Vascular Proliferation Is Important for Clinical Progress of Endometrial Cancer

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Abstract

Angiogenesis is essential for tumor growth, invasion, and metastatic spread. Whereas microvessel density (MVD) has been widely used as a measure of tumor-associated angiogenesis, we now wanted to examine the significance of other angiogenic markers, especially vascular proliferation (by Ki-67/factor VIII staining) and the degree of pericyte coverage [by α-smooth muscle actin (α-SMA)/factor VIII staining], in a large and population-based series of endometrial carcinoma with complete follow-up. Due to limited information on the role of lymphangiogenesis in these tumors, lymphatic vessel density (LVD) by LYVE-1 staining was also determined, as well as selected angiogenic factors [vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D and basic fibroblast growth factor (bFGF)], which could possibly be related to vascular proliferation and lymphangiogenesis. The information on angiogenic phenotype was related to clinicopathologic features and disease progress. Median vascular proliferation, as estimated by vascular proliferation index (VPI), was 3.9% and high VPI was associated with features of aggressive tumors and decreased survival. The prognostic effect of VPI was superior to that of MVD. Presence of pericyte coverage, as estimated by the α-SMA index (SMAI), was 35% and low SMAI was significantly associated with vascular invasion by tumor cells and impaired prognosis. Peritumoral lymphatic vessels (LVD-pt) were found in 39.5% of the cases and high LVD-pt was significantly associated with aggressive tumor features and decreased survival. In multivariate survival analysis, only the extent of vascular proliferation had independent prognostic effect, in addition to well-known clinicopathologic factors, whereas MVD did not have significant prognostic value. In conclusion, our study indicates that vascular proliferation is a meaningful variable in assessing the angiogenic phenotype of endometrial carcinoma. (Cancer Res 2006; 66(6): 3303-9)

Introduction

In 1945, Algire et al. (1) concluded that "the rapid growth of tumor explants is dependent on the development of a rich vascular supply." This was further proposed by Folkman (2) in 1971, suggesting that the growth of malignant tumors depends on the process of angiogenesis and that tumors can be treated by attacking their blood supply. Formation of new vessels represents a complex process which might be stimulated or inhibited by multiple regulators (3). Several trials of antiangiogenic regimens are being done and the clinical effect of anti-vascular endothelial growth factor (VEGF) treatment on metastatic colorectal cancer was recently reported (4). Still, more data are needed on the applicability of tissue-based angiogenic markers for prognostic evaluation and whether these might also be used to predict or monitor clinical response to various treatments. Especially, the limitations of microvessel density (MVD), which has been widely used in prognostic studies, have been discussed (5, 6). Previous reports have failed to show a predictive effect of MVD on the effect of chemotherapy (7, 8) and a more detailed profiling of tumor-associated angiogenesis in human tumors could possibly reveal novel markers and therapeutic targets.

Tumor-associated vessels are structurally and functionally abnormal, with increased permeability, delayed maturation, and potential for rapid proliferation (9, 10). These vessel defects may also facilitate hematogenous spread of tumor cells (11). Further, recent studies have provided significant insight into the molecular mechanisms of lymphatic vessel development and the role of lymphangiogenesis in the spread and progress of malignant tumors (12–14). Among angiogenic factors, VEGF-A is a strong stimulator of endothelial cell proliferation (15) whereas VEGF-C and VEGF-D are considered more important for lymphangiogenesis although their role in various human tumors is not clear (12, 16, 17).

In endometrial carcinoma, which is a common malignancy of the female genital tract, subgroups show an aggressive and therapy-resistant behavior. Previous studies have reported reduced survival in tumors with high MVD (18, 19) but there are no data on other aspects of the angiogenic phenotype in these tumors.

