

Importance of Vascular Phenotype by Basic Fibroblast Growth Factor, and Influence of the Angiogenic Factors Basic Fibroblast Growth Factor/Fibroblast Growth Factor Receptor-1 and Ephrin-A1/EphA2 on Melanoma Progression

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The expression of several angiogenic factors and receptors was examined in a series of vertical growth phase cutaneous melanomas using high-throughput tissue microarray technology and immunohistochemistry. The results were correlated with microvessel density, clinicopathological features, and patient survival. Expression of basic fibroblast growth factor (bFGF) was significantly associated with increased microvessel density. Also, we found an independent prognostic importance of vascular phenotype by endothelial cell expression of bFGF; cases with positive vessels had the best prognosis and these tumors revealed a low frequency of vascular invasion (14%) when compared with bFGF-negative vessels (47%). This bFGF-negative phenotype was significantly increased in metastatic lesions. Strong tumor cell expression of FLT-4, ephrin-A1, and EphA2 was associated with increased melanoma thickness, and ephrin-A1 staining was related to decreased survival ($P = 0.039$). Expression of EphA2 in tumor cells was associated with increased tumor cell proliferation (Ki-67 positivity), indicating possible autocrine growth stimulation. Thus, our findings indicate the presence of phenotypic diversity among tumor-associated vessels, and subgroups defined by bFGF expression may be of clinical importance. bFGF was associated with microvessel density, whereas the ephrin-A1/EphA2 pathway might also be important for tumor cell proliferation and patient survival. (*Am J Pathol* 2002, 160:1009–1019)

Extensive vascularization must occur if a tumor mass is to exceed 1 mm in diameter.¹ The process of tumor-associated angiogenesis, which is vital also for invasion and metastatic spread, is regulated by networks of proangiogenic and anti-angiogenic molecules.^{2,3} Recent studies have focused on this complex balance, and the possibil-

ity of effective anti-angiogenic treatment is presently being considered.^{4,5} Microvessel density (MVD), a commonly applied estimate of tumor angiogenesis, has proved effective as a prognostic indicator in several types of malignant tumors, such as breast cancer,⁶ endometrial cancer,⁷ and prostate cancer,⁸ whereas its importance in malignant melanoma has been more controversial.^{9–14} Furthermore, new concepts such as vasculogenic mimicry^{15–18} and mosaic tumor vessels,¹⁹ as well as the impact of tumor-associated lymphangiogenesis,^{20–22} are being examined.

In general, several growth factors are important for endothelial cell proliferation and migration. Vascular endothelial growth factor (VEGF) seems to have a fundamental role in tumor vessel formation,²³ and VEGF expression has been associated with increased angiogenesis in clinical^{24–27} and experimental studies.²⁸ The VEGF receptors FLT-1 and KDR are restricted primarily to vascular endothelium,^{23,29,30} although expression has also been found on tumor cells^{31–33} such as malignant melanoma,^{14,34–36} indicating the possibility of autocrine growth stimulation.

Other important factors for neoplastic progression and angiogenesis are the basic fibroblastic growth factor (bFGF) and its receptors,^{37–39} and interleukin (IL)-8.^{40–42} VEGF-C and the receptor protein FLT-4 are thought to be important growth regulators for lymphatic endothelial cells,^{21,43–46} and the relative importance of lymphangiogenesis has been focused.^{21,22,45} The EPH family, which is the largest subfamily of receptor tyrosine kinases,^{47,48} were originally isolated with unknown ligands⁴⁹ and found to have roles in the regulation of neurons and neural crest cells.⁵⁰ The first ligand to be identified, ephrin-A1, was up-regulated in activated endothelial cells after cytokine stimulation.⁵¹

Regarding malignant melanoma, previous studies have indicated that several angiogenic growth factors and receptors might be important, both for tumor-asso-

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ciated angiogenesis, and possibly also acting as autocrine or paracrine growth factors on tumor cells.^{36,52–56} Increased expression of VEGF has been associated with malignant progression in melanocytic tumors,^{13,57,58} and one study found that VEGF increased the proliferation of KDR-positive melanoma cells *in vitro*.⁵² Further, bFGF and its receptor FGFR-1 are important for melanoma angiogenesis,³⁸ and several studies indicate that these factors might also be of importance for autocrine growth control and melanoma progression.^{59–63} Studies also indicate that IL-8 can act as an autocrine factor for melanoma cells,⁶⁴ and IL-8 mRNA expression was associated with increased tumor progression in cutaneous melanoma.⁶⁵ In experimental studies, IL-8 was found to enhance invasive growth and metastatic potency of melanoma cells by various mechanisms.^{41,66,67}

Recently, ephrin-A1 was found to be a melanoma growth factor,⁶⁸ and it was up-regulated during melanoma progression and possibly implicated in angiogenesis.⁵³ Its receptor EphA2 might be important for aggressive behavior and vasculogenic mimicry properties in melanoma cell lines.¹⁷

