Proliferation of Immature Tumor Vessels Is a Novel Marker of Clinical Progression in Prostate Cancer

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Abstract

Nestin (neuroepithelial stem cell protein) is expressed in immature endothelial cells, and we here introduce coexpression of Nestin and Ki-67 as a novel angiogenesis marker on tissue sections. Including vascular endothelial growth factor (VEGF)-A and hypoxia-inducible factor-1α (HIF-1α) expression, we studied relation to disease progression in prostate cancer. Different patient series were included. Sections from 104 radical prostatectomies with long follow-up, 33 castration-resistant prostate cancers, 28 nonskeletal metastases, 13 skeletal metastases, and 41 benign prostatic hyperplasias were immunostained for Nestin/Ki-67, VEGF-A, and HIF-1α. Vascular proliferation by Nestin/Ki-67–positive vessels was counted within "hotspot" areas. Median vascular proliferation counts were 4- to 5-fold higher in castration-resistant prostate cancers and metastases versus localized cancers and prostatic hyperplasias ($P < 0.0005$). Among localized cancers, high vascular proliferation was significantly related to adverse clinicopathologic features and was a strong and independent predictor of biochemical failure ($P < 0.005$), clinical recurrence ($P = 0.005$), and skeletal metastasis ($P = 0.025$) in multivariate analysis. Castration-resistant cancers were characterized by reduced VEGF-A and increased HIF-1α expression, and vascular proliferation was associated with reduced patient survival in this group. Thus, vascular proliferation was of independent prognostic importance among prostate cancers. When compared with localized cancers, vascular proliferation was significantly increased in castration-resistant cases and metastatic lesions. The castration-resistant tumors exhibited weak VEGF-A but strong HIF-1α expression. These novel data might have an effect on clinical evaluation and treatment of prostate cancer patients. [Cancer Res 2009;69(11):4708–15]

Introduction

Angiogenesis is important for the growth and spread of malignant tumors (1). In prostate cancer, the second most frequent cause of male cancer deaths, angiogenesis is androgen dependent in early stages of tumor development (2–4) and known to influence patient survival (5–7). Although microvessel density has been used as a marker of tumor-associated angiogenesis in prognostication of malignant tumors, this method has several limitations. Mostly, it reflects the metabolic demand of tissues rather than angiogenesis as pointed out by Hlatky and colleagues (8). Improved markers of active angiogenesis are needed to better predict patient prognosis and response to therapy. This might be especially important after the introduction of antiangiogenesis treatment. In a recent study of endometrial cancer, we proposed that vascular proliferation, the number of vessels with evidence of dividing endothelial cells given by dual staining of factor VIII and Ki-67 antigens, may represent a better angiogenesis indicator than overall microvessel density (9).

We here suggest that coexpression of Nestin (neuroepithelial stem cell protein) and Ki-67 might be used as a novel angiogenesis marker in prostate cancer. Nestin, which has been reported as a marker of proliferating and immature microvessels (10–15), is a class VI intermediate filament protein forming heterodimers mostly with vimentin as an integral part of the cytoskeleton in immature endothelium (16). Consequently, Nestin-positive vessels show higher proliferation rates than $CD34^+$ vessels (12). By combining Nestin expression and the proliferation marker Ki-67, we propose that the most actively expanding and immature parts of the vasculature can be visualized on tissue sections. We show that vascular proliferation, as estimated by Nestin/Ki-67 coexpression, is a superior and independent prognostic factor in localized prostate cancer when compared with standard microvessel density. Notably, vascular proliferation was significantly increased among castration-resistant cancers and in metastatic lesions.

Tumor endothelial marker 7 (TEM7) is a promising indicator of tumor-associated endothelium. It was discovered by using serial analysis of gene expression technology on colon cancer tissue, and TEM7 expression was absent in endothelium from benign tissues (17). By immunohistochemistry, we here found that TEM7 was expressed in tumor-related vessels but also in the epithelium of prostate and colon cancers, making vessel counts difficult.

Vascular endothelial growth factor (VEGF)-A, an endothelial cell mitogen acting on its main receptor VEGF receptor-2, is a pivotal factor of the angiogenic switch in malignant tumors (18). Recently, some studies have explored its prognostic significance in prostate cancer (19–23). Furthermore, tissue hypoxia is also a critical factor during the establishment of tumor angiogenesis and has been associated with increased tumor cell migration, metastasis, and poor patient outcome (24, 25). Hypoxia-inducible factor-1α (HIF-1α; refs. 26, 27) has been studied in localized prostate cancer with biochemical failure as an endpoint, but the results have been conflicting (28, 29). Our present findings indicate that castration-resistant prostate cancers might switch to HIF-1α-driven and VEGF-A-independent angiogenesis. HIF-1α is known to activate >100 genes promoting cell survival under hypoxic conditions (25) and may be an important characteristic of the phenotype of aggressive castration-resistant prostate cancers.

