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Selective immunohistochemical staining shows significant prognostic influence of lymphatic and blood vessels in patients with malignant melanoma

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Abstract

Few data on the influence of lymphatic microvessel density (LMVD) on survival in patients with melanoma are available. The aim of this study was to assess LMVD and blood microvessel density (MVD) in tissue samples from 120 patients with melanoma. LMVD was stained with an antibody staining for podoplanin, and blood MVD was assessed by CD31 (PECAM-1)-immunostaining. Survival was determined using univariate and multivariate analysis. A significant association between a high CD31 MVD (but not LMVD) and the presence of lymph node metastases ($P=0.007$) was observed. Patients with a high LMVD had a significant shorter overall (OS) ($P=0.0436$) and disease-free survival (DFS) ($P=0.0249$) in univariate analysis. The survival analysis showed CD31 MVD was a strong prognostic factor for OS and DFS in both uni-and multivariate analyses. Our results demonstrate LMVD as a prognostic factor in malignant melanoma, although its prognostic relevance is much smaller compared with blood MVD.

Keywords: Lymphatic microvessel density; Angiogenesis; Melanoma; Prognosis; Podoplanin; CD31 (PECAM-1)

1. Introduction

Blood and lymphatic microvessels (LMV) play an important role in human cancer, since the capacity of malignant tumours to stimulate angiogenesis is a prerequisite for tumour progression and metastasis [1]. The extent of tumour angiogenesis is commonly estimated by the density of microvessel density (MVD), using pan-endothelial markers and correlations between high MVD and the occurrence of metastasis are established prognostic factors in several malignancies [2–4]. Data on the impact of lymphatic microvessel density (LMVD) on prognosis in patients with cancer are scarce since, until recently, there were no reliable markers for the lymphatic endothelium. Blood endothelial cells can be easily highlighted by immunostaining for the pan-endothelial marker CD31, utilising antibodies against platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) [4,5]. The discovery of new membrane proteins, expressed uniquely on lymphatic endothelia, made selective staining of lymphatic vessels possible, e.g. hyaluronan receptor LYVE-1 [6,7], the homeobox protein Prox 1 [8], the β-chemokine receptor D6 [9], expressed only on a subset of lymphatics, and podoplanin, an approximate 38-kDa membrane mucoprotein [10,11]. Initially, the vascular endothelial growth factor receptor-3 (VEGFR-3) was considered to be a selective lymphatic marker, but has later been found in the fenestrated blood vessels of normal tissues, in the angiogenic blood vessels of the retina, of wounds and of tumours [12,13].

Using immunostaining for podoplanin, lymphatic vessels have been identified in paraffin-embedded samples of cervical, ovarian and breast cancers in previous studies [14–17].

Dilated lymphatic vessels are commonly observed at the periphery of malignant tumours, including melanoma [18,19] and dissemination to regional lymph nodes via afferent lymphatics is one of the first steps in

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melanoma metastasis. A recent report has shown that intratumoral lymphatics are not functional in contrast to those in the tumour's margin, suggesting that lymphatics in the tumour's margin alone are responsible for metastasis [19].

The published reports on the prognostic significance of MVD and/or LMVD in malignant melanoma are controversial [18,20–25], partly due to the lack of a reliable method of specific staining for lymphatic endothelia and partly due to the limited number of cases studied.

In the present study, we therefore present data on the prognostic value of LMVD and MVD in samples of malignant melanoma types, using a specific marker for lymphatic vessels, i.e. podoplanin. In addition, blood MVD was determined using CD31 immunostaining. Although CD31 stains some lymphatic vessels [26], it is considered to be relatively specific for blood vessels.

Table 1
Clinical parameters and MVD

Parameter	Median LMVD	Median CD31 MVD
Lymph nodes		
Negative ($n=113$)	10.3 (5–24)	31 (9–81)[a]
Postive ($n=7$)	12.7 (6–19.7)	45.7 (35.7–12.3)[a]
Clark's level		
0 ($n=6$)	9.2 (5.7–12)	25.5 (13.7–39.7)
1 ($n=1$)	16.3	29.3
2 ($n=14$)	10.3 (6.3–16.7)	23.5 (9–31.7)[a]
3 ($n=45$)	9.7 (5–22.7)	28.3 (11.3–78)[a]
4 ($n=51$)	12 (5.7–24)	38.3 (15.7–123.33)[a]
5 ($n=3$)	10 (9.7–15)	41.7 (37–56.3)
Stage		
Melanoma *in situ* ($n=1$)	12	39.7
1 ($n=72$)	9.5 (5–16.3)[a]	27 (9–72.7)[a]
2 ($n=31$)	14 (6–24)[a]	37.3 (15.7–81)[a]
3 ($n=16$)	12.8 (6.7–20.3)	43.3 (20.6–123.3)[a]

