
Skin (nodular melanoma lesions)	11/11
Skin (acrolentiginous melanoma lesions)	3/3
Skin (melanoma metastasis lesions)	9/9

<sup>a</sup> Positivity was defined as staining (as shown in Figs. 1 and 3) by the mAb 9D9 (anti-VEGFR-3) of PAL-E<sup>+</sup> or CD34<sup>+</sup> vessels.

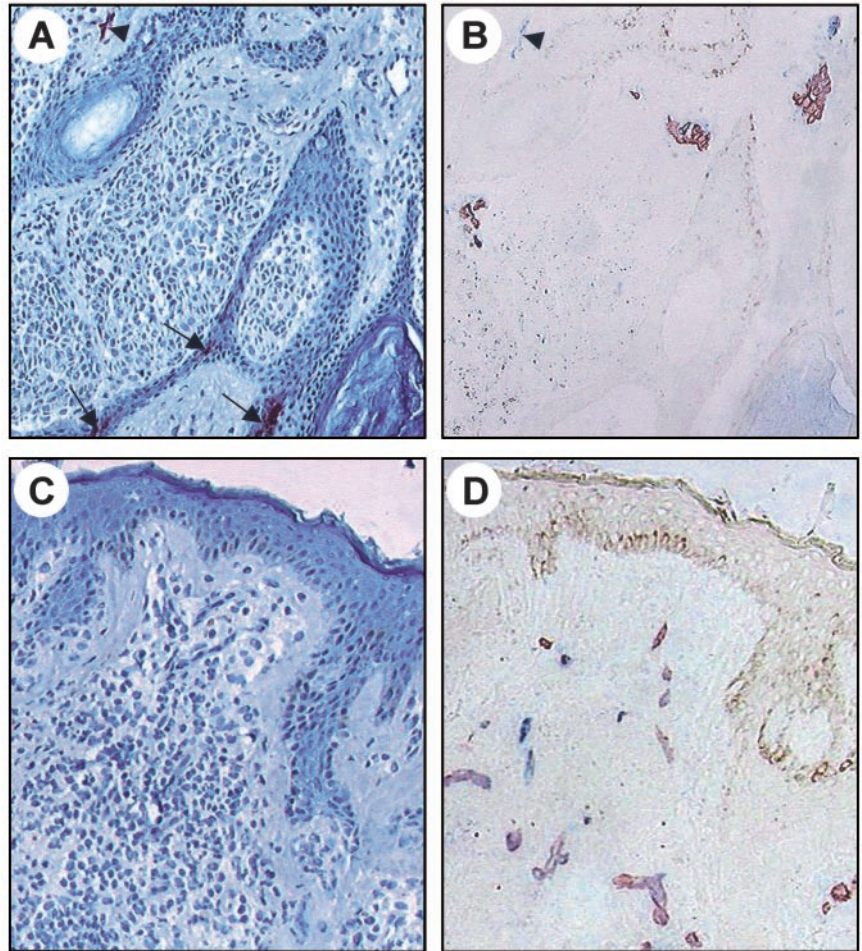


Fig. 2. Immunohistochemical analysis of the VEGFR-3 expression using the 9D9 mAb (A and C). VEGFR-3 expression was localized on the vasculature by comparison to a serial section stained by the PAL-E/CD31 double staining protocol (B and D) in the common acquired melanocytic nevus (A and B) and dysplastic nevus (C and D). Blood vessels are stained red by mAb PAL-E; lymph vessels and arteries are stained blue by anti-CD31 (B and D). VEGFR-3 is present on lymphatic endothelium (corresponding arrowheads, A and B), whereas blood vessels are negative (A–D). VEGFR-3 positivity of Langerhans cells is indicated by the arrows (A). Sections in A and C were counterstained with Harris' hematoxylin. Magnification:  $\times 100$ .

contained blood vessels expressing VEGFR-3 (24 of 35 tumors) compared with the number of melanomas in HGP (7 of 25;  $P = 0.008$ , Fisher's exact test). Next to growth phase classification (*i.e.*, HGP and VGP), which relates to the pattern of tumor invasion, we related vascular VEGFR-3 expression to the anatomical level of invasion, as defined by Clark *et al.* (34). As shown in Fig. 5, vascular VEGFR-3 expression is absent in noninvasive melanoma (Clark I) and the majority of minimal invasive tumors (Clark II), whereas vascular VEGFR-3 becomes present in the majority of tumors filling up the papillary or invading the reticular dermis (Clark III–IV;  $P < 0.0001$ ,  $\chi^2$  test).