On this background, we sought to explore in more detail the angiogenic profile of aggressive endometrial carcinoma in a large and population-based series with complete follow-up. We hypothesized that other markers besides MVD could be even more important prognostically. Our specific aims were to examine the presence and prognostic effect of vascular proliferation (20) and to study the degree of pericyte coverage as measured by α-smooth muscle actin (α-SMA) positivity in tumor-associated vessels (21). Because there is only limited information about the role of lymphangiogenesis in endometrial cancer (22), lymphatic vessel density was included. A panel of selected angiogenic markers was examined to see whether they were related to vessel proliferation and patient prognosis. Our study provides novel data indicating that vascular proliferation is an independent prognostic factor and stronger than MVD and other angiogenic markers. Decreased pericyte coverage by α-SMA staining was associated with vascular invasion by tumor cells

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and clinical progress. These findings may improve the histology-based prognostic assessment of vascular phenotype in human tumors and might also be relevant for prediction of treatment response in future studies.

Materials and methods

Patients

All patients diagnosed with endometrial carcinoma in Hordaland County, Norway, during 1981-1990 have been studied ($n = 316$). Hordaland County has ~450,000 inhabitants (~10% of the Norwegian population) and a similar age-adjusted incidence rate of endometrial cancer (23). The treatment protocol was constant during the study period and consisted of abdominal hysterectomy with bilateral salpingo-oophorectomy as initial treatment (24). Adjuvant radiation therapy was recommended for all patients with myometrial tumor infiltration without distant metastases. Paraffin-embedded tumor tissue was available for further studies in 286 cases (96%).

Basic clinicopathologic characteristics known to be of prognostic importance were included in accordance with established criteria, such as histologic type (25), histologic grade according to Fédération Internationale de Gynécologie et d'Obstétrique (FIGO; ref. 25), tumor necrosis (defined as areas of necrotic tumor cells immediately adjacent to viable tumor tissue; ref. 26), and vascular invasion (recorded as no vessel involvement, tumor cells within one vascular space, or tumor tissue in more than one vessel). Tumor stage (FIGO) was also included (27).

The median follow-up time for survivors was 9 years (range, 4-16 years) and no patients were lost due to insufficient follow-up. Information on survival was obtained from medical records, The Cancer Registry of Norway, and The Registry of Deaths of Statistics Norway. The Norwegian Data Inspectorate and the Regional Ethical Committee (Health Region III) have approved this study.

Tissue Microarray

The tissue microarray technique has been described and validated previously (28, 29). Briefly, the area of highest tumor grade was identified on H&E-stained slides and three tissue cylinders (0.6 mm in diameter) were punched from the selected area(s) and mounted into recipient paraffin blocks using a precision instrument (Beecher Instruments, Silver Spring, MD).

Immunohistochemistry

Five-micrometer sections of formalin-fixed and paraffin-embedded archival tissue were used. Standard slides were applied for double staining and LYVE-1 staining whereas tissue microarray slides were used for VEGF-A, VEGF-C, VEGF-D, and basic fibroblast growth factor (bFGF) staining. Details on the immunohistochemical procedures are given in Supplementary Table S1. Sufficient amount of tumor tissue with acceptable staining was available in 281 cases (Ki-67/factor VIII), 273 cases (α-SMA/factor VIII and LYVE-1), and 274 cases (tissue microarray slides). Data on tumor cell proliferation (Ki-67 staining) were included from previous studies for comparison (30).

Variables

Microvessel density. The average number of microvessels within selected tumor areas (MVD) has been widely used as a measure of tumor-associated angiogenesis since introduced in 1991 (31) and was therefore included. As recommended, sections were first examined at low magnifications (×25, ×100) to identify the most vascular areas of the tumors ("hotspots," i.e., the area(s) with most intense factor VIII staining and apparently the highest density of microvessels; ref. 31). Then, as a rule, 10 fields (×250; field size, 0.424 mm^2) were examined, except in a few cases where less tumor tissue was available; the counts were expressed as the average of all fields examined (MVD_{mean}; vessels per mm^2).

Vascular proliferation index. This variable was included as a measure of "active" angiogenesis (20). Proliferating endothelial cells were recognized by their morphology, localization, and distinct Ki-67/factor VIII coexpression. Positive nuclei outside the endothelial cell layer, or within the vessel lumen, were avoided. Vascular proliferation index (VPI) was determined by calculating the ratio between the number of proliferating microvessels with Ki-67-positive endothelial cells (regardless of the number of positive endothelial cells per vessel) and the total number of factor VIII positive microvessels (%), as examined within the selected hotspot areas. In a subset of the tumors ($n = 10$), the vascular proliferation (VPI) of vessels in myometrial stroma was estimated.