The aim of our study was to establish improved angiogenesis markers in human prostate cancer and to examine the significance of VEGF-A and HIF-1α expression, two important angiogenic

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Table 1. Associations between vascular proliferation (Nestin/Ki-67), cytoplasmic VEGF-A, nuclear HIF-1α, and clinicopathologic features in 104 patients with clinically localized prostatic adenocarcinoma (radical prostatectomies)

Variable	Nestin-Ki-67			VEGF-A			HIF-1α		
	Low	High	P*	Low	High	P*	Low	High	P*
	n (%)	*n* (%)		*n* (%)	*n* (%)		*n* (%)	*n* (%)	
Gleason score †									
≤3+4	35 (71)	14 (29)	0.11	42 (88)	6 (12)	0.06	34 (72)	13 (28)	0.82
≥4+3	31 (56)	24 (44)		40 (73)	15 (27)		38 (70)	16 (30)	
Diameter ‡									
≤31 mm	53 (68)	25 (32)	0.07	63 (82)	14 (18)	0.29	54 (70)	23 (30)	0.64
>31 mm	12 (48)	13 (52)		18 (72)	7 (28)		18 (75)	6 (25)	
Extraprostatic extension									
Absent	26 (81)	6 (19)	0.01	28 (90)	3 (10)	0.07	23 (77)	7 (23)	0.43
Present	40 (56)	32 (44)		54 (75)	18 (25)		49 (69)	22 (31)	
Seminal vesicle invasion									
Absent	49 (71)	20 (29)	0.02	56 (82)	12 (18)	0.33	46 (69)	21 (31)	0.41
Present	17 (49)	18 (51)		26 (74)	9 (26)		26 (77)	8 (23)	
Pathologic stage§									
pT_2	25 (83)	5 (17)	0.007	26 (90)	3 (10)	0.11	21 (75)	7 (25)	0.60
$\geq pT_3$	41 (55)	33 (45)		56 (76)	18 (24)		51 (70)	22 (30)	
Lymph node infiltration‖									
Absent	63 (65)	34 (35)	0.25	78 (81)	18 (19)	0.14	67 (71)	27 (29)	0.99
Present	3 (43)	4 (57)		4 (57)	3 (43)		5 (71)	2 (29)	

*Pearson's χ^2 test.
†Gleason score in radical prostatectomy specimens.
‡Largest tumor dimension in prostatectomy specimen (by top quartile).
§Pathologic stage, International Union Against Cancer tumor-node-metastasis classification of malignant tumors, 1992 (37).
‖Pelvic lymph node infiltration at radical prostatectomy.

factors, in prostatic tissues. Our findings may be important for the treatment and follow-up of prostate cancer patients.

Materials and Methods

Patients and Tissues

Different tissues were collected for the present study. As described, a consecutive series of 104 men (median, 62.0 years) treated by radical prostatectomy for clinically localized cancer [1988-1994; in part before the prostate-specific antigen (PSA) screening era in Norway; ref. 30] with complete follow-up was included (5, 30–32). In addition, 33 castration-resistant prostate cancers (median, 77.3 years), 13 skeletal metastases, 33 soft tissue metastases (28 from lymph nodes), and 41 cases of benign prostatic hyperplasia were included.

Clinicopathologic Variables

Among the 104 radical prostatectomies, largest tumor dimension (median) was 28 mm (range, 10-45 mm). Other clinicopathologic characteristics are shown in Table 1. The median preoperative serum PSA (s-PSA) was 11.2 ng/mL (range, 1.8-70.0). Tumor cell proliferation (Ki-67; ref. 33) and other biomarkers (30, 32) were included from previous studies for comparison.

Tissue Microarrays

The tissue microarray technique has been used previously (30–32). Briefly, three tissue cores (diameter 0.6-1.0 mm) were obtained from representative areas of highest tumor grade (35). Tissue microarray sections were used for VEGF-A and HIF-1α immunohistochemical staining (regular sections were used for the 13 skeletal metastases).

Immunohistochemistry

Staining was done on formalin-fixed and paraffin-embedded tissue using 5 μm sections (32).

Nestin/Ki-67. Regular sections were used for this marker. After microwave antigen retrieval (boiling 20 min at 350 W) in citrate buffer (pH 6.0), slides were incubated for 1 h in room temperature with both monoclonal mouse Nestin antibody 10C2 (sc-23927; Santa Cruz Biotechnology) diluted 1:50 and monoclonal rabbit Ki-67 antibody clone SP6 (Neomarkers) diluted 1:100. Staining was done on a DAKO Autostainer Instrument (DAKO) using goat anti-rabbit IgG (Southern Biotech) diluted 1:100 in horseradish peroxidase goat anti-mouse EnVision (DAKO) for 30 min at room temperature. The alkaline phosphatase was localized by Fast Blue BB F-3378 (Sigma) for 19 min at room temperature and horseradish peroxidase by AEC K3469 (DAKO) for 17 min at room temperature. Counterstaining was not done.

VEGF-A. After boiling in Tris-EDTA (pH 9.0) for 15 min at 350 W, sections were incubated for 1 h at room temperature with the monoclonal mouse antibody VEGF-A (R&D Systems) diluted 1:30 and stained with horseradish peroxidase EnVision (DAKO) for 30 min at room temperature.