Seven of 17 most thin superficial spreading melanomas (thickness  $\leq 0.5$  mm) contained VEGFR-3-positive blood vessels. Five of these positive melanomas were classified as VGP and the remaining 2 as HGP, which both showed clear signs of regression (Fig. 3, E and F). The remaining 10 thin VEGFR-3-negative melanomas were all classified as HGP. In addition, in 4 of the positive tumors, evident signs of immunological regression were present. In total, 7 of 9 superficial spreading melanomas with evident signs of regression contained VEGFR-3-positive blood vessels.

The MVD in VGP lesions (mean density between 1.5–3.4 vessels/field, mean 2.1 vessels/field) was significantly increased compared with the HGP lesions (mean density between 0.85–2.4 vessels/field; mean, 1.4 vessels/field; Mann-Whitney  $t$  test,  $P = 0.03$ ), confirming earlier results (18) and indicating a process of hemangiogenesis. In tumors containing vascular VEGFR-3 expression, the MVD lesions (mean density between 1.6–3.4 vessels/field; mean, 2.0 vessels/field) was also significantly increased compared with tumors without

VEGFR-3 expression lesions (mean density between 0.85–2.7 vessels/field; mean, 1.4 vessels/field; Mann-Whitney  $t$  test,  $P = 0.02$ ).

Data whether metastasis occurred were available in 57 (of 74) primary superficial spreading, nodular, and acrolentiginous melanoma lesions [follow-up time,  $4.1 \pm 2.2$  years (mean  $\pm$  SD)]. In this subset of 57 lesions, vascular VEGFR-3 expression was not related to the rate of metastasis ( $P = 0.22$ , Fisher's exact test), although in 6 of 7 metastatic lesions, VEGFR-3 was expressed on tumor blood vessels.

## DISCUSSION

In the past, it has been difficult to study lymphatic vasculature because of a lack of specific endothelial markers (5). Here, we further extended the use of PAL-E/CD31 double staining in different normal and tumor tissues. Our results confirmed earlier studies (18, 30, 35) on endothelial specificity of these markers. Identification of lymphatics by the PAL-E/CD31 double staining protocol depends on the lack of lymphatic staining by the PAL-E mAb. Because of the absence of PAL-E staining on arterial vessels, antigen is present on all blood vessels with exception of arteries (30, 36), PAL-E-negative vasculature could either be of lymphatic or arterial origin. Fortunately, these types of vasculature can easily be distinguished from each other by their morphological properties. The expression of CD31 by infiltrating cells may also hamper lymphatic detection in areas of extensive infiltration. So, with some limitations, the PAL-E/CD31 double staining is a reliable technique to classify vessel types. In agreement with previous studies (12, 15, 17), CD34 was not detected on lymphatic channels in normal tissues. Although variable CD34 immunostaining