In our study of angiogenesis in vertical growth phase melanomas,¹⁴ we found that MVD, as estimated by two different endothelial cell markers (F-VIII and CD105/endothelin), was an independent prognostic factor, although of only moderate strength. Most cases were positive for VEGF, but there was no strong association with MVD or survival for VEGF and its receptors. Possible autocrine loops were suggested by co-expression of VEGF and its two receptors in tumor cells, and by a significant correlation between KDR and tumor cell proliferation (Ki-67). On this background, the present study was performed to examine the importance of other angiogenic factors and some of their receptors, such as VEGF-C, VEGFR-3 (FLT-4), bFGF, FGFR-1, IL-8, ephrin-A1, and EphA2. It was of particular interest to see whether these regulators correlated with MVD and indicators of tumor growth, as well as with survival, in advanced primary melanomas. We especially focused on vascular phenotype by endothelial cell expression of bFGF. The study was performed using high-throughput tissue microarray (TMA) technique with sensitive immunohistochemistry protocols, and expression data were related to clinicopathological variables and follow-up information.

Materials and Methods

Patients

The patient series is described in detail elsewhere.⁶⁹ Briefly, 202 vertical growth phase melanomas occurring during 1981 to 1997 were included. The presence of a vertical growth phase, and the lack of a radial growth phase, ie, adjacent *in situ* or microinvasive component, were used as inclusion criteria for the present study.⁷⁰ In addition, 68 separate biopsies of local (skin; $n = 17$), regional (lymph nodes; $n = 44$), or distant ($n = 7$) metastases from 58 patients with recurrent disease were available for analyses.

Complete information on patient survival and time and cause of death was available in all 202 cases. Last date of follow-up was December 18, 1998, and median follow-up time for all survivors was 76 months (range, 13 to 210 months). During this period, 69 patients died of malignant melanoma. Clinical follow-up (with respect to recurrences) was not performed in 14 (mostly older) patients, and 21 patients were not treated with complete local excision. Thus, recurrence-free time could be studied in 167 patients.

TMA

The technique of TMA was recently introduced⁷¹ and validated by independent studies of several tumor markers.^{72,73} TMA slides were used for most markers in this study (VEGF-C, FLT-4, FGFR-1, IL-8, ephrin-A1, and EphA2), whereas bFGF was examined on standard slides. For TMA construction,^{71,73} representative tumor areas were identified on hematoxylin and eosin slides. Tissue cylinders with a diameter of 0.6 mm were then punched from selected areas of the donor block and mounted into a recipient paraffin block using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD). Sections of the resulting TMA blocks (5 μm) were then made by standard technique. In our experience, TMA blocks with ~300 samples and standard sectioning gave better results than using the tape transfer technique (to support cohesion of samples) on sections from recipient blocks with larger number of cylinders. As recommended,⁷² three parallel tissue cylinders were sampled from each case, and these were taken from the suprabasal areas of the primary tumors. For internal validation, TMA sections from 50 randomly selected cases were stained for Ki-67 as previously described,⁶⁹ and the labeling index (percent positive tumor cell nuclei) was determined. We found a highly significant correlation between results from TMA sections and standard slides ($P < 0.0005$; $r = 0.69$; $\kappa = 0.76$).

Immunohistochemistry

The immunohistochemical staining was performed on formalin-fixed and paraffin-embedded archival tissue (5 μm sections). In some cases, a sufficient amount of tumor tissue was not available in the remaining paraffin blocks. For bFGF, sections from 176 cases could be included and 147 primary tumors and 56 metastases were available using the TMA technique. There was no significant difference regarding MVD or survival between the 147 cases included and those without sufficient material left for the TMA technique. Regarding staining procedures, the conditions were optimized for each antibody, and some important steps in the respective protocols are summarized in Table 1. All negative controls were negative. The staining procedures and evaluation of VEGF, FLT-1, KDR, TSP-1, p16, p53, and Ki-67 expression, as well as MVD estimates, have been described previously.^{14,69} The results on these markers have been in-

Table 1. Immunohistochemical Staining Methods

Antibody	Provider	Epitope retrieval	Dilution	Incubation	Controls
pAb SC-1881, VEGF-C	Santa Cruz	4 × 5 minute MW in Target retrieval solution (TRS, DAKO)	1:75	Overnight, RT	Pos: Adult heart muscle Neg: Blocking peptide
pAb SC-321, Flt-4	Santa Cruz	4 × 5 minute MW in citrate buffer (pH = 6) at 500 W	1:800	1 hour, RT	Pos: Liver Neg: Blocking peptide
mAb GF22, bFGF	Oncogene	4 × 5 minute MW in citrate buffer (pH = 6) at 500 W	1:200	Overnight, RT	Pos: Colon carcinoma Neg: Irrelevant mouse MoAb
pAb SC-121, Flg (FGFR-1)	Santa Cruz	4 × 5 minute MW in citrate buffer (pH = 6) at 500 W	1:100	1 hour, RT	Pos: Colon carcinoma Neg: Blocking peptide
pAb AF-208-NA, IL-8	R&D	4 × 5 minute MW in Target retrieval solution (TRS, DAKO)	1:50	Overnight, RT*	Pos: Granulocytes Neg: Blocking peptide
pAb SC-911, Ephrin-A1	Santa Cruz	4 × 5 minute MW in citrate buffer (pH = 6) at 500 W	1:250	1 hour, RT	Pos: Colon carcinoma Neg: Blocking peptide
pAb SC-924, EphA2	Santa Cruz	4 × 5 minute MW in citrate buffer (pH = 6) at 500 W	1:100	1 hour, RT	Pos: Colon carcinoma Neg: Blocking peptide

MW, microwave treatment; RT, room temperature.
 *All steps incubated with 0.1% Saponin.

cluded in the present study for comparisons (see Results).