Vascular smooth muscle actin index. This variable was included to explore the association between structural aspects of tumor-associated vessels and clinical progress. The degree of pericyte coverage, as estimated by α-SMA-positive cells, was studied (21). Within hotspot areas with highest MVD, a maximum of 10 fields (×400; field size, 0.158 mm^2) were examined. α-SMA index (SMAI) was determined by calculating the ratio between the number of microvessels with α-SMA positivity, using a α-SMA/factor VIII double staining, and the total number of microvessels (%).

Lymphatic vessel density. This variable was included because recent studies have indicated an importance of lymphatic vessels for metastatic spread and patient prognosis. Sections were examined at low magnifications (×25, ×100) to identify tumor area(s) with LYVE-1-positive vessels and hotspot areas if present. Five fields (×250; field size, 0.424 mm^2) were examined and mean values per field were recorded (12). Only LYVE-1-positive vessels with a distinct lumen or clusters of LYVE-1-positive cells were counted. Counts for peritumoral lymphatic vessels (LVD-pt; immediately outside of the tumor mass) and intratumoral lymphatic vessels (LVD-it) were recorded separately. In this study, intratumoral was defined as vessels within the main tumor mass, surrounded by tumor cells, but not considering the exact distance between endothelial cells and tumor cells. Vessels more than one high-power field (×250; field diameter, 0.73 mm) away from the invasive tumor front were not counted. Lymphatic vessels in the deeper part of the myometrium were used as positive internal controls.

Expression of angiogenic factors. The protein expression of selected angiogenic factors (VEGF-A and bFGF) and factors suggested to be important for lymphangiogenesis (VEGF-C and VEGF-D) was examined by immunohistochemistry using established antibodies. For these markers, cytoplasmic staining intensity and the proportion of positive tumor cells were recorded and a staining index (values of 0-9) was calculated as the product of staining intensity (0-3) and area of positive staining (1, <10%; 2, 10-50%; 3, >50%; ref. 32).

Statistical Analysis

Associations between categorical variables were examined by Pearson's χ^2 test. Comparisons of mean values of continuous variables (not following the normal distribution) were evaluated by nonparametric tests [i.e. Mann-Whitney (two groups) or Kruskal-Wallis (more than two groups) test]. Linear association between two continuous variables was evaluated by linear regression analysis or Spearman's correlation coefficient. Univariate survival analyses (using death from endometrial carcinoma as end-point or event; other events were censored) were done using the product-limit procedure (Kaplan-Meier method) with the log-rank test using time of primary operation as entry date (starting point). Variables with effect on survival in univariate analyses ($P \leq 0.15$) were examined by log-log plot to determine whether they could be incorporated in Cox proportional hazards regression models (backward elimination procedure, likelihood ratio test). Tests for interactions were carried out for the variables that were significantly related to survival in Cox regression analysis. Data were analyzed using the SPSS software package (33).

In statistical analyses, cutoff values for staining index categories were based on median or quartile values, also considering the frequency distribution, as well as size of the subgroups and number of events. In survival analyses, subgroups with similar prognosis were merged. Cases were divided in two by the upper quartile for MVD (cutoff value, 83.2), VPI (cutoff value, 7.6), and SMAI (cutoff value, 57.1). For LVD, the median value was used (0 versus >0). For VEGF-A, VEGF-C, VEGF-D, and bFGF, the median value was initially used as cutoff value between high and low expression. Based on survival analyses, cases with the strongest VEGF-A expression (by upper quartile, staining index = 9) had significantly decreased survival compared with the other categories and this cutoff value for VEGF-A was used in selected analyses.

Results

Associations between angiogenic markers (MVD, vascular proliferation, vascular α-SMA status, LVD, and expression of angiogenic factors) and several variables are presented to explore how these angiogenic markers relate to clinicopathologic phenotype and disease progression. For the most part, negative findings will not be referred to.

