VEGFR1 and NRP1 Endothelial Expressions Predict Distant Relapse after Radical Prostatectomy in Clinically Localized Prostate Cancer

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Abstract. *Prostate cancer can usually be treated at a clinically localized stage by radical prostatectomy. Unfortunately, within 10 years following surgery, 30% of patients experience local or distant relapse. Few data exist on the association of markers of angiogenesis and distant relapse after radical prostatectomy. By immunohistochemistry in tissue microarray, we compared the expression pattern of hypoxia inducible factor 1, alpha subunit (HIF1α) and vascular endothelial growth factor (VEGF) and its receptors in 45 patients with distant relapse and 68 patients without relapse after radical prostatectomy. Expressions of HIF1α and VEGF were assessed in prostate tumor cells and those of VEGFR1, VEGFR2 and neuropilin 1 in tumor and endothelial cells. The five molecules studied were expressed by all tumors, with the exception of neuropilin 1 in endothelial cells for one tumor. Strong endothelial expression of VEGFR1 appeared to be an independent predictor of distant relapse. A moderate to strong endothelial expression of neuropilin 1 was in turn an independent predictor of absence of distant relapse. No significant difference was found for HIF1α, VEGF, VEGFR1, VEGFR2 and neuropilin 1 expression in tumor cells, nor for VEGFR2 in endothelial cells, between the two groups. To our knowledge, this is the first study to evaluate the prognostic value of VEGFR1, VEGFR2 and neuropilin 1 in endothelial cells in prostate cancer after radical prostatectomy. The evaluation by immunohistochemistry of endothelial expression of neuropilin 1 and VEGFR1 could be an additional tool in the assessment of tumor aggressiveness of clinically localized prostate cancer to better identify patients at high risk of distant relapse.*

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Prostate cancer is the third leading cause of cancer-related death among men in developed countries (1). The generalization of its screening with digital examination and serum prostate-specific antigen (PSA) level led, not only, to a significant increase in its incidence, but also, in most cases, to treatment of the disease at a stage clinically localized to the prostate accessible to radical prostatectomy. Validated prognostic factors such as tumor-nodes-metastasis (TNM) status, Gleason score, serum PSA concentration before treatment associated with surgical margin status allow the risk of relapse after radical prostatectomy to be assessed (2). However, these tools lack precision. Thus, within 10 years following surgery, about 30% of the patients experience relapse locally or at distance, initially revealed by a serum PSA rise (biochemical failure) (3). While local relapse can be treated with good results by salvage radiotherapy, distant relapse is associated with a worse prognosis; disease progression is inevitable despite the initial efficacy of hormonotherapy and chemotherapy (4). New biomarkers are needed to identify patients at high risk of distant relapse (5). The identification of such markers, potential therapeutic targets, would help to better understand the molecular mechanisms underlying metastatic spread and, possibly, enable these patients to be offered neo-adjuvant or adjuvant targeted treatments.

Angiogenesis plays an important role in tumor growth and metastasis (6). Among the multiple signaling pathways involved in angiogenesis, the main and best characterized is that of vascular endothelial growth factor (VEGF) and its receptors VEGFR1, VEGFR2 and neuropilin 1 (NRP1) (7). VEGFR1 is a high affinity receptor for VEGF but also for placenta growth factor (PGF) and VEGFB, two other members of the VEGF family (8, 9). It is present on vascular endothelial cells and tumor cells, including prostate cells (10), and on monocytes, hematopoietic stem cells and endothelial progenitor cells (7). VEGFR2, also expressed by prostate tumor cells (10), is considered as the receptor providing the majority of the biological effects of VEGF on endothelial cells (7). NRP1 can act not only as a co-receptor for VEGFR1 but also as a full-fledged receptor (11). It is expressed by arterial endothelial cells (12) and prostate tumor cells (13). Intratumoral hypoxia *via* the transcription factor hypoxia inducible factor-1 (HIF1) is the main mechanism initiating angiogenesis (14). HIF1 consists of two subunits: β, constitutively expressed, and α, rapidly degraded under normoxic conditions. HIF1 leads to the transcription of VEGF in tumor cells and of its receptors in tumor and endothelial cells, allowing its paracrine (angiogenic) and autocrine (pro-tumor direct) actions (15). While VEGF overexpression is correlated with a poorer prognosis for several tumor types, including colorectal (16), breast (17) and lung cancer (18), its prognostic value in clinically localized prostate cancer, particularly in terms of relapse after radical prostatectomy, is not consistent in the few published studies (19-25).