Staining was performed on a DAKO TechMate 500 slide processing equipment (DAKO, Copenhagen, Denmark), using the standard avidin-biotin method. Finally, the peroxidase was localized by the 3-amino-9-ethylcarbazole-peroxidase reaction with Harris hematoxylin as counterstain.

To control for the possibility of misinterpreting bFGF-negative stromal cells as endothelial cells in cases with uncertain morphology, sections from 10 cases were double stained for bFGF and Factor-VIII. An indirect/indirect simultaneous method was used. bFGF (1:50) and Factor-VIII (pAb A-082, 1:800; DAKO) was incubated overnight at room temperature, after epitope retrieval by both proteinase K and microwave treatment. Factor-VIII was detected by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (P0448, DAKO) at 1:50 for 30 minutes followed by 3-amino-9-ethylcarbazole for 10 minutes. bFGF was detected by a biotinylated goat anti-mouse secondary antibody (E0433, DAKO), followed by a 30-minute incubation with a streptavidin/biotinylated alkaline phosphatase (AP) complex (K0391, DAKO), and 20 minutes with Fast blue as chromogen for AP.

Evaluation of Staining Results

For all markers, both staining intensity and positive area were recorded. A staining index (values 0 to 9), obtained as a product of staining intensity (0 to 3) and proportion of immunopositive cells of interest ($\leq 10\% = 1$, 10 to $50\% = 2$, $>50\% = 3$), was calculated. For bFGF, both tumor cell expression (staining index) and staining in tumor-associated endothelial cells (absent or present) was determined. For other markers, only tumor cell expression was evaluated on the TMA sections. For statistical purposes, cut points for continuous variables and staining index categories were based on the distribution of the values.

Statistics

Analyses were performed using the statistical package SPSS.⁷⁴ Associations between different categorical variables were assessed by Pearson's chi-square test. Continuous variables not following the normal distribution were compared between two or more groups using the Mann-Whitney U or Kruskal-Wallis H tests. Wilcoxon signed ranks test was used to compare related samples. Univariate analyses of time to death because of malignant melanoma or time to recurrence (recurrence-free survival) were performed using the product-limit procedure (Kaplan-Meier method), with date of histological diagnosis as the starting point. Patients who died of other causes were censored at the time of death. Differences between categories were tested by the log-rank test. The influence of covariates on patient survival was analyzed by the proportional hazards method,⁷⁵ including all variables with a *P* value ≤ 0.15 in univariate analyses, and tested by the likelihood ratio (l-ratio) test. Model assumptions were tested by log-minus-log plots, and significant variables were tested for interactions. Estimated hazard ratio, 95% CI for hazard ratio, and *P* values are given in the tables. Prognostic information on standard variables, which has been presented elsewhere,¹⁴ was included for comparison in multivariate analyses (see Results).

Results

Expression results for each individual marker were recorded, with reference to staining pattern, ie, cytoplasmic or nuclear staining signals, and intensity and positive area of expression (staining index). Also, results from our previous study on VEGF, VEGFR-1 (FLT-1), VEGFR-2 (FLK-1/KDR), p16, p53, Ki-67, TSP-1, and MVD were included for comparison.¹⁴ Table 2 summarizes the associations between the angiogenic markers studied and MVD, proliferative rate, and tumor thickness.

Table 2. Microvessel Density (MVD), Proliferative Rate (Ki-67), and Tumor Thickness Related to Angiogenic Markers in Patients with Vertical Growth Phase Melanoma

Marker	No	MVD*	<i>P</i> [†]	Ki-67	<i>P</i> [†]	Tumor thickness	<i>P</i> [†]
VEGF-C [‡]							
Weak/absent	42	118	0.24	31%	0.72	4.2 mm	0.33
Moderate/strong	105	125		28%		3.7 mm	
FLT-4 [‡]							
Weak/absent	46	125	0.77	30%	0.41	3.4 mm [§]	0.62
Moderate/strong	99	125		29%		4.0 mm	
bFGF [‡] - tumor cells							
Weak/absent	124	118	0.022	28%	0.17	3.8 mm	0.91
Moderate/strong	52	138		27%		3.3 mm	
bFGF [¶] - endothelium							
Absent	38	115	0.045	28%	0.82	4.3 mm	0.30
Present	138	128		27%		3.7 mm	
FGFR-1 [‡]							
Weak/absent	54	121	0.44	32%	0.18	4.2 mm	0.060
Moderate/strong	91	125		26%		3.6 mm	
IL-8 [‡]							
Weak/absent	49	125	0.57	34%	0.46	3.8 mm	0.52
Moderate/strong	95	125		28%		4.0 mm	
Ephrin-A1							
Weak/moderate	123	121	0.077	29%	0.64	3.7 mm	0.017
Strong	23	138		26%		4.8 mm	
EphA2							
Weak/moderate	122	125	0.87	28%	0.049	3.7 mm	0.066
Strong	23	125		37%		4.8 mm	

*Number of microvessels per mm².