MVD

Median MVD (Fig. 1*A*) was 60 microvessels/mm^2 (mean, 67; range, 8-189). Table 1 shows the associations between MVD and different clinicopathologic variables. MVD showed no significant association with tumor cell proliferation as estimated by Ki-67 expression (data not shown). Increased MVD was significantly associated with reduced tumor cell expression of the cell adhesion marker E-cadherin (ref. 24; data not shown).

VPI

Median VPI (Fig. 1*B*) was 3.9% (mean, 5.3%; range, 0-21%). In myometrial stroma, mean VPI was 0.02% (median, 0.01%; range, 0-0.06%). VPI was significantly associated with MVD (Spearman's rank correlation, $R = 0.15$, $P = 0.012$). High VPI was associated with presence of tumor necrosis and high FIGO stage (Table 1). Presence of necrosis was also found to be associated with increased tumor cell proliferation by Ki-67, with 19.5% (median, value) in tumors without necrosis compared with 24.0% in tumors with necrosis ($P = 0.018$, Mann Whitney test).

Vascular SMAI

Median SMAI (Fig. 1*C* and *D*) was 35% (mean, 41%; range, 2-99%) and increased MVD was significantly associated with lower SMAI (Spearman's rank correlation, $R = -0.48$, $P < 0.0001$). Low SMAI was more frequent in tumors with vascular invasion by tumor cells and also showed a borderline association with presence of tumor necrosis (Table 1).

LVD

Peritumoral lymphatic vessels (LVD-pt; Fig. 1*E* and *F*) were present in 39.5% of the cases ($n = 88$) with a median LVD-pt of 1.91 vessels/mm^2 in positive cases (mean, 4.30; range, 0.48-25.33); 30.6% of the tumors showed intratumoral lymphatic vessels (LVD-it) especially in the periphery (median, 1.43 vessels/mm^2 for positive cases; mean, 3.4; range, 0.48-42.06). LVD-pt and LVD-it were significantly correlated (Spearman's rank correlation, $R = 0.40$, $P < 0.0001$) whereas LVD-pt showed stronger associations with other variables than did LVD-it. Within the tumors, LVD was significantly associated with a diffusely infiltrative growth pattern (data not shown). Multiple significant associations with clinicopathologic features were found for LVD-pt (Table 2). However, no significant relationships were found between LVD-pt and MVD or VPI.

Coexpression of LYVE-1 and Ki-67 (proliferating lymphatic vessels) was occasionally observed with a mean lymphatic proliferation rate of 0.12% in peritumoral areas (median, 0.037%; range, 0-0.38%) whereas intratumoral areas had a lower mean proliferation rate of 0.08% (median, 0.027%; range, 0-0.23%).

Expression of Angiogenic Factors

VEGF-A. High expression was significantly associated with several clinicopathologic variables and angiogenic markers, such as histologic type and grade, tumor necrosis, VPI, and LVD-pt (Table 3).