MVD, microvessel density; LMVD, lymphatic MVD.
[a] Significant differences ($P<0.05$) in LMVD/MVD within the groups; assessed by Kruskal–Wallis test and/or (subsequent) Mann–Whitney tests, as appropriate.

2. Patients and methods

2.1. Patients and tissues

A hundred and twenty formalin-fixed, paraffin-embedded surgical specimens of patients presenting with cutaneous melanoma at the University Hospital Vienna between 1991 and 2000 were included in our study. Special care was taken to include only specimens with sufficient amounts of normal tissue surrounding the invasive melanoma cells.

The median age of the patients was 57.13 years (range 25.79–87.79) years at the time of surgery. Tumours were staged according to the new classification system of the American Joint Commitee on Cancer (AJCC) staging system for cutaneous melanoma [27]. Clinical data are compiled in Table 1.

The patients were initially evaluated by clinical and ultrasound examination (lymph nodes, abdomen) and chest X-ray. Treatment consisted of radical surgery with resection margins of 0.5, 1 or 2 cm, according to the invasion depth and/or interferon-alpha-2a for patients with a Breslow index of >1.5 mm. The patients were followed at 3-monthly intervals by clinical examination and appropriate imaging studies at 6-monthly intervals.

2.2. Immunohistochemistry

Rabbit anti-human podoplanin IgG was raised against the recombinant human homologue of the rat 43-kDa glycoprotein podoplanin as previously described in Refs. [10,11]. Affinity purification of rabbit serum was performed using nitrocellulose strips containing recombinant protein [28]. Histological slides, 4 μm in thickness, were deparaffinised in xylol. The slides were heated in 0.01 M citrate buffer for 16 min in a microwave oven. After cooling for 20 min and washing in phosphate-buffered solution (PBS), endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min, followed by incubation with PBS containing 10% normal goat serum for 30 min. For immunohistochemical detection of podoplanin, specimens were incubated at a room temperature with the polyclonal rabbit antibody in a dilution of 1:2000 for 1 h.

Immunohistochemical detection of CD31 antigen was performed on a separate slide from the same block using the monoclonal antibody JC/70 (DAKO, Glostrup, Denmark).

Detection of positive staining for both antigens was performed using the ChemMate kit (DAKO, Glostrup, Denmark) and 3-amino-9-ethylcarbazole (BioGenex, San Ramon, CA, USA). Counterstaining was performed using haematoxylin. As positive control, a sample of breast cancer with a known high LMVD and MVD was used. As negative controls, primary antibodies were replaced with normal rabbit serum or irrelevant mouse IgG.

2.3. Microscopy

Determination of LMVD (assessed by immunostaining for podoplanin) and CD31 MVD (assessed by immunostaining for CD31) was performed as suggested by Weidner in Ref. [29]. Briefly, after scanning the immunostained section at low magnification (40×), the area of tissue with the greatest number of distinctly highlighted microvessels (hot spots) was selected. Hot spots were determined by two independent investigators, who were blinded to the clinical course of the

patients. Microvessel counts were made using the Axio-HOME microscope (Zeiss, Germany), consisting of a computer attached to the microscope. The technical specifications have been previously described in detail [30]. Hot spots of CD31 MVD were counted in the tumour, hot spots of LMVD at the edge of the tumour. Highest LMVD was less than 1.4 mm away from the tumour.

Due to weak cross-reactions with fibroblasts in some cases and with basal keratinocytes, only vessels with a typical morphology (lumen) were considered lymphatic microvessels. In slides stained for CD31, any positive cell was considered an endothelial cell and countable microvessel, even when no lumen was visible. Three hot spots were counted in slides immunostained for podoplanin and in slides immunostained for CD31. For all calculations, the mean (L)MVD of the three hot spots was used.