[†]Mann-Whitney *U* test.

[‡]Weak/absent: staining index <4.

[§]See Results for weak/moderate vs. strong expression.

[¶]Staining in tumor associated endothelium.

^{||}Weak/moderate: staining index <9 (see Materials and Methods).

VEGF-C

All cases showed some tumor cell positivity for VEGF-C, and the staining pattern was predominantly cytoplasmic, with some nuclear reactivity in most cases. Low-grade staining (index < 4) was present in 28.6% of the cases, whereas 29 cases (19.7%) revealed strong staining (index = 9) (Figure 1a).

There was no significant association with MVD. Increased staining of VEGF-C (low grade versus high grade) was associated with reduced frequency of vascular invasion (*P* = 0.010). Also, a significant co-expression was found between VEGF-C expression and staining for FLT-1 (*P* = 0.009) and KDR (*P* = 0.04) in tumor cells.

FLT-4

Staining for FLT-4 was found in all tumors, and most cases showed mixed cytoplasmic and nuclear staining. Low-grade positivity (index < 4) was observed in 31.7%, whereas strong expression (index = 9) was observed in 20 cases (13.8%). Looking at nuclear FLT-4 staining only, 11.7% of the cases were negative, whereas low-grade positivity (index < 4) was found in 49.7% of the tumors. Strong nuclear expression (index = 9) was found in 14.5% of the cases (Figure 1b).

Strong FLT-4 expression (index = 9) was significantly associated with increased histological tumor thickness (*P* = 0.043, Mann Whitney test), as well as with histological tumor ulceration (*P* = 0.014). FLT-4 revealed no

significant association with VEGF-C; in contrast, VEGF expression was related to FLT-4 staining (*P* = 0.021). Regarding nuclear staining, this was significantly associated with expression of ephrin-A1 (*P* = <0.0005), FGFR-1 (*P* = 0.007), and tumor ulceration (*P* = 0.001).

bFGF

Tumor cell staining for bFGF was negative or showed only minimal reactivity in 70.5% of all examined cases, whereas 29.5% of the tumors were positive and showed distinct nuclear staining. Also, 6.8% showed moderate to strong expression (index 6 to 9) (Figure 1c).

bFGF expression in tumor cells showed a significant association with increased MVD, when cases with no or minimal staining (index 0 to 1) were compared with the others (*P* = 0.022, Mann Whitney test). Positive cases showed increased MVD, 138 versus 118 mm⁻². This relationship was especially evident in the subgroup of thick melanomas, ie, greater than median value 3.55 mm (*P* = 0.023, Mann Whitney test), as well as in tumors without microscopic ulceration (*P* = 0.022). Cases with co-expression of bFGF (index > 1) and VEGF (index > 4) showed increased MVD (*P* = 0.019, Mann Whitney test) and tumor cell proliferation (*P* = 0.030, Mann Whitney test). Lack of p16 staining showed a significant association with increased expression of bFGF (*P* = 0.047).

Expression of bFGF in tumor-associated endothelial cells was found in 78.4% of the cases, whereas 21.6%