VEGFR1 and VEGFR2 are expressed by prostate tumor cells and endothelial cells within the stroma (10). Two studies from the same team suggested that VEGFR1 expression in tumor cells was not associated with relapse after radical prostatectomy (23, 26). The expression of NRP1 was found to be higher in metastatic than in localized prostate cancer (13, 27). However, the association between the expression of NRP1 and the occurrence of biochemical failure after prostatectomy has never been evaluated to our knowledge. HIF1α is overexpressed in prostate cancer (28). One study found an association between HIF1α overexpression and relapse after prostatectomy (20), but such an association was not confirmed by two other articles (24, 25). Based on these data, the role of angiogenesis in the occurrence of distant relapse after radical prostatectomy needs to be clarified. Therefore, by immunohistochemistry, we compared expression of HIF1α, VEGF and its receptors in tumor and endothelial cells in 45 patients with distant relapse and 68 patients relapse-free after radical prostatectomy.

Materials and Methods

Study population. This retrospective case control study included 113 patients with clinically localized prostate cancer (pT1-3, N0 or Nx, M0 or Mx – pTNM classification 2009) (29) treated by radical prostatectomy at the Brest University Hospital between 2002 and 2009. Patients were divided into two groups according to presence or absence of disease relapse, defined as serum PSA concentration greater than 0.2 ng/ml and rising on two consecutive measurements (30). The first group (relapse-free patients) included 68 men with an undetectable serum PSA lasting six years or more after prostatectomy. The second group (patients with distant relapse) included 45 men with a serum PSA concentration greater than 0.2 ng/ml rising on two consecutive measurements and with at least one of the three following items: (i) histologically proven distant relapse [regional lymph node metastasis (pN1) or distant metastasis (pM1)]; (ii) one or more predictors of distant relapse (rising PSA one year or more after prostatectomy, PSA doubling time of six months or less, seminal vesicle invasion (pT3b), Gleason score ≥8 (31-34); (iii) failure of salvage radiotherapy.

No patient started adjuvant or neo-adjuvant treatment (before PSA failure). Data on clinicopathology, follow-up, and survival were available for all patients. After surgery, patients were followed up with physical examination and measurements of serum PSA concentration every three months during the first year, every six months until the fifth year and then annually. Date of relapse was set as the date of first PSA test greater than 0.2 ng/ml. Written informed consent was obtained from all patients prior to study.

Tissue microarray. The tissue microarray (TMA) blocks were prepared using Tissue-arrayer® (Beecher Instruments, Alphelys, Plaisir, France). Areas representative of the tumor with the highest Gleason score were marked. For each case, six cores (0.6 mm diameter) of tumor were transferred from the selected areas to the recipient block. Sections of 3 μm were cut on a microtome and transferred to glass slides. Tissue samples used as positive controls for immunohistochemical techniques were included in the same way.

Immunohistochemistry. Immunohistochemistry was performed on tissue sections from the TMA blocks, using the following antibodies: VEGF (polyclonal rabbit, clone A-20 sc-152; dilution 1:25; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HIF1α (monoclonal mouse, clone 1α67 sc-53546; dilution 1:50; Santa Cruz Biotechnology), VEGFR1 (polyclonal rabbit, RP077; dilution 1:50; Clinisciences, Nanterre, France), VEGFR2 (polyclonal rabbit, RP076; dilution 1:50; Clinisciences) and NRP1 (polyclonal rabbit, clone H-286 sc-5541; dilution 1:50; Santa Cruz Biotechnology). Incubation with the primary antibody was performed either for 24 h at 4˚C, or for 1 h at room temperature. Negative controls were obtained after omission of the primary antibody. Samples from other tissues known to express each marker were used as positive controls, including glioblastoma for HIF1α and cutaneous angiosarcoma for VEGF, VEGFR1, VEGFR2 and NRP1.