2.4. Statistical analysis

Mann–Whitney test, Kruskal–Wallis test and Spearman's coefficient of correlation were used as appropriate. Overall survival (OS) was defined from the day of surgery until the death of the patient. Death from a cause other than melanoma or survival until the end of the observation period were considered censored observations. Disease-free survival (DFS) was defined from the end of primary therapy until the first evidence of progression of disease, if the patient showed no evidence of disease after primary therapy. Univariate analysis of OS and DFS was performed as outlined by Kaplan and Meier [31] or by univariate Cox-Regression. The Cox proportional-hazards model was used for the multivariate analysis.

LMVD/CD31 MVD, patients' age at the time of diagnosis, tumour stage, Breslow index, Clark's level and lymph node status were entered into the Cox-regression. For all tests, two-tailed P values given are for mean values ± standard deviations (S.D.), if not otherwise stated.

3. Results

Mean LMVD was 11.34 ± 4.39 microvessels/field, mean CD31 MVD was 35.07 ± 18.1 microvessels/field. Median LMVD was 10.33 (range 5–24) lymphatic microvessels/field, while median CD31 MVD was 32.33 (range 9–123.3) microvessels/field.

Fig. 1 shows samples of immunostaining.

There was a weak correlation between LMVD and CD31 MVD ($P < 0.001$, $r = 0.499$, Spearman's coefficient of correlation). Patients with positive lymph nodes had significantly higher CD31 MVD ($P = 0.007$, Mann–Whitney test), but not LMVD ($P = 0.373$) (Table 1).

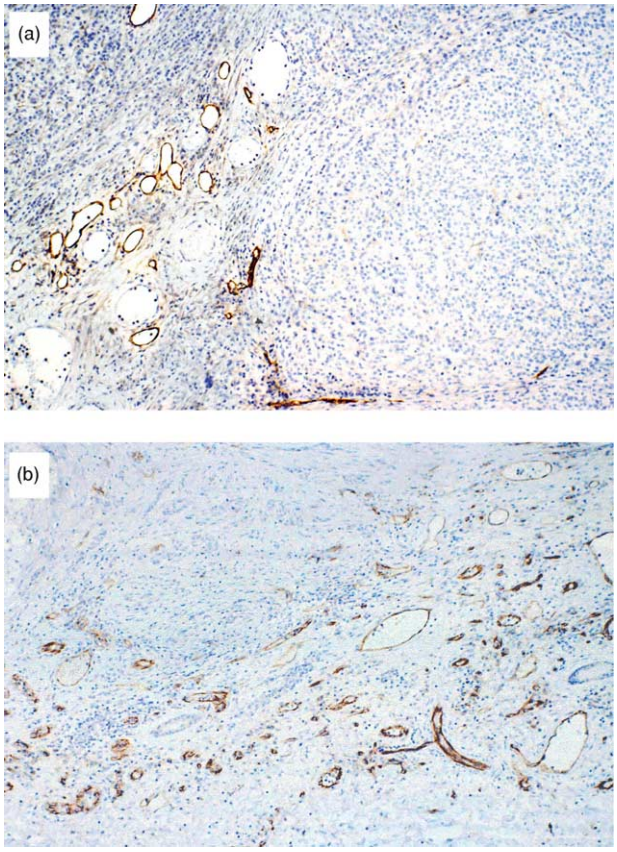

Fig. 1. (a) A sample of a nodular melanoma with high lymphatic microvessel density (LMVD), assessed by anti-podoplanin immunostaining. Note the unstained blood vessels. Original magnification 200×; (b) A sample of a nodular melanoma with high microvessel density (MVD), assessed by anti-CD31 immunostaining. Original magnification 200×.

High CD31 MVD (but not LMVD) was also associated with a high Clark's level ($P < 0.001$, Kruskal–Wallis test) (Table 1). Advanced tumour stage was associated with high LMVD ($P = 0.004$, Kruskal–Wallis test) and CD31 MVD ($P < 0.001$, Kruskal–Wallis test) (Table 1).

A good correlation of CD31 MVD with the Breslow index was noted ($r = 0.573$, $P < 0.001$, Spearman's coefficient of correlation), but only a weak correlation of LMVD and the Breslow index was observed ($r = 0.33$, $P < 0.001$, Spearman's coefficient of correlation).