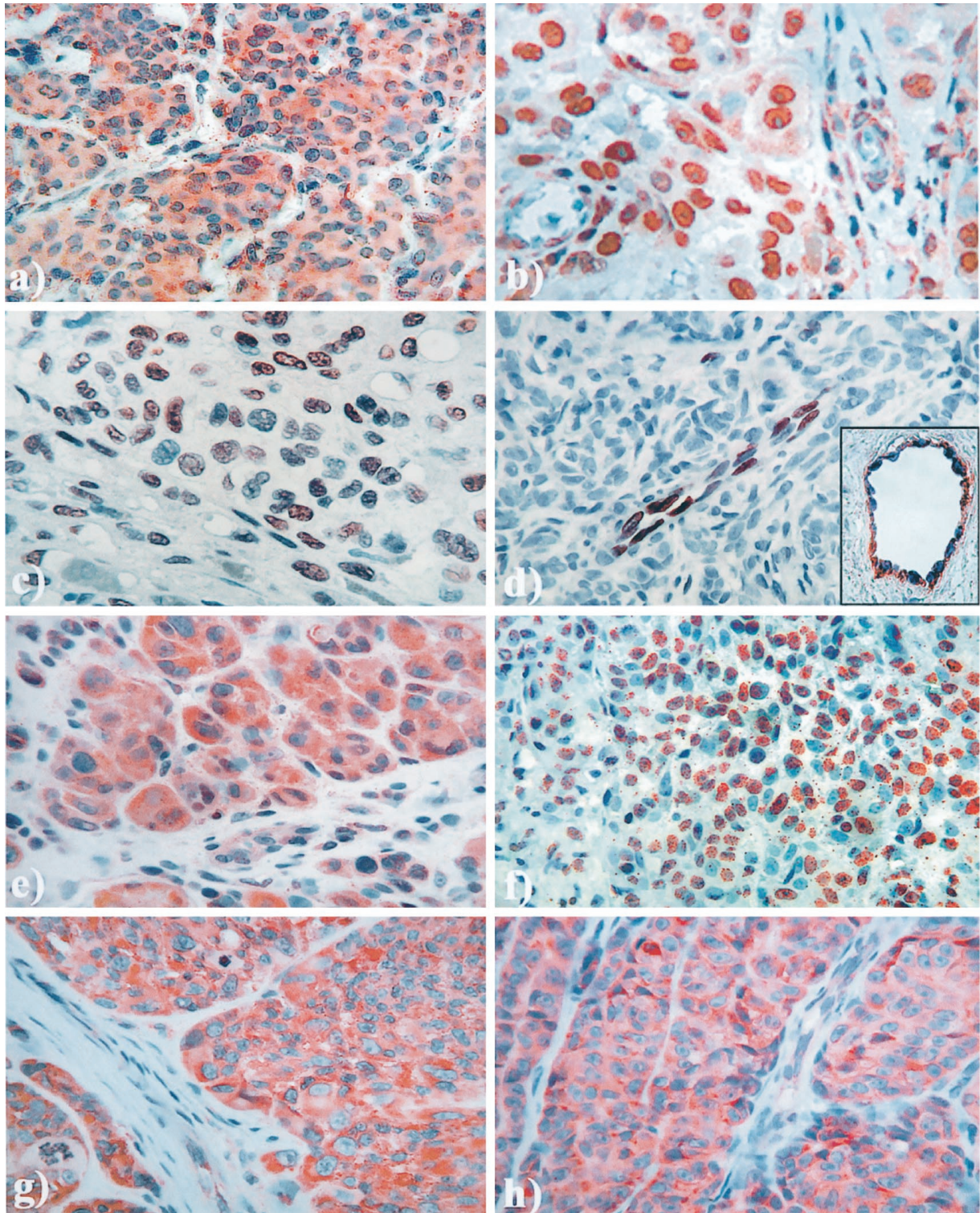


Figure 1. Immunohistochemical staining pattern of VEGF-C (a), VEGF-C receptor FLT-4 (b), bFGF in tumor cells (c), and bFGF in tumor-associated endothelial cells (d); inset, double staining of bFGF (Fast Blue) and Factor-VIII (3-amino-9-ethylcarbazole, red) in tumor-associated endothelial cells, FGFR-1 (e), interleukin-8 (f), ephrin-A1 (g), and ephrin receptor EphA2 (h). Original magnifications, $\sim \times 400$.

were negative (Figure 1d). Double staining for bFGF and Factor-VIII confirmed the presence and absence of bFGF staining related to endothelial cells (Figure 1d, inset). Positive cases showed significantly increased MVD, 128 versus 115 mm^{-2} in negative tumors ($P = 0.04$, Mann

Whitney test). There was also a significant association between expression of bFGF in endothelial cells and tumor cells ($P = 0.012$). In endothelial cells, bFGF and KDR were significantly co-expressed ($P = 0.019$), and positive endothelial cell staining of bFGF was significantly

associated with FGFR-1 expression in tumor cells ($P = 0.022$). Also, bFGF staining in endothelial cells was inversely associated with vascular invasion; negative cases showed 47% vascular invasion, compared with 14% in positive cases ($P < 0.0005$).

FGF Receptor (FGFR-1)

Five cases were completely negative, whereas the rest showed various degrees of positive cytoplasmic staining in the tumor cells. Some positive nuclei were observed in a few cases, and nuclear staining was also found in some stromal cells. Low-grade expression (index < 4) was present in 37.2% of the cases, whereas 17 tumors (11.7%) revealed strong expression (index = 9) (Figure 1e).

There was no simple association with MVD. However, co-expression of FGFR-1 and bFGF in tumor cells was associated with increased MVD ($P = 0.021$, Mann-Whitney test).

IL-8

All cases except one tumor showed some positive staining, which was granular and both cytoplasmic and nuclear. Low-grade expression (index < 4) was found in 34.0% of the cases. Strong staining (index = 9) was present in eight cases (5.6%) (Figure 1f).

No association with MVD was found. Increased expression was significantly associated with tumors on the head/neck area and the extremities, when compared with the trunk ($P = 0.002$). Also, females revealed significant stronger expression ($P = 0.011$). Other associations were not found.

Ephrin-A1

All cases showed some tumor cell staining of ephrin-A1, although it varied considerably. The positivity was mainly cytoplasmic. Low-grade staining (index < 4) was present in 39.0% of the cases, whereas the rest revealed stronger expression. Twenty-three cases (15.8%) showed the strongest staining (index = 9) (Figure 1g).

The cases with strong expression (index = 9) were significantly associated with increased histological thickness ($P = 0.017$, Mann-Whitney test), advanced Clark's level of invasion ($P = 0.004$) and histological tumor ulceration ($P = 0.006$). In thin melanomas (below median value, 3.55 mm), there was a significant relation between ephrin-A1 expression and MVD ($P = 0.013$, Mann-Whitney test; Figure 2).