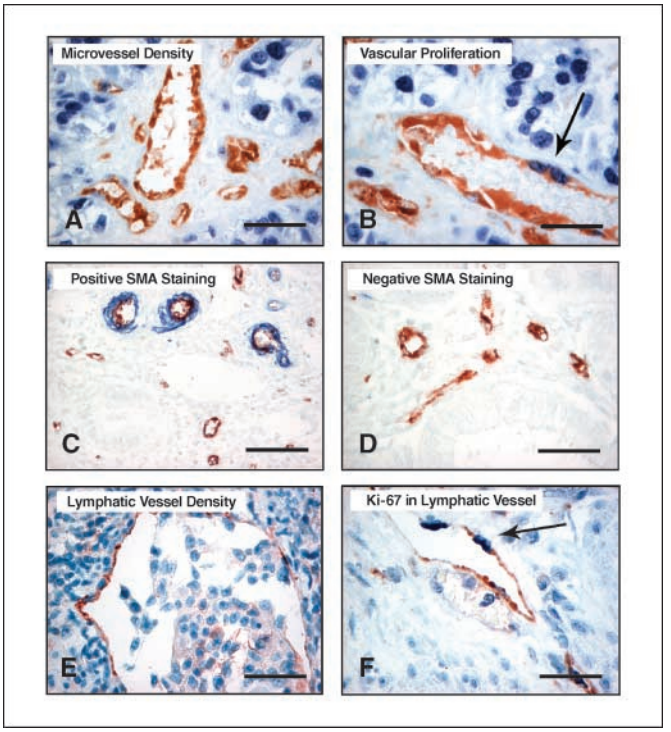

Figure 1. Immunohistochemical staining. *A,* microvessels (*red*; magnification, ×400; bar, 50 μm); *B,* double staining: endothelial cells (*red*) with positive staining for Ki-67 as a marker of proliferation (*blue, arrow*; magnification, ×400; bar, 50 μm); *C,* double staining: microvessels with positive (*blue*) α-SMA staining (magnification, ×200; bar, 100 μm); *D,* vessels negative for α-SMA (magnification, ×400; bar, 50 μm); *E,* LYVE-1-positive lymphatic vessels in peritumoral area (magnification, ×400; bar, 50 μm); *F,* proliferating lymphatic vessel by double staining for LYVE-1 (*red*) and Ki-67/MIB-1 (*blue*); positive staining is seen in nuclei of lymphatic endothelial cells (*arrow*; magnification, ×630; bar, 32 μm).

VEGF-C. High expression was significantly associated with increased histologic grade, presence of necrosis, vascular invasion by tumor cells, and high MVD, but not with LVD (Table 3).

VEGF-D. High expression was significantly associated with increased histologic grade, presence of tumor necrosis (Table 3), and high tumor cell proliferation by Ki-67 (not shown). There was no association with LVD.

bFGF. High expression was significantly associated with several variables, such as histologic grade, presence of tumor necrosis, and vascular invasion (Table 3).

Univariate Survival Analysis

Patient survival was significantly decreased in cases (by upper quartile) with highest values for MVD ($P < 0.0001$; Fig. 2*A*) and VPI ($P = 0.0015$; Fig. 2*B*) and in the patient group with lowest values (by upper quartile) for SMAI ($P = 0.007$; Fig. 2*C*). Similar survival results were obtained when using absolute counts (or counts per mm^2) for proliferating vessels or α-SMA negative vessels (data not shown). Presence of glomeruloid microvascular proliferation, recorded as previously described (34), was significantly associated with decreased survival in this extended set of tumors (data not shown). Five-year survival in cases with no LVD-pt was 83%, compared with 69% in LVD-pt positive cases ($P = 0.019$; Fig. 2*D*). LVD-it was not significant ($P = 0.079$).

For VEGF-A, patients with strong expression (by upper quartile, staining index = 9; $n = 49$) had significantly worse prognosis

Table 1. MVD, VPI, and SMAI related to histopathologic variables in 281 patients with endometrial carcinoma

Variable	No. patients	MVD*	P †	VPI ‡	P †	SMAI§	P †
Histologic type			0.3		0.1		0.6
Endometrioid	252	60		3.8		35	
Serous/clear cell	29	60		5.3		28	
Histologic grade			0.001‖		0.2‖		0.1‖
1	52	59		3.7		36	
2	121	58		3.4		37	
3	108	70		4.7		30	
Tumor necrosis			0.04		0.006		0.08
Absent	117	56		3.4		39	
Present	164	61		4.9		31	
Vascular invasion			0.001		0.5		0.002
0-1 vessel	179	58		3.9		39	
>1 vessels	102	69		4.3		28	
FIGO stage			0.012		0.03		0.3
I/II	226	60		3.7		36	
III/IV	54	72		5.6		32	

*Median values.
†Mann-Whitney U test.
‡Number of Ki-67-positive vessels in % of total number of vessels.
§Number of α-SMA-positive vessels in % of total number of vessels.
‖Kruskal-Wallis test.

compared with the rest (P = 0.011; Supplementary Fig. S1). The other three markers did not influence survival significantly.