3.1. Survival analysis

Median follow-up time was 44.6 months (range 1–94.4 months). During this observation period, 37 patients (31%) developed recurrent disease, i.e. melanoma metastases and 33 patients (28%) died from malignant melanoma.

For survival analysis, the median values of LMVD ($\leqslant 10.33$ versus > 10.33 lymphatic microvessels/field) and CD31 MVD ($\leqslant 32.33$ versus 32.33 CD31 microvessels/

field) were used as cut-off-values defining groups with low and high LMVD or CD31 MVD, respectively.

At univariate analysis of OS, tumour stage ($P<0.0001$, log-rank test), lymph node status ($P<0.0001$, log-rank test), Breslow index ($P<0.001$, univariate Cox-Regression), and Clark's level ($P=0.0065$, log-rank test) influenced the prognosis of patients.

At univariate analysis of DFS, tumour stage ($P<0.0001$, log-rank test), lymph node status ($P=0.0001$, log-rank test), Breslow index ($P<0.001$, univariate Cox-Regression), and Clark's level ($P=0.0005$, log-rank test) influenced the prognosis of patients.

No influence of patients' age ($\leqslant 57.13$ versus >57.13 years) on OS and DFS was observed in the univariate analysis ($P>0.05$, log-rank test).

For analysis of the prognostic impact of LMVD and CD31 MVD, the median values were used as cut-offs (LMVD $\leqslant 10.33$ versus >10.33 lymphatic microvessels/field; CD31 MVD $\leqslant 32.33$ versus >32.33 microvessels/field). Patients with a high LMVD (>10.33) ($n=66$) had a significant shorter OS ($P=0.0436$, log-rank test) and DFS ($P=0.0249$, log-rank test) in the univariate analysis (Fig. 2), compared with those with $\leqslant 10.33$ lymphatic microvessels ($n=54$).

In multivariate analysis of OS with regard to LMVD, only tumour stage remained an independent prognostic factor, and for DFS tumour stage and Breslow index remained independent prognostic factors (Table 2).

Survival analysis with regard to CD31 MVD showed this parameter as a very strong prognostic factor: Patients with a high CD31 MVD (>32.33) ($n=59$) had a significant shorter OS ($P<0.0001$, log-rank test) and DFS ($P<0.0001$, log-rank test) in univariate analysis (Fig. 3), compared with those with $\leqslant 32.33$ microvessels/field ($n=61$).

At multivariate analysis of OS, CD31 MVD and tumour stage remained independent prognostic factors, while in the analysis of DFS, only CD31 MVD was an independent prognostic factor (Table 3). When LMVD and CD31 MVD were both included into a multivariate analysis of OS and DFS, CD31 MVD, but not LMVD, remained an independent prognostic factor ($P<0.05$, Cox regression).

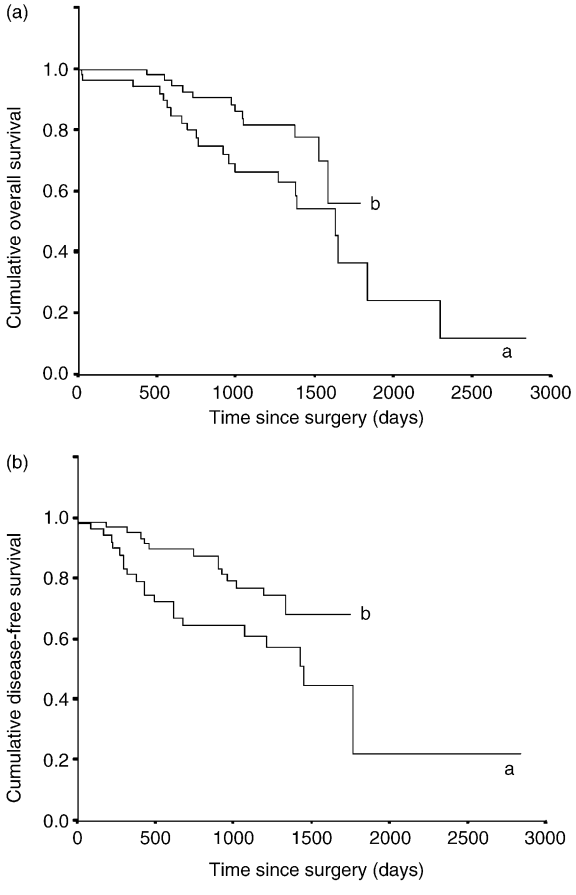

Fig. 2. (a) Cumulative (a) overall survival (OS), (b) disease free survival (DFS) of 120 melanoma patients with a >10.33 and b $\leqslant 10.33$ lymphatic microvessels/field.