HIF-1α. After microwave antigen retrieval (boiling 20 min at 350 W) in citrate buffer (pH 6.0), slides were incubated overnight at 4°C with a monoclonal mouse HIF-1α antibody clone H1α67 (sc-53546; Santa Cruz Biotechnology) diluted 1:40 and stained with MACH 3 Mouse Horseradish Peroxidase Polymer Detection kit (Biocare Medical). Colon cancer with perinecrotic nuclear positivity served as a positive control.

TEM7. Sections were boiled in Tris-EDTA (pH 9.0) for 15 min at 350 W and incubated for 30 min at room temperature with the monoclonal mouse (IgG_1) antibody TEM7 (Imgenex) diluted 1:1,500 and stained with EnVision anti-mouse/rabbit K5007 (DAKO) for 30 min at room temperature.

For VEGF-A, HIF-1α, and TEM7, horseradish peroxidase was localized by the diaminobenzidine tetrachloride peroxidase reaction and sections were counterstained with Mayer's hematoxylin. Negative controls were obtained using either isotypic mouse immunoglobulin (IgG_{2B}) or antibody diluent omitting the primary antibody.

Evaluation of Staining Results in Prostate Tissues

Blue Ki-67-positive nuclei were identified in coexpression with Nestin-positive tumor vessels. Number of Nestin-positive vessels (microvessel density) and number of Ki-67–positive vessels (Ne/Ki-67) were recorded in 10 high-power fields (×400; total 1.6 mm^2) within selected "hotspot" areas. Immature vessels were defined as elongated, branched, or curved Nestin-positive structures with or without a lumen. Single cells in the tumor stroma with a weaker staining (possibly immature fibroblasts, nerve cells, or stem cells) were not counted.

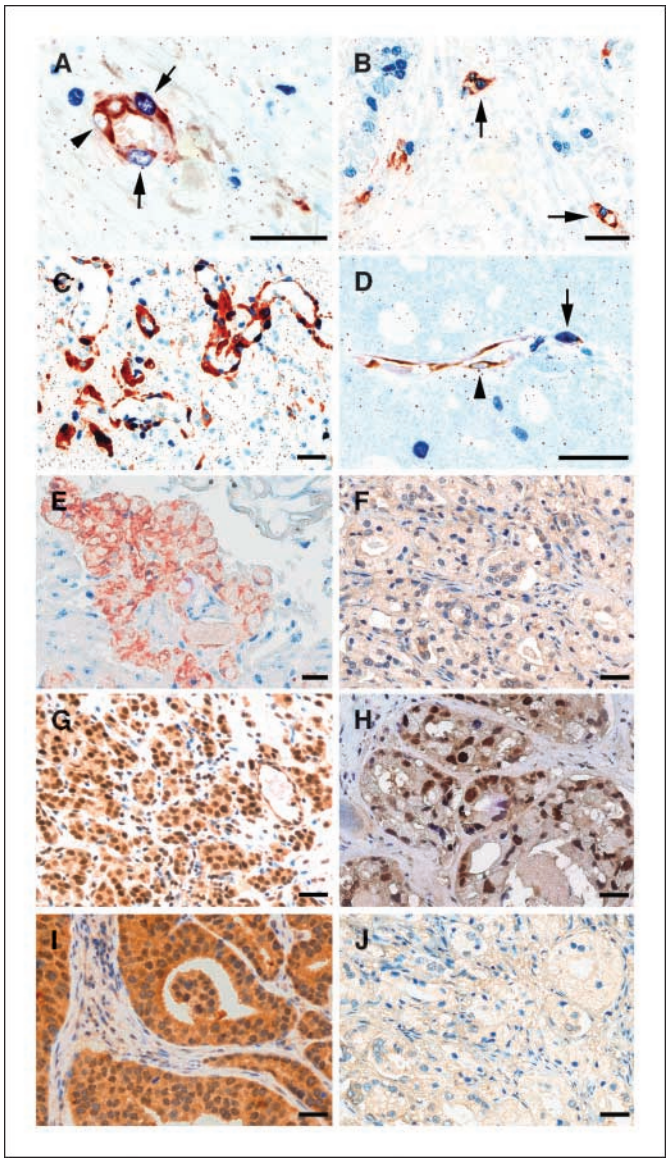

Figure 1. Vascular proliferation in prostate cancer by Nestin (*red*) and Ki-67 (*blue*) dual immunostaining from areas with high proliferation (*A-E*) and HIF-1α and VEGF-A expression (*F-J*) in prostate cancer. *A,* prostatic hyperplasia, Nestin-positive vessel with two Ki-67–positive endothelial nuclei (*arrows*) and two negative nuclei (*arrowhead*). *B,* localized prostate cancer, Ki-67–positive proliferating vessels highlighted (*arrows*). *C,* castration-resistant cancer with marked vessel proliferation. *D,* poorly differentiated skeletal metastasis with Ki-67–positive endothelial nucleus (*arrow*) and Ki-67–negative nucleus (*arrowhead*), both within Nestin-positive vessel. *E,* castration-resistant cancer with glomeruloid microvascular proliferation. *F,* weak nuclear HIF-1α expression in localized prostate cancer. *G* to *H,* strong nuclear HIF-1α staining in castration-resistant cancer and skeletal metastasis, respectively. *I* and *J,* strong and weak VEGF-A expression in localized cancers, respectively. *Bar*, 30 μm.