Ephrin Receptor A2 (EphA2)

All cases except one tumor showed some positive tumor cell staining for this marker, mainly in the cytoplasm. Low-grade expression (index < 4) was found in 37.9% of the cases. Twenty-three cases (15.9%) showed the strongest staining (index = 9) (Figure 1h). There was also a significant co-expression between ephrin-A1 and its

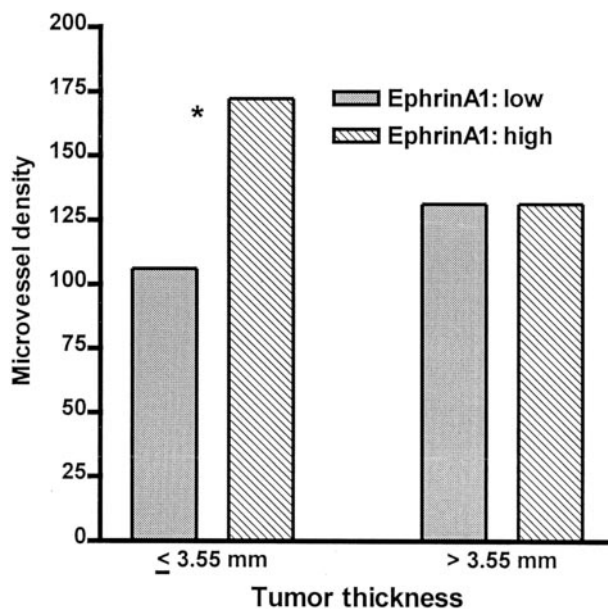


Figure 2. The correlation between ephrin-A1 and MVD in subgroups according to median tumor thickness. *, The difference is statistically significant (Mann-Whitney, $P = 0.013$).

receptor EphA2 (index < 4 versus the rest, for both markers; $P = 0.007$).

The cases with strong expression (index = 9) tended to be associated with increased histological thickness ($P = 0.066$, Mann-Whitney test), and histological tumor ulceration ($P = 0.009$). Also, there was a significant association between this strong expression and proliferation in tumor cells, as estimated by Ki-67 expression ($P = 0.049$, Mann-Whitney test).

Expression in Metastases

Using pairwise testing (Wilcoxon signed ranks test), we found that expression of VEGF-C tended to be increased in the metastases when compared with corresponding primary tumors ($P = 0.07$), whereas the staining of FLT-4 was significantly increased ($P < 0.0005$). Expression of bFGF was not different, whereas FGFR-1 was increased ($P = 0.005$). However, the staining of bFGF in tumor-associated endothelial cells was found to be significantly decreased ($P = 0.009$). Also, there was a significant association between bFGF expression in endothelial cells and reduced tumor cell proliferation (Ki-67) in metastases ($P = 0.03$, Mann-Whitney test). The other angiogenic factors (IL-8, ephrin-A1, EphA2) showed no significant differences in expression between primary tumors and metastatic lesions.

Survival Analyses

A nonsignificant tendency between increased bFGF expression and reduced survival was observed ($P = 0.12$, log rank test). In contrast, lack of bFGF expression in tumor-associated endothelial cells was associated with a significantly reduced survival ($P = 0.012$, log rank test;

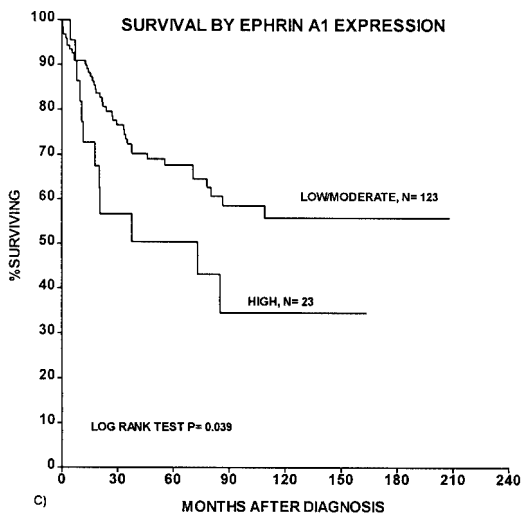
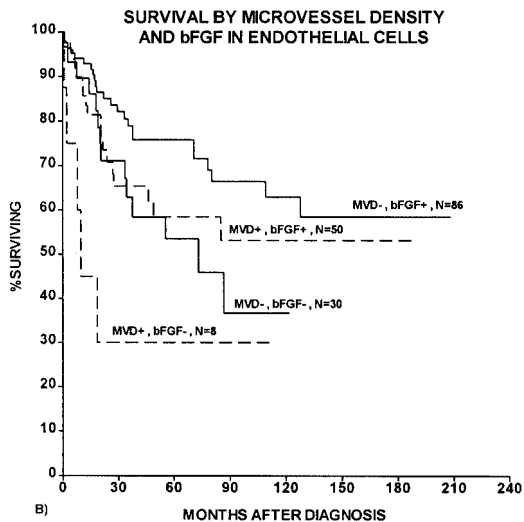
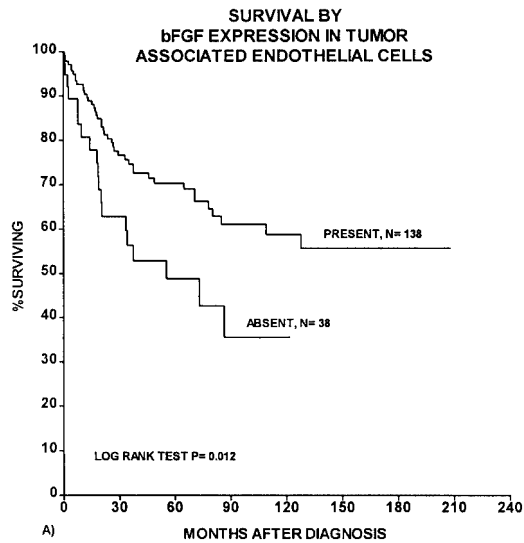