4. Discussion

The research on tumour angiogenesis has traditionally focused on blood vessels, partially due to the lack of specific markers for lymphatic endothelia, although the relevance of the lymphatic system is evident by the role of lymphatic vessel invasion for dissemination of tumour cells. In malignant melanoma, a variety of studies investigated the role of blood vessel angiogenesis, with often unequivocal results [20–25], but only few studies deal

Table 2
Multivariate survival analysis pertaining to LMVD (Cox regression)

	Significance	95% Confidence Interval	Relative Risk
Overall survival			
LMVD	0.764	–	–
Patients' age ($\leqslant 57.13$ versus >57.13 years)	0.702	–	–
Tumour stage	0.008	1.272–5.161	2.562
Lymph node status (positive versus negative)	0.323	–	–
Breslow index	0.06	–	–
Clark's level	0.894	–	–
Disease-free survival			
LMVD	0.232	–	–
Patients' age ($\leqslant 57.13$ versus >57.13 years)	0.954	–	–
Tumour stage	0.043	1.022–3.922	2.002
Lymph node status (positive versus negative)	0.994	–	–
Breslow index	0.04	1.009–1.446	1.208
Clark's level	0.463	–	–

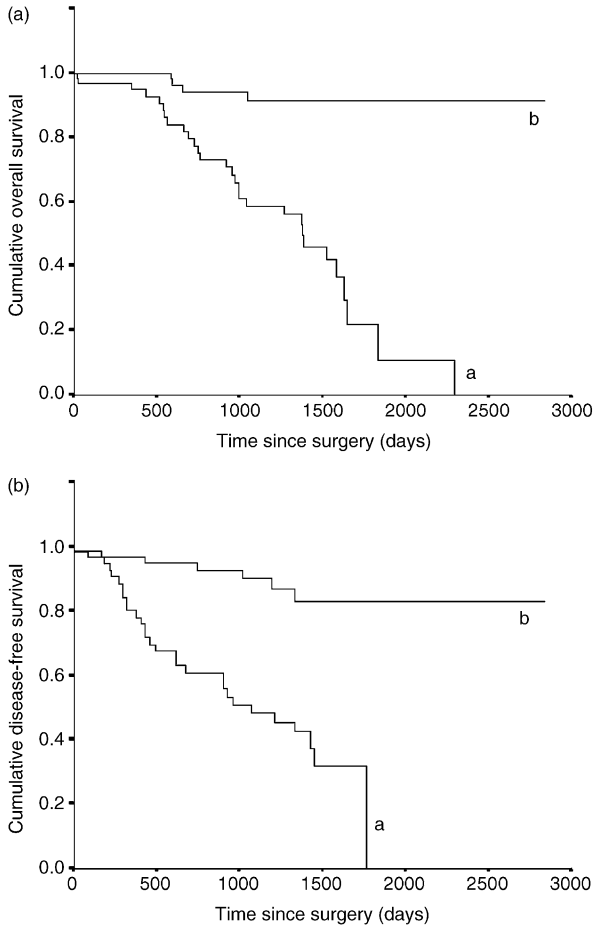

Fig. 3. (a) Cumulative (a) OS, (b) DFS of 120 melanoma patients with a >32.33 and b $\leqslant 32.33$ CD31 + microvessels/field.

Table 3
Multivariate survival analysis pertaining to CD31 MVD (Cox regression)

	Significance	95% Confidence Interval	Relative Risk
Overall survival			
CD 31 MVD	0.015	1.323–14.133	4.324
Patients' age ($\leqslant$57.13 versus $>$57.13 years)	0.495	–	–
Tumour stage	0.029	1.084–4.339	2.169
Lymph node status (positive versus negative)	0.336	–	–
Breslow index	0.072	–	–
Clark's level	0.414	–	–
Disease-free survival			
CD31 MVD	0.009	1.379–9.966	3.707
Patients' age ($\leqslant$57.13 versus $>$57.13 years)	0.819	–	–
Tumour stage	0.087	–	–
Lymph node status (positive versus negative)	0.936	–	–
Breslow index	0.066	–	–
Clark's level	0.909	–	–

with lymphatic vessels, which could only be assessed by quite complicated methods [18], hampering the investigation of large collectives of patients. Due to the recent development of specific lymphatic markers, the lymphatic system in human tumours has become more accessible and so, currently, great interest exists in this topic.