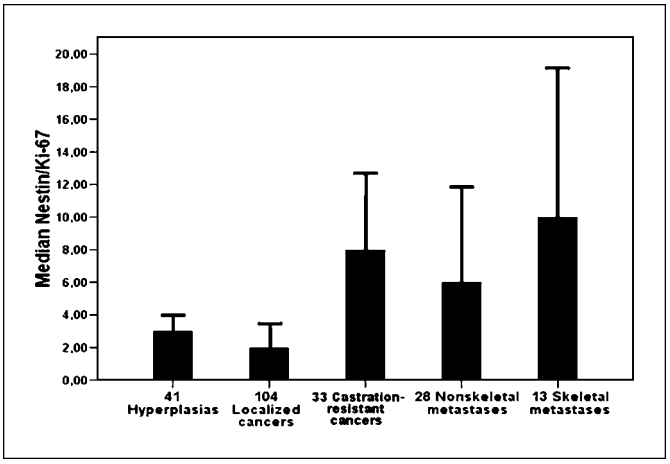

Figure 2. Median vascular proliferation by Nestin/Ki-67 coexpression (absolute number of Ki-67 and Nestin-positive vessels recorded in 10 high-power fields, ×400; total 1.6 mm^2) within selected "hotspot" areas in different prostatic tissues showed 4- to 5-fold increase in advanced (castration-resistant) prostate cancer versus localized cancer and hyperplasia ($P < 0.0005$). *Columns,* median; *bars,* 75 percentiles.

For VEGF-A and HIF-1α expression, a staining index (SI; values 0-9) was calculated as a product of a subjectively determined staining intensity (grades 0-3) and proportion of positive tumor cells (0%, 0; 1-10%, 1; 11-50%, 2; and >50%, 3; ref. 36).

Cutoff points for SI categories were based on the frequency distribution for each marker (median, tertile, and quartile), also considering the number of cases and events in each subgroup; categories with similar survival estimates were merged. Absolute number of Ki-67–positive vessels (Ne/Ki-67) was divided into high (greater than median) or low (median or less). VEGF-A staining was categorized by top quartile as strong (SI = 9) or weak (SI = 0-6), nuclear HIF-1α by top quartile as strong (SI ≥ 6) or weak (SI = 0-4), and cytoplasmic HIF-1α by median as strong (SI ≥ 6) or weak (SI = 0-4). All photomicrographs were taken using a Leica DMLB microscope with an Olympus camera with Olympus DP 5-0 software.

Follow-up

For the 104 patients treated by radical prostatectomy, postoperative s-PSA, locoregional tumor recurrences, distant metastases, and patient survival were recorded (5). Time from surgery until biochemical failure (defined as persistent or increasing s-PSA level of ≥0.5 ng/mL in two consecutive blood samples) was noted. Tumors in the prostatic fossa or evidence of distant metastasis on bone scan, X-ray, or magnetic resonance imaging was recorded as clinical recurrence. The last date of follow-up was December 31, 2001 (median follow-up time, 95 months). No patients were lost to follow-up; 67 patients experienced biochemical failure; 31 had clinical recurrence (15 with skeletal metastasis and 26 locoregional recurrences); and 9 patients died of prostate cancer. Patients with nonskeletal (n = 28) and skeletal metastases (n = 13) did not receive androgen ablation before tissue sampling.

Among 33 patients with castration-resistant cancer, time (in weeks) from resistance to death was recorded (maximum follow-up, 180 weeks). Castration resistance was defined as disease progression during androgen ablation therapy (which did not include antiandrogens). Most patients had clinical progression and increasing s-PSA on consecutive measurements. In 9 patients diagnosed before the PSA era, disease progression was based on physical examination or imaging results.

Statistics

Associations between variables were assessed by Pearson's χ^2 test, Mann-Whitney *U* test, or Kruskal-Wallis test when appropriate. Univariate survival analysis was done by the Kaplan-Meier method (log-rank test), and multivariate survival analysis was done using the proportional hazards method and likelihood ratio test. Model assumptions were examined by log-log plots. Interobserver variability was examined by Spearman's ρ. The SPSS statistical package version 15.0 (SPSS) was used.