Multivariate Survival Analysis

Initially, the angiogenic markers MVD, VPI, and SMAI were entered in addition to basic prognostic factors in endometrial cancer (histologic type, histologic grade, vascular invasion, and FIGO stage; ref. 35). VPI had significant and independent prognostic effect (hazard ratio, 1.7, P = 0.028; Table 4) whereas the variables histologic type, MVD, and SMAI were not significant in this model. In a subgroup analysis of the predominant endometrioid type of carcinomas, VPI confirmed its independent prognostic significance (hazard ratio, 2.2, P = 0.008), together with histologic grade, vascular invasion, and FIGO stage. MVD was not significant in these models. When using absolute counts (see above), similar results were found in multivariate analysis with vascular proliferation as the only significant angiogenic variable (data not shown). LVD-pt had no independent prognostic value. No interactions were found between the significant variables.

Discussion

In this study of endometrial carcinoma, increased vascular proliferation was associated with features of aggressive tumors, such as presence of necrosis and high tumor stage, and further showed an independent prognostic effect in multivariate survival analysis. Structural changes in tumor vessels, as indicated by reduced pericyte coverage, were associated with increased frequency of vascular invasion by tumor cells and decreased survival. In addition, high LVD in peritumoral areas was a marker of high-grade tumors with impaired prognosis. These findings provide novel data on the prognostic features of tumor-associated angiogenesis. Only vascular proliferation was found to be of independent prognostic importance among the angiogenic markers studied.

Proliferation of tumor-associated endothelial cells was generally low, with a median of 4% of tumor vessels showing evidence of endothelial Ki-67 expression, ranging from 0% to 21%. The vascular proliferation in myometrial stroma outside the tumor was clearly

Table 2. LVD-pt (mean; vessel counts per mm^2) related to clinicopathologic variables in patients with endometrial carcinoma

Variable	No. patients*	LVD-pt	P †
Histologic type			0.14
Endometrioid	206	0.51	
Serous/clear cell	17	3.90	
Histologic grade			<0.0001 ‡
1	40	0.32	
2	103	1.31	
3	80	2.88	
Tumor necrosis			0.002
Absent	92	0.86	
Present	131	2.28	
Vascular invasion			<0.0001
0-1 vessel	135	0.93	
>1 vessel	88	2.87	
FIGO stage§			0.54
I/II	184	1.62	
III/IV	38	2.11	

*223 cases available for LVD-pt.
†Mann-Whitney U test when otherwise not specified.
‡Kruskal-Wallis test.
§Data missing in one case.

lower (median, 0.02%). Previous studies of different human cancers have reported a vascular proliferation ranging from 0.15% to 17% using different methods (36–38). In our study, vascular proliferation (VPI) was increased in high-grade tumors with presence of necrosis and advanced tumor stage. The association with necrosis might indicate a relationship with tumor hypoxia and induction of VEGF. Our findings support this as strong tumor cell expression of VEGF-A was associated with both necrosis and increased vascular proliferation.

MVD was associated with patient survival in univariate analysis, in line with what we (30) and others (18) have previously found for endometrial carcinoma, and multiple other tumors (39, 40). Further, high MVD correlated with aggressive tumor features such as presence of tumor necrosis and vascular invasion by tumor cells. It was of special interest to see whether increased vascular proliferation, as a measure of activated angiogenesis, was a stronger prognostic marker than MVD, which is by far the most commonly used histology-based indicator of tumor-associated angiogenesis. Vascular proliferation has, to our knowledge, not been previously examined in prognostic studies of human cancer. In multivariate analysis, vascular proliferation was an independent predictor of patient survival whereas MVD was not significant. This observation supports the arguments of Hlatky et al. (6) on the limitation of MVD as a sole marker of tumor-associated angiogenesis.

There is considerable interest in the structural integrity of tumor vessels and its functional importance (10, 11, 41, 42). The recruitment of mural cells (pericytes and smooth muscle cells) is suggested by some authors to reflect the degree of vessel maturity (21). However, very few studies on human tumors have been presented and endometrial carcinomas have not been studied. We found that 35% of tumor vessels showed presence of α-SMA-positive perivascular cells. Interestingly, low SMA index (pericyte coverage) was associated with increased vascular invasion by tumor cells. In univariate analyses, SMA index showed a significant effect on survival whereas vascular proliferation was the only significant factor of these two in multivariate analysis. Regarding prognosis, one previous study of lung cancer (43) reported a better outcome for tumors with high vascular maturation, in contrast to a study on breast cancer where no difference was found (44). In both studies, the basement membrane antibody LH39 was applied as a maturation marker. Thus, differences might be found between various tissues and tumor types (20).