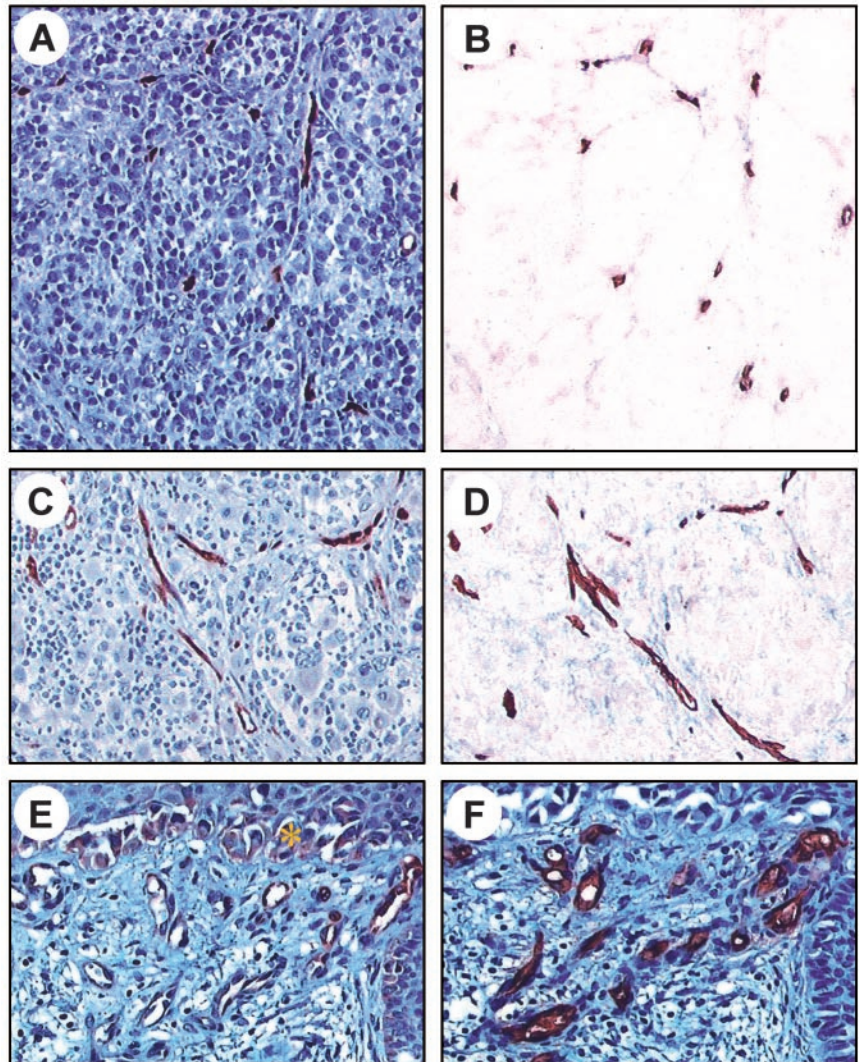


Fig. 3. Immunohistochemical analysis of the VEGFR-3 expression using the 9D9 mAb (A, C, E). VEGFR-3 expression was localized on the vasculature by comparison to a serial section stained by the PAL-E/CD31 double staining protocol (B and D) and anti-CD34 MAb (F). Blood vessels are stained red by mAb PAL-E; lymph vessels and arteries are stained blue by anti-CD31 (B and D). In primary nodular melanoma lesions (A and B) and cutaneous metastatic melanoma lesions (C and D), VEGFR-3 is present on the blood vasculature. VEGFR-3 is expressed on blood vessels in areas of regression in a superficial spreading melanoma (HGP; E and F). Melanoma cells (E; \*) sometimes expressed VEGFR-3 as well. Sections in A, C, E, and F were counterstained with Harris' hematoxylin. Magnification:  $\times 100$  (A and B);  $\times 200$  (C–F).

of lymphatics was observed by others in a variety of vascular and lymphatic neoplasms (16, 17, 20), this may be explained by their use of conventional histological criteria for vessel classification (15–17). No direct comparison with a specific lymphatic staining was performed in these studies. We therefore suppose that these presumed lymphatics may have been, in fact, blood vessels. By combining the PAL-E/CD31 and CD34 stainings, lymphatics and blood vessels can reliably be detected in both normal and tumor tissues.

Recently, an elevated number of VEGFR-3-positive blood vessels was found in different tumor types (19, 23, 24). By using the double staining protocol (18) that differentially highlights the lymphatic and blood vasculature, we have now confirmed these earlier results (19, 23, 24) in a panel of different tumor types, including breast carcinoma, melanoma, squamous cell carcinoma of the larynx and adenocarcinoma of colon, kidney, and liver. These earlier and our present results suggest that induction of blood vessel VEGFR-3 expression may be a general phenomenon in malignant human solid tumors, which makes it a potential marker for tumor blood vessel endothelium.

Recent studies (6–9) have provided strong experimental evidence that tumors are capable of activating tumor lymphangiogenesis. These studies indicate that tumor-induced lymphangiogenesis may occur, which was recently confirmed in head and neck cancer patients (37). In our study, in a number of lesions, lymphatics were predominantly present between tumor cells in the stromal areas. The limited number

of the samples/tumor type studied did not allow for a clear distinction between preexistent or tumor-induced lymphatic neovasculature. A higher lymphatic density in areas directly surrounding the tumor could not be determined. However, it is remarkable that we did not observe

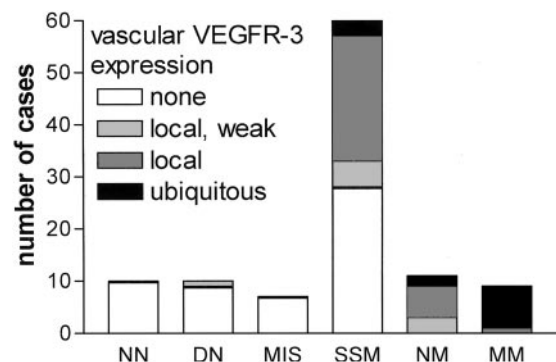


Fig. 4. Bar diagram showing the distribution of vascular VEGFR-3 expression in the melanoma progression series, which includes common acquired melanocytic nevi (MN), dysplastic nevi (DN), melanoma *in situ* (MIS), superficial spreading melanoma (SSM), nodular melanoma (NM), and metastatic melanoma (MM). VEGFR-3 blood vessel expression was classified as absent, locally and weakly present, and locally present and ubiquitously present. For additional details, see text. Blood vessel VEGFR-3 expression was defined as staining (as shown in Figs. 2 and 3) by the mAb 9D9 (anti-VEGFR-3) of PAL-E<sup>+</sup> or CD34<sup>+</sup> vessels.