Results

Vascular Proliferation (Nestin/Ki-67) in Different Prostatic Tissues

Nestin stained mainly small tumor vessels appearing as vascular clusters ("hotspot" areas) within both tumor and benign tissue (Fig. 1*A-D*). The Nestin-positive vascular clusters were separated by Nestin-negative areas. Glomeruloid microvascular proliferation was observed mainly in castration-resistant carcinomas (Fig. 1*E*). Larger and mature vessels were negative or weak, and nerve structures showed moderate staining. Vascular proliferation (number of Ne/Ki-67–positive vessels per 10 high-power field; total area, 1.6 mm^2) was, on average, 4- to 5-fold higher in 33 castration-resistant prostate cancers (median, 8.0; mean, 16.0; range, 0-180), 13 skeletal metastases (median, 10.0; mean, 13.5; range, 0-55), and 28 soft-tissue metastases (median, 6.0; mean, 7.8; range, 0-25), when compared with 104 localized cancers (median, 2.0; mean, 2.8; range, 0-15) and 41 benign prostatic hyperplasias (median, 3.0; mean, 4.9; range, 0-32; $P < 0.0005$; Fig. 2).

Among the 104 localized cancers, high vascular proliferation (above median) was associated with presence of extraprostatic tumor extension ($P = 0.012$), seminal vesicle invasion ($P = 0.025$), and high pathologic stage ($\geq pT_3$; $P = 0.007$; Table 1). Further, vascular proliferation was related to high VEGF-A expression ($P = 0.001$). Ne/Ki-67 was also associated with markers of epithelial-to-mesenchymal transition from our previous study (30), such as

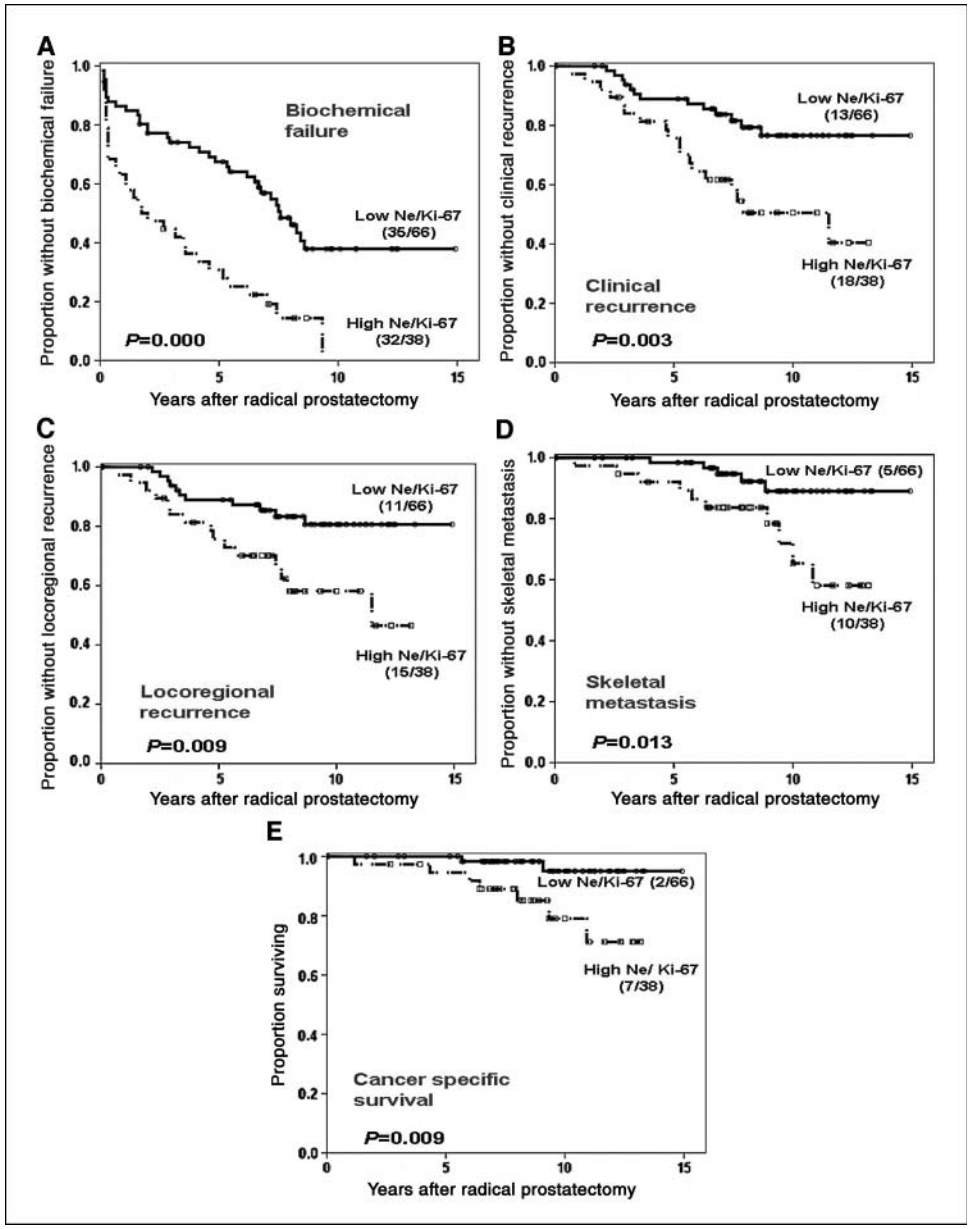

Figure 3. Univariate survival analyses (Kaplan-Meier method) by vascular proliferation (Nestin/Ki-67 coexpression) in localized prostate cancer with (*A*) biochemical failure, (*B*) clinical recurrence, (*C*) locoregional recurrence, (*D*) skeletal metastasis, and (*E*) cancer-specific patient survival as endpoints.