Straume and colleagues [32] described recently LMVD in a series of 202 nodular melanomas by immunohistochemical staining with LYVE-1 and podoplanin, but evaluated two different lymphatic areas around the tumour: one peritumoral and one intratumoral area. Increased peritumoral LMVD was associated with decreased tumour thickness and absence of vascular invasion, whereas decreased LMVD in intratumoral and peritumoral areas both predicted improved survival rates in multivariate analyses. These results are in contrast to our findings, since we observed that LMVD at the tumour base or in the capsule of nodular melanomas was significantly higher compared with counts in the central area of the nodular tumours, where lymphatic vessels were even absent in most cases. This correlates with findings in other studies [15,18,19]. Straume and colleagues [32] observed intratumoral lymphatic microvessels using LYVE-1 antibody for analysis. According to Padera and colleagues [19], it has been shown recently that approximately 10% of LYVE-1-positive vessels are indeed blood vessels, suggesting that LYVE-1 alone is not suitable for the detection of functional lymphatics [19]. The difference in results between our study and the one by Straume and colleagues might be explained by the fact that some intratumoral LYVE-1-positive vessels are in reality blood vessels. In any case, further research on the specificity of various lymphatic markers is urgently needed.

In our series of melanomas, LMVD was assessed by immunostaining for podoplanin, blood MVD by immunostaining for CD31, and both mean values were correlated to different clinico-pathological data and to patient survival. While it has been a matter of discussion if tumours can actively induce lymphangiogenesis, evidence exists today that tumours can actively induce the growth of a lymphatic network by secretion of various lymphatic growth factors [33,34]. This active inducement is reflected by the fact that we found an association of tumour stage and LMVD in our collective. This also contrasts with the findings of de Waal and colleagues [18], although their study was not based on a selective lymphatic marker, but rather on a complicated double staining method and only on 27 cases. In our observation, only a weak correlation between LMVD and CD31 MVD was found. Although we can not exclude the possibility that this correlation might occur due to the fact that CD31 is also expressed on some lymphatic vessels, this finding indicates that lymphangiogenesis seems to be independent of blood vessel

angiogenesis, as shown in a variety of studies [33–35]. Anyway, blood vessel neoangiogenesis seems to be much more prominent in melanoma compared with lymphangiogenesis, evident by the far higher number of blood vessels compared with lymphatic vessels. Interestingly, the situation seems to be similar in cervical and ovarian cancers [15–17].

It seems logical that if the number of lymphatic microvessels increases, the 'lymphatic window' for tumour cells to enter the lymphatic system also increases, and so an association of high LMVD and high prevalence of lymph node metastases might be expected.

Apparently, this does not seem not to be the case in malignant melanoma, since we found no significant association between lymph node status and LMVD. This correlates with the findings in cervical and ovarian cancers, where no such association was also found [15,16], although a significant association between high CD31 MVD and the presence of lymph node metastases was observed. One possibility is that pre-existing lymphatics can be invaded by tumour cells when the tumour reaches these vessels by outgrowth driven by strong angiogenesis. In this case, angiogenesis, reflected by a high MVD, may increase lymphatic spread indirectly.

According to our findings, the amount of lymphangiogenesis, assessed by LMVD, is of prognostic relevance in melanoma. Nevertheless, the prognostic influence is smaller, compared with the strong relevance of blood vessel neoangiogenesis on clinical outcome. Currently, antilymphangiogenic therapeutic-approaches are under discussion, but our data indicate that such therapies would most probably have only a minor impact on the progression of malignant melanoma.

Although blood vessel angiogenesis seems to be of relevance, it has recently been shown that melanoma cells are able to mimic the activities of endothelial cells, by formation of blood vessels, independent of endothelial cell angiogenesis [36]. Further studies will be needed to evaluate how this phenomenon, called 'vasculogenic mimicry', contributes to tumour progression and to prove the value of antiangiogenic therapy in patients with malignant melanoma.

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