Table 3. Expression of angiogenic factors VEGF-A, VEGF-C, VEGF-D, and bFGF in relation to clinicopathologic variables and the angiogenic markers MVD, VPI, SMAI, and LVD-pt

Variable	No. patients	VEGF-A expression*		*P*	VEGF-C expression		*P*	VEGF-D expression		*P*	bFGF expression		*P*
		Low(%)	High(%)		Low(%)	High(%)		Low(%)	High(%)		Low(%)	High(%)	
Histologic type				0.009			0.09			0.15			0.12
Endometrioid	246	84	16		45	55		50	50		40	60	
Serous/clear cell	28	64	36		29	71		36	64		25	75	
Histologic grade				<0.0001			0.001			0.001			0.01
Grade 1	51	96	4		65	35		69	31		55	45	
Grade 2	118	88	12		44	56		51	49		39	61	
Grade 3	105	69	31		32	68		36	64		30	70	
Tumor necrosis				0.003			0.02			0.018			0.007
Absent	114	90	10		52	48		57	43		48	52	
Present	160	76	24		37	63		43	58		32	68	
Vascular invasion				0.2			0.01			0.61			0.04
0-1 vessel	173	85	16		49	51		50	50		43	57	
>1 vessel	101	78	22		34	66		46	54		30	70	
FIGO stage				0.5			0.7			0.57			0.27
I/II	220	83	17		43	57		49	51		40	60	
III/IV	53	79	21		45	55		45	55		32	68	
MVD †				0.3			0.005			0.3			0.5
Median value	273	60.0	66.9		63.0	71.4		66.6	68.8		67.4	68.1	
VPI †				0.005			0.3			0.4			0.9
Median value	273	3.6	6.2		5.1	5.6		5.2	5.6		5.3	5.4	
SMAI †				0.1			0.4			0.9			0.5
Median value	268	35.3	25.6		41.7	39.1		39.7	40.7		41.7	39.6	
LVD-pt †				0.04			0.5			0.2			0.2
Mean value	217	1.4	3.3		1.4	1.9		1.7	1.8		1.3	2.0	

NOTE: Percent of the cases with low or high expression of each angiogenic factor is given for all categories of histologic type, histologic grade, tumor necrosis, vascular invasion, and FIGO stage; for MVD, VPI, SMAI, and LVD-pt, median or mean values are given for low or high categories of each angiogenic factor. χ^2 test when otherwise not specified.

*Cutoff point: upper quartile.

†Mann-Whitney *U* test.