reduced E-cadherin expression (P = 0.001), positive membranous N-cadherin expression (P = 0.025), reduced p120^{CTN} staining (P = 0.004), strong P-cadherin staining (P = 0.033), and the EN-cadherin switch (ref. 30; low E-cadherin, positive N-cadherin; P < 0.0005). Ne/Ki-67 was not associated with tumor cell proliferation by Ki-67 expression (P = 0.13) but was significantly related to vascular proliferation given by factor VIII/Ki-67 (ref. 32; P = 0.008).

Using Ne/Ki-67–positive vessels as a percentage of all Nestin-positive vessels (vessel proliferation index) gave comparable results, but associations tended to be somewhat weaker than when using the absolute count of Ne/Ki-67–positive vessels (data not shown).

Univariate survival analyses. High vascular proliferation was strongly associated with shorter time to biochemical failure (P < 0.0005), clinical recurrence (P = 0.003), locoregional recurrence (P = 0.009), skeletal metastasis (P = 0.013), and cancer specific death (P = 0.009; Fig. 3). In contrast, microvessel density by Nestin single stain (microvessel density) was not associated with patient survival or clinicopathologic variables.

Within the series of castration-resistant prostate cancers (n = 33), patients with low Ne/Ki-67 (bottom tertile) showed better survival using time from castration resistance to cancer-specific death as endpoint (P = 0.063, log-rank test; P = 0.052, univariate Cox analysis; Fig. 4).

Expression of VEGF-A in Different Prostatic Tissues

VEGF-A variably stained the cytoplasm in benign and malignant prostatic epithelium (Fig. 1*I-J*). VEGF-A expression was decreased in castration-resistant cancers (median SI, 3) when compared with localized cancers (median SI, 6), skeletal metastases (median SI, 6), and nonskeletal metastases (median SI, 4.5), whereas expression in benign hyperplasias (median SI, 3) was also weak (P < 0.0005, Kruskal-Wallis test).

Among localized carcinomas, strong VEGF-A expression tended to be associated with higher Gleason score, extraprostatic extension (Table 1), and high tumor cell proliferation by Ki-67 (P = 0.041). Maximum Nestin microvessel density counts were related to stronger VEGF-A expression (P = 0.042). Strong VEGF-A staining was associated with several epithelial-to-mesenchymal transition markers, such as low membrane p120^{CTN} expression (P = 0.024), membrane N-cadherin staining (P = 0.046), and the EN-cadherin switch (ref. 30; P = 0.076).

Univariate survival analysis. Among localized cancers, strong VEGF-A was associated with shorter time to biochemical failure (P = 0.042) and clinical recurrence (P = 0.030).

Expression of HIF-1α in Different Prostatic Tissues

HIF-1α showed both cytoplasmic and nuclear positivity (Fig. 1*F-H*). Moderate to strong nuclear staining of HIF-1α (SI ≥ 4) was observed in 88% (29 of 33) of castration-resistant carcinomas (median SI, 6) and 62% (8 of 13) of skeletal metastases (median SI, 6) when compared with only weak and sporadic staining in 104 localized carcinomas (median SI, 3), nonskeletal metastases (median SI, 3), and benign prostatic hyperplasias (median SI, 2; P < 0.0005, Kruskal-Wallis test).

Among localized carcinomas, nuclear HIF-1α expression was not related to clinicopathologic variables (Table 1), but strong expression was associated with epithelial-to-mesenchymal transition markers such as weak membrane β-catenin expression (P = 0.003), weak p120^{CTN} (P = 0.055), and positive nuclear β-catenin staining (P = 0.022). Strong nuclear staining of HIF-1α was associated with stromal staining of basic fibroblast growth factor (P = 0.013) using data from our previous study (32).

Univariate survival analyses. Among localized cancers, strong nuclear expression of HIF-1α was related to increased risk of biochemical failure (P = 0.041), clinical recurrence (P = 0.083), and locoregional recurrences (P = 0.050). No differences were observed for cytoplasmic HIF-1α among the 104 localized carcinomas.

Observer Variation for Vascular Proliferation (Ne/Ki-67), VEGF-A, and HIF-1α

Twenty-five randomly selected cases stained for Ne/Ki-67, VEGF-A, and HIF-1α, respectively, were reevaluated independently by two observers (K.G. and O.J.H.), both blinded from earlier registrations and clinical data. Observations were significantly associated between the two observers for vascular proliferation by Ne/Ki-67 (Spearman's ρ = 0.80; P < 0.0001), VEGF-A (Spearman's ρ = 0.45; P = 0.024), and HIF-1α (Spearman's ρ = 0.67; P < 0.0001).