Figure 3A); cases with bFGF+ vessels had a 59% 10-year survival, compared with 35% for patients with the bFGF- vascular phenotype. Figure 3B shows the estimated survival curves for subgroups defined by combinations of endothelial cell bFGF expression and MVD. The cases with strong ephrin-A1 expression showed significantly reduced survival ($P = 0.04$, log rank test; Figure 3C), whereas no significant survival differences were present for EphA2. No survival differences were found for VEGF-C, FLT-4, FGFR-1, or IL-8.

In multivariate analysis of patient survival (Cox' proportional hazards method), including tumor variables with significant impact on survival in univariate analyses (tumor thickness, Clark's level of invasion, tumor ulceration, vascular invasion, tumor cell proliferation by Ki-67 expression), both endothelial staining of bFGF ($P = 0.02$, l-ratio test) and MVD ($P = 0.03$, l-ratio test) were found to be of independent prognostic importance, in addition to Clark's level of invasion, tumor ulceration, and tumor cell proliferation (Table 3). Estimates of hazard ratio showed an equally strong influence in the final model of these two angiogenesis related variables. Prognostic information on standard variables has been presented elsewhere.¹⁴

Discussion

We previously found a more than 10-fold difference between low-grade and high-grade angiogenesis in vertical growth phase cutaneous melanoma,⁷⁶ and MVD was a significant prognostic factor of moderate strength in multivariate analysis.¹⁴ However, no marked associations between angiogenesis and expression of VEGF and its receptors FLT-1 and KDR were found. In the present study, a panel of other angiogenic factors and some of their receptors have been examined with reference to MVD, tumor cell proliferation, and survival in vertical growth phase melanoma. As the only factor, bFGF showed a clear association with MVD in these tumors. Our findings support previous experimental data that bFGF is an important factor for melanoma angiogenesis.^{38,77} However, melanoma vascularity is not a very strong prognostic factor,¹⁴ and expression of bFGF was not significantly associated with survival in this study, in accordance with others.⁷⁸ Still, studies indicate that bFGF is important for melanoma growth and progression, and this is probably related to autocrine growth stimulation^{59-63,79} in addition to angiogenesis.

Endothelial cell expression of bFGF in tumor vessels showed a significant association with improved patient survival, when compared with cases having a bFGF-negative vascular phenotype. Other studies suggest that bFGF might influence not only endothelial cell proliferation, invasion, and migration, but also vascular morphogenesis.⁸⁰ Thus, bFGF and Angiopoietin-1, which promotes vessel maturation and integrity, was found to be

Figure 3. Survival curves were estimated according to the Kaplan-Meier method with death because of melanoma as end point. **A:** Survival by bFGF expression in tumor-associated endothelial cells. **B:** Survival by combinations of vascular phenotype by bFGF expression and MVD (MVD+, MVD >67th percentile; MVD-, MVD ≤67th percentile). **C:** Survival by ephrin-A1 expression.

Table 3. Multivariate Survival Analysis of Tumor-Associated Characteristics, According to the Proportional Hazards Method for Patients with Vertical Growth Phase Melanoma, Using Death from Melanoma as End Point

Variable	Categories	n	HR*	P value†
Level of invasion (Clark)	II, III, IV	138	1	<0.0001
	V	33	3.3	
Tumor ulceration	Absent	96	1	0.02
	Present	75	1.9	
Ki-67 expression	Low‡	42	1	0.03
	High	129	2.6	
bFGF, endothelial cells	Absent	36	2.0	0.02
	Present	135	1	
MVD	Low§	115	1	0.03
	High	56	1.9	

Only patients with information on all variables were included, *n* = 171.

*Hazard ratio.

†L-ratio test.

‡≤16% (25th percentile).

§≤144 vessels/mm² (67th percentile).

co-expressed in MCF-7 tumor cells.⁸¹ The bFGF-negative vascular phenotype was associated with a strikingly increased frequency of vascular invasion, supporting a functional significance of bFGF expression in tumor vessels. Further, lack of bFGF expression in endothelial cells showed an independent prognostic impact in multivariate analysis of patient survival, when compared with MVD, which is by far the most commonly applied indicator of tumor-associated angiogenesis in clinical studies. A similar inverse relation between vessel bFGF expression and metastasis has also been reported in non-small cell lung cancers.⁸² The proportion of this aggressive bFGF-negative vascular phenotype was increased in metastases, further supporting its importance in melanoma progression. Our findings indicate the presence of phenotypic diversity among tumor-associated vessels, and different subgroups or vascular differentiation grades defined by endothelial cell expression patterns may be of clinical importance.