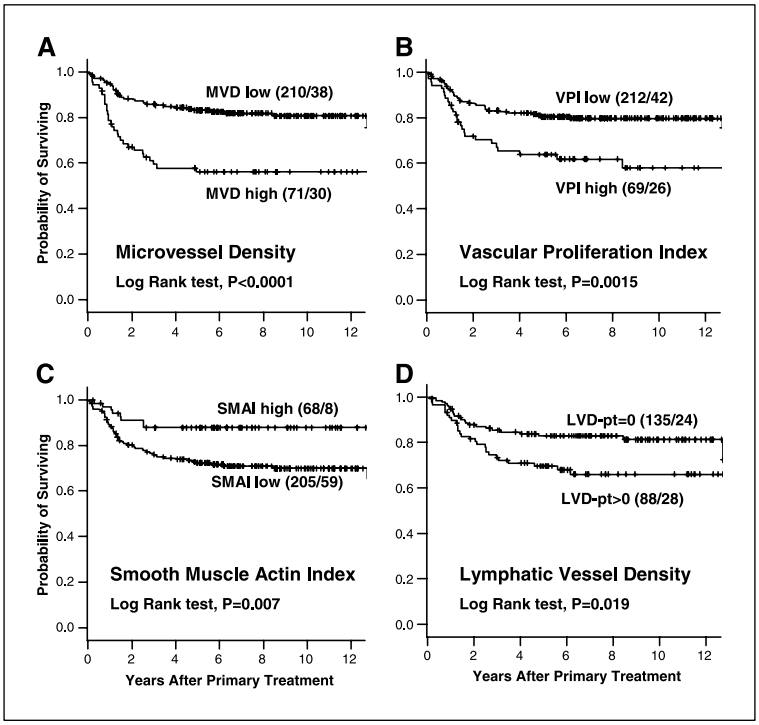

Figure 2. Estimated survival for patients with endometrial carcinoma according to MVD (*A*), VPI (*B*), vascular SMAI (*C*), and LVD-pt (*D*). Survival curves are estimated according to the Kaplan-Meier method with death due to endometrial carcinoma as end point. For each category: number of cases / number of cancer deaths.

Vascular invasion by tumor cells is a strong predictor of metastatic spread and prognosis in malignant tumors (35). In our study, vascular involvement was increased in cases with high MVD, supporting a connection between these features (45). One explanation could be that newly formed tumor vessels are less mature and more rendered to angioinvasion by tumor cells. This is supported by our data showing that high MVD was associated with lower pericyte coverage and cases with lower SMA index were associated with the presence of vascular invasion. Similar results have been shown in experimental studies (11) but the finding has, to our knowledge, not been reported in human tumors.

Table 4. Multivariate survival analysis (Cox proportional hazards method) of angiogenesis markers and clinicopathologic variables in patients with endometrial carcinoma

Variable	Categories	No. patients*	Hazard ratio	P †
Histologic grade	Grade 1-2	167	1	0.001
	Grade 3	105	2.6	
Vascular invasion	0 or 1 vessel	173	1	0.009
	>1 vessels	99	2.0	
FIGO stage	I/II	220	1	<0.0001
	III/IV	52	7.3	
Vascular proliferation ‡	Low	205	1	0.028
	High	67	1.7	

*Only patients with information on all values are included (n = 272).
†Likelihood ratio test (backward stepwise procedure).
‡Number of Ki-67-positive vessels in % of total number of vessels.

Recently, focus has been given to the importance of lymphangiogenesis for tumor spread (14). In our study, presence of peritumoral lymphatic vessels was evident in 40% of the cases. High LVD presented with multiple and strong associations with features of aggressive endometrial carcinomas like high histologic grade, presence of necrosis, and vascular invasion by tumor cells. An association between high LVD and vascular invasion has previously been reported for pancreatic tumors (17). LVD was significantly associated with endometrial cancer deaths in univariate analysis, in line with a few studies of other human tumors (13, 46). Our data on low lymphatic proliferation (0.12%) are consistent with reports on other tumors (12, 17). Although our findings indicate that peritumoral LVD might contribute to the clinical progress of endometrial cancers, other factors are even stronger from a prognostic point of view.

There is considerable discussion about the presence and biological significance of lymphatic vessels within tumors (47). Whereas some studies have suggested a true formation of intratumoral lymphatic vessels (16, 17), others have proposed that co-option of preexisting vessels by invading tumor cells could be an alternative explanation (48). In our study, the finding of lymphatic vessels within the tumor mass, especially in the periphery, could in part be explained by co-option because LVD within the tumors was significantly associated with a diffusely infiltrative growth pattern (data not shown).

Expression of angiogenic factors (VEGF-A, VEGF-C, VEGF-D, and bFGF), as well as MVD and vascular proliferation, was positively associated with the presence of tumor necrosis. This up-regulation of angiogenic factors could be a result of hypoxia and mediated by hypoxia-inducible factor 1α as previously reported in breast cancer (49). The presence of necrosis was also strongly associated with higher density of lymphatic vessels and the presence of hypoxia and activated VEGF-A could be involved (50).

In conclusion, our data indicate that several features of angiogenesis and lymphangiogenesis are associated with clinical

progression of endometrial cancer and might be examined in pathology specimens for outcome information. Of special importance, vascular proliferation was found to be the strongest angiogenic marker in this study independent of other prognostic factors. Decreased pericyte coverage was significantly associated with vascular invasion by tumor cells and reduced patient survival. These angiogenic markers, especially vascular proliferation, should be further studied to examine their role in prognostication and prediction of clinical response to treatment.

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