Expression of TEM7 in Different Prostatic Tissues

TEM7 preferentially stained tumor-associated vessels, but a strong cross-reactivity in prostate and colon cancer epithelium was noted, making this marker unsuitable for vessel counts in tumor stroma. TEM7 was therefore not further studied.

Multivariate Survival Analysis of Localized Prostate Carcinomas

Variables with P values < 0.15 in univariate survival analyses were included together with basic factors such as preoperative s-PSA, Gleason score (≤3+4 versus ≥4+3), and pathologic stage (≥pT$_3$ versus pT$_2$; ref. 37). Vascular proliferation (above median) consistently showed a strong and independent prognostic effect together with Gleason score using biochemical failure, clinical recurrence, or skeletal metastasis as endpoints.

When VEGF-A expression was included in addition to the basic factors, strong VEGF-A showed only a borderline significance [hazard ratio (HR), 2.0; P = 0.098] in addition to Gleason score (HR, 4.7; P = 0.001) with respect to clinical recurrence, whereas only Gleason score predicted biochemical failure and skeletal metastasis.

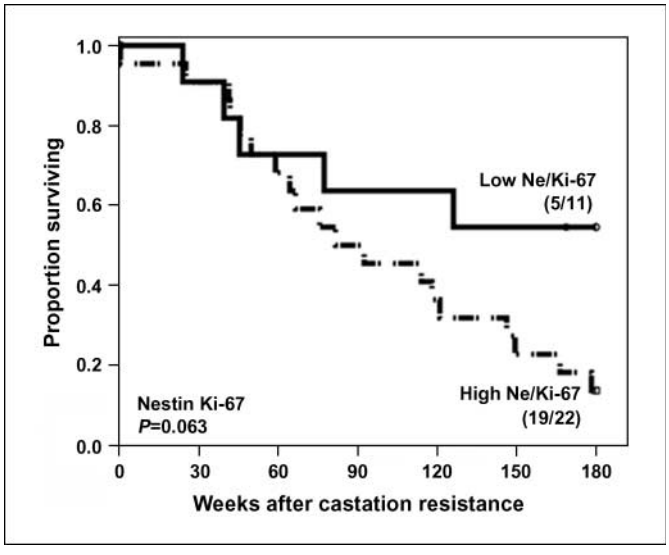

Figure 4. Univariate survival analysis (Kaplan-Meier method) by vascular proliferation (Nestin/Ki-67 coexpression) in castration-resistant prostate cancers with cancer-specific survival as endpoint.

Table 2. Multivariate survival analysis according to Cox's proportional hazards method for patients with clinically localized prostate cancer using biochemical failure, clinical recurrence, and skeletal metastasis as endpoints

Variable	*n*	HR (95% confidence interval)	*P**
Biochemical failure			
Gleason score †			
≤3+4	41	1.0	<0.0005
≥4+3	54	3.5 (2.0-6.4)	
Nestin/Ki-67 ‡			
Low	60	1.0	<0.0005
High	35	2.7 (1.6-4.7)	
Pathologic stage			
pT_2	28	1.0	0.051
≥pT_3	67	1.9 (1.0-3.8)	
Preoperative s-PSA (ng/mL)§			
≤20	71	1.0	0.030
>20	24	1.9 (1.1-3.5)	
HIF-1α			
Low	67	1.0	0.048
High	28	1.8 (1.0-3.0)	
Clinical recurrence			
Gleason score			
≤3+4	41	1.0	<0.0005
≥4+3	54	5.0 (1.7-14.3)	
Nestin/Ki-67 ‡			
Low	60	1.0	0.003
High	35	3.1 (1.5-6.7)	
Skeletal metastasis			
Gleason score			
≤3+4	41	1.0	0.006
≥4+3	54	8.3 (1.1-63.6)	
Nestin/Ki-67 ‡			
Low	60	1.0	0.010
High	35	4.2 (1.3-13.5)	

*Likelihood ratio test.
†Gleason score in radical prostatectomy specimens.
‡Nestin/Ki-67 counts, cutoff by median value.
§Six cases lacking information on preoperative s-PSA.

When HIF-1α expression was included in addition to the basic factors, strong nuclear HIF-1α (HR, 1.8; $P = 0.038$), Gleason score (HR, 3.5; $P < 0.0005$), and pT (HR, 2.3; $P = 0.012$) were independent predictors of biochemical failure; Gleason score (HR, 5.4; $P < 0.0005$) remained in the model using clinical recurrence as endpoint, whereas nuclear HIF-1α (HR, 2.0; $P = 0.087$) was of borderline importance.

In final models using different endpoints, including the basic factors s-PSA, Gleason score, pT, vascular proliferation, VEGF-A, and HIF-1α in the first step, both Gleason score and Ne/Ki-67 consistently showed a strong and independent prognostic effect using biochemical recurrence, clinical recurrence, or skeletal metastasis as endpoints (Table 2).

Multivariate Survival Analysis of Castration-Resistant Carcinomas

Including vascular proliferation and VEGF-A in the model with time from castration resistance to cancer-specific death as endpoint, high vascular proliferation was an independent predictor of cancer specific death (HR, 2.7; $P = 0.036$), whereas low VEGF-A was of borderline significance (HR, 2.5; $P = 0.068$).