There was no strong influence of VEGF-C, FLT-4, IL-8, ephrin-A1, and its receptor EphA2 on melanoma angiogenesis, as estimated by MVD. This lack of association with most angiogenic factors is in line with our recent findings that VEGF and its receptors FLT-1 and KDR showed no marked relationship with angiogenesis and survival,¹⁴ and the same has been found for other tumors such as breast cancer.⁸³ Previously, VEGF has been associated with malignant progression in melanocytic lesions.^{13,57,58} In our recent study, VEGF was up-regulated in smaller tumors, whereas weak expression was found in thicker and more angiogenic lesions,¹⁴ suggesting that a lower baseline level of VEGF might be sufficient for an established vascular system, with VEGF acting as a survival factor for endothelial cells.⁸⁴ In these advanced primary melanomas, the impact of molecular cross-talk and synergistic effects of several factors such as VEGFs, bFGF, IL-8, and ephrins, might possibly be more important for angiogenesis and survival than single growth factors, and different regulatory subgroups may be present. This is supported by experimental data indicating that vessel formation in poorly angiogenic melanomas is promoted solely by VEGF, whereas multiple factors are involved in highly vascularized melanomas.⁵⁵ bFGF was

most clearly associated with increased angiogenesis (MVD) in the thicker tumors.

VEGF-C, which is thought to be a relatively specific lymphatic endothelial growth factor,⁸⁵ showed strong tumor cell expression in ~20% of the primary melanomas, and only few tumors were completely negative. Expression of VEGF-C has been found in other tumors like breast cancer,⁴³ and its up-regulation in tumor cells may act as an angiogenic factor for blood vessels.⁸⁶ Similarly, the VEGF-C receptor FLT-4, which was considered a predominantly lymphatic marker,⁸⁷ has been found on blood vessels.⁸⁶ This indicates that VEGF-C and FLT-4 expression may promote tumor-associated angiogenesis, although a direct relationship to MVD was not found in the present study. Significant associations between VEGF-C and FLT-1 or KDR, as well as between tumor cell expression of VEGF and FLT-4, further support the functional importance of angiogenic cross-talk and cross-over interactions in the VEGF family of multiple ligands and receptors.

Several of the angiogenic factors were related to indicators of tumor growth. Increasing evidence supports the importance of VEGF receptors in nonendothelial cell types^{31,32,35,36,88-90}, and functional evidence showing increased proliferation of KDR-positive melanoma cells has been published.⁵² Growth stimulation by possible autocrine loops was suggested by co-expression of VEGF and its two receptors in tumor cells, and by a significant correlation between KDR and tumor cell proliferation (Ki-67).¹⁴ In our study, tumor thickness was related to expression of ephrin-A1 and EphA2. The association between EphA2 receptors on melanoma cells and increased tumor cell proliferation, as indicated by Ki-67 expression, support the existence of autocrine or paracrine growth stimulation. Whereas ephrin-A1 showed an angiogenic effect among thinner tumors, the relationship between EphA2 and tumor cell proliferation indicates a dual role for the ephrin-A1/EphA2 system. Thus, our previous¹⁴ and present data suggest that certain factors might switch from angiogenic action to autocrine growth stimulation of tumor cells in these advanced primary melanomas. As suggested by the recent vasculogenic mimicry concept, the appearance of endothelial cell markers

on tumor cells might indicate a reversion to more embryonic-like phenotypes, possibly also promoting the formation of tubular structures by tumor cells and thereby enhancing perfusion by extra-angiogenic networks.^{16,17} These properties seem to indicate increasing aggressiveness in melanoma cell lines as well as in human tumors.⁹¹

The presence of tumor ulceration was significantly associated with increased expression of angiogenic factors such as FLT-4, ephrin-A1, and EphA2, indicating that ulceration might co-activate these regulators. We previously found that the relationship between level of TSP-1 expression and MVD was also different in ulcerated and nonulcerated tumors,¹⁴ supporting a possible interaction with angiogenic factors associated with ulceration. Alternatively, these factors might stimulate tumor growth and indirectly increase the risk for microscopic ulceration.

In conclusion, we found that vascular phenotype by endothelial cell expression of bFGF showed a significant association with patient survival in this series of human vertical growth phase melanomas of the skin. Cases with bFGF+ vessels had the best prognosis, and these tumors also revealed a strikingly low frequency of vascular invasion (14%), when compared with bFGF- vessels (47%). Expression of bFGF in tumor cells was significantly associated with MVD, whereas tumor cell bFGF was in itself not a significant prognostic factor. In multivariate survival analyses, both vascular phenotype by bFGF status and MVD had an independent prognostic importance. Also, the expression of receptors such as EphA2 and KDR on tumor cells, being associated with increased tumor cell proliferation (Ki-67), indicate a regulatory role of autocrine or paracrine growth stimulation.

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