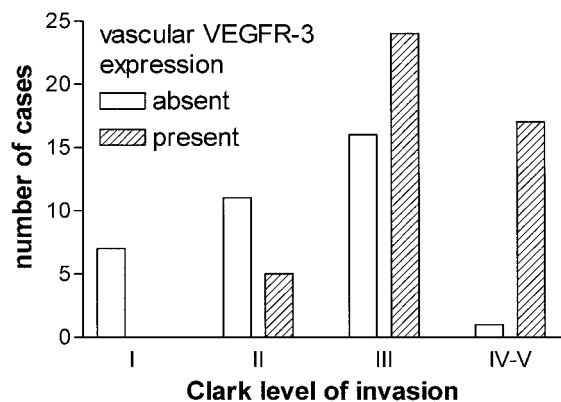


Fig. 5. Bar diagram showing the distribution of vascular VEGFR-3 expression in relation to the anatomical level of invasion according to Clark *et al.* (34) Only one nodular melanoma lesion containing VEGFR-3-positive blood vessels invaded the s.c. tissue (Clark V).

evident differences in localization and lymphatic vessel density during melanoma progression. This observation might suggest that the lymphatic vasculature may not be involved in the progression of benign melanocytic lesions to malignant melanoma. In addition, data of this study seems to confirm our previous finding that lymphangiogenesis does not occur in primary cutaneous melanoma (18). However, to evaluate lymphangiogenesis in human cancer, comparison of lymphatic density to that in normal tissue (in which the tumor arose) is absolutely necessary (18). This comparison requires the presence of both normal and tumor tissue in one biopsy, which was also often not the case in our series.

Despite that our data strongly suggest that blood vessel VEGFR-3 expression is a melanoma progression marker, it remains questionable whether blood vessel VEGFR-3 expression is predictive of clinical and therapeutic outcome. Evident expression of VEGFR-3 by blood vessel endothelial cells was confined to the malignant lesions, *i.e.*, those with dermal invasion and metastatic potential. In contrast, melanoma *in situ*, which by definition does not invade the basal membrane and lacks metastatic potential, lacked VEGFR-3 expression. Moreover, in general, thin microinvasive superficial spreading melanoma lesions (*i.e.*, <0.60 mm) appeared to express no VEGFR-3 on their vessels, whereas thicker superficial spreading and nodular melanomas did. The majority of thin superficial spreading melanoma lesions containing VEGFR-3-positive blood vessels showed signs of increased aggressiveness such as a VGP and regression (38). Finally, the localized VEGFR-3 expression in primary melanoma lesions compared with the ubiquitous expression in melanoma metastatic lesions also supports the hypothesis that VEGFR-3 expression gradually increases as a tumor becomes more malignant. So, following this hypothesis, evaluation of VEGFR-3 expression in melanoma lesions may be an interesting candidate for additional clinical evaluation. For example, similar as recently suggested for the presence of VEGF in cutaneous melanoma (39), detection of VEGFR-3 expression may be helpful in those cases when differential diagnosis of benign and malignant lesions by conventional diagnostic methods (H&E staining) is difficult (which is often the case in the differential diagnosis of Spitz nevi and melanoma). Finally, the presence of blood vessel VEGFR-3 expression would have major therapeutic advances: by targeting VEGFR-3 expression, tumor vasculature may selectively be affected.

Recently, an association between a negative clinical outcome and expression of P- and E-selectin by endothelium of intratumoral vessels in cutaneous melanoma has been reported (40). Together with our data, showing that primary and metastatic melanoma cells can cause

VEGFR-3 expression on blood vessels in the skin and in distant tissues, these data point at an evident interaction of melanoma cells and their microenvironment as discussed previously (41). In this respect, the question arises which factor induces VEGFR-3 expression on blood vessels. In breast carcinoma, blood vessels expressing VEGFR-3 were located adjacent to islets of tumor cells by which VEGF-C was expressed (24). The fact that VEGFR-3 expression is restricted to the direct vicinity of tumor nests suggests that tumor-derived factors induce the appearance of VEGFR-3 in a paracrine way. Obviously, members of the VEGF-family are first target of investigation. So, thus far, most recent studies have assessed characteristics of melanoma cells themselves to predict clinical outcome. However, it is very likely that functional and morphological evaluation of the interaction of melanoma cells and surrounding stroma may yield data of substantial biological and clinical relevance as well.

In conclusion, for lymphatic staining the PAL-E/CD31 double staining combined with CD34 immunohistochemistry is well suited. The presence of VEGFR-3 on tumor blood and lymphatic vessels is likely to play a role in mediating VEGF-C-induced hemangiogenesis and, perhaps, lymphangiogenesis. Our data suggest that induction of blood capillary and venous VEGFR-3 expression may be a general phenomenon in cutaneous melanoma, and it may therefore be a marker for tumor endothelium. Finally, this induction may correlate with the degree of malignancy in human cutaneous melanoma.

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## Induction of Vascular Endothelial Growth Factor Receptor-3 Expression on Tumor Microvasculature as a New Progression Marker in Human Cutaneous Melanoma

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