Discussion

Angiogenesis is important for the growth of primary tumors, vascular spread, and expansion of metastases (1). Whereas microvessel density has been applied as an estimate of tumor-associated angiogenesis, we here focus on a novel angiogenesis marker. Proliferation of immature tumor vessels is estimated by immunohistochemical coexpression of Nestin and Ki-67 on tissue sections of tumors. Immature vessels are outlined by Nestin staining, whereas proliferating endothelial cells in vascular structures are delineated by the proliferation marker Ki-67. We find that vascular proliferation (by Ne/Ki-67) is significantly increased in castration-resistant prostate cancer and various metastatic lesions when compared with localized cancers and benign prostatic hyperplasias. Within the group of localized carcinomas, vascular proliferation was found to be an independent prognostic marker using different endpoints of disease progression, such as biochemical failure, clinical recurrence, and skeletal metastases. Among the highly aggressive castration-resistant carcinomas, cases with lower vascular proliferation had the best prognosis. These findings support the view that angiogenesis as estimated by vascular proliferation is important in predicting clinical progression of prostate cancer and appears to be especially increased from localized to castration-resistant tumors.

In localized prostate cancers, vascular proliferation and Gleason score were the two strongest independent predictors of biochemical failure, clinical recurrence, or skeletal metastasis. High vascular proliferation was also associated with adverse features such as increased tumor diameter, advanced pathologic stage (pT_3), and seminal vesicle invasion. Although tumor cell expression of both VEGF-A and HIF-1α was associated with patient outcome, vascular proliferation was the strongest prognostic factor in multivariate analysis. In previous studies of this series, microvessel density by factor VIII staining showed only modest prognostic value within the subgroup of moderately differentiated carcinomas. In the present study, we show that microvessel density by Nestin monostaining had no prognostic value. It thus appears that proliferation of immature tumor vessels is a novel and superior angiogenesis marker in prostate cancer.

Elevated vascular proliferation was observed in benign prostatic hyperplasia when compared with localized cancers. Benign hyperplasia in the transition zone is characterized by hyperplastic nodules frequently being larger than malignant tumors in the peripheral zone. Hyperplastic tissue could therefore have a proangiogenic ability equal to or higher than localized cancers. Others have found that microvessel density in hyperplasia is comparable with that of cancers and higher than in benign tissue from the peripheral zone (38).

Among castration-resistant prostate cancers, expression of VEGF-A was weaker than in localized carcinomas. In marked contrast, the opposite was found for nuclear HIF-1α expression, which was very weak among localized carcinomas, whereas almost all castration-resistant cancers were strongly positive. Our findings indicate that castration-resistant tumors might switch to different angiogenic factors than VEGF-A, and this is supported by experimental data. In the androgen-dependent prostate cancer cell line LNCaP, testosterone (dihydrotestosterone) induces HIF-1α

and VEGF-A expression (4). In clinical studies, androgen ablation has been shown to have an antiangiogenesis effect (3). Low VEGF-A levels have been found in many androgen-independent cell lines (39–41) as well as castration-resistant human prostate cancer tissue (42). In contrast, plasma levels of VEGF-A is increased with metastatic disease (42–44), similar to what we found, but not further elevated after castration-resistant metastatic disease (45). Several other proangiogenic factors than VEGF-A can be stimulated by HIF-1α, among them basic fibroblast growth factor, platelet-derived growth factor-β, plasminogen activator inhibitor-1, angiopoietins 1 and 2, and matrix metalloproteinase-2 and -9 as well as VEGF receptors (Flt-1 and Flk-1) and Tie-2 receptor (25, 41, 46–48). One study showed up-regulation of VEGF-D and down-regulation of VEGF-A in castration-resistant tissues when compared with localized cancer (42). Factors other than VEGF-A might be present and explain the increased vascular proliferation observed in castration-resistant cancers.

Our present findings might be relevant for angiogenesis-based treatment of prostate cancer at different stages. Several agents are being developed as HIF-inhibitors, among them topotecan (49) and 2-methoxyestradiol (50), and these might be of interest in castration-resistant cancers. There are ongoing trials with bevacizumab treatment, and one with topotecan treatment for patients with castration-resistant prostate cancers.[3] To speculate, estimates of vascular proliferation as well as expression of angiogenic factors such as VEGF-A and HIF-1α should be examined with respect to response prediction in these settings.

In summary, proliferation of tumor-associated vessels was significantly increased from localized prostate cancers to castration-resistant tumors and metastatic lesions. Within the group of localized cancers, vascular proliferation, as estimated by Nestin/Ki-67 coexpression, is an independent prognostic factor and superior to microvessel density. Our findings on VEGF-A and HIF-1α expression indicate that castration-resistant prostate cancers might switch to VEGF-A–independent angiogenesis, whereas VEGF-A may be more important for localized carcinomas. These results might be relevant for the clinical evaluation and treatment of prostate cancer patients.

[3] www.clinicaltrials.gov

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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