Semiquantitative scoring of antibody staining was established by two pathologists in a blinded fashion (Figures 1-3). Cytoplasmic staining for VEGF was evaluated in tumor cells as follows: 0=no staining, 1=weak, 2=moderate, 3=strong (21, 23, 24). Cytoplasmic and membranous staining for VEGFR1, VEGFR2 and NRP1 were evaluated in tumor and endothelial cells as 0=no staining, 1=weak, 2=moderate, 3=strong (13, 23, 26). Nuclear staining for HIF1α was evaluated in tumor cells, as proposed and adapted from Zhong *et al*. (28): 0=no staining, 1=less than 10% of cells, 2=10-50% of cells, 3=>50% of cells. In patients who had scoring heterogeneity between

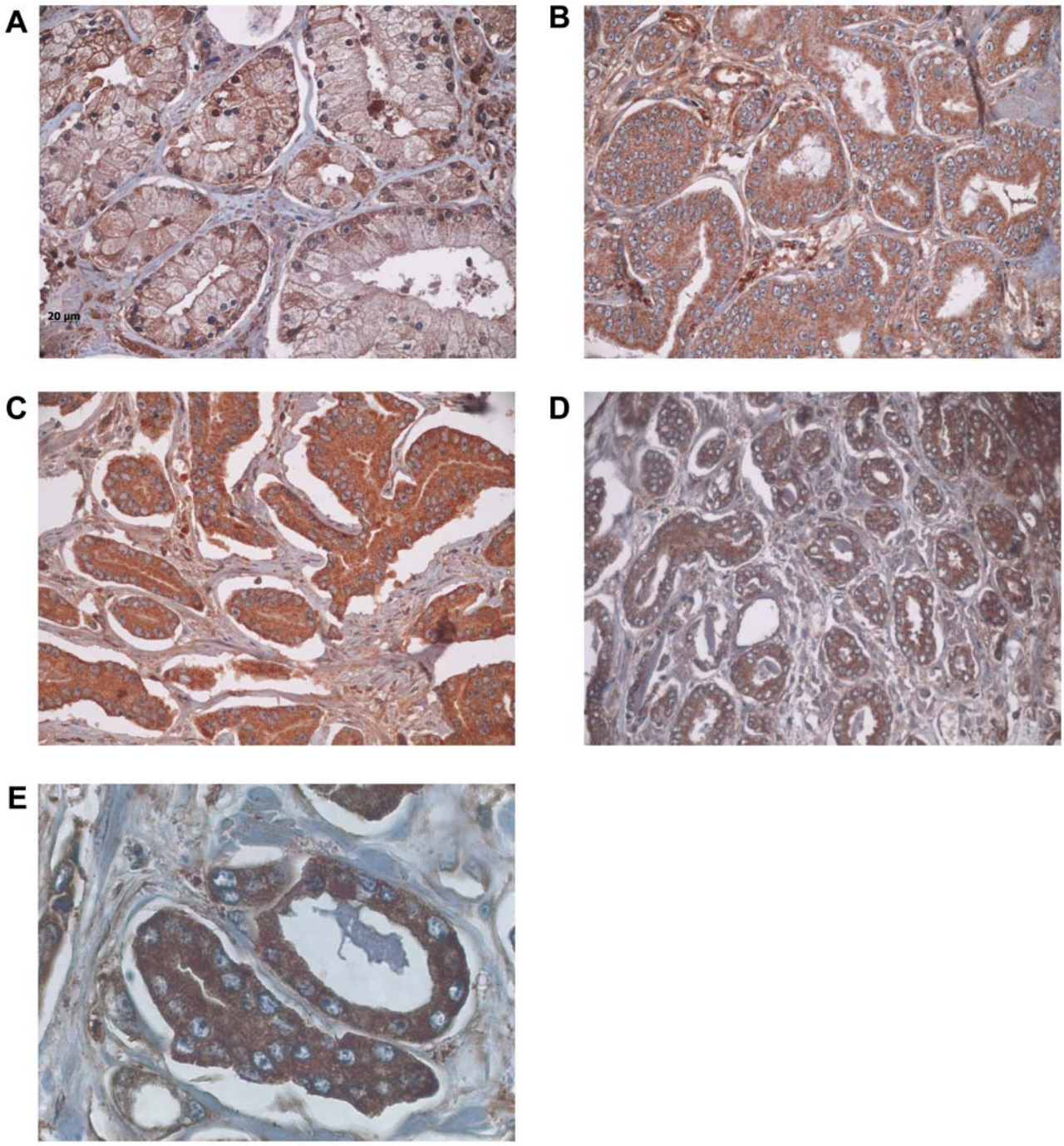

Figure 1. *Scoring of (VEGF) and (HIF1α) immunohistochemical staining in prostate tumor cells. Cytoplasmic VEGF staining of weak intensity (A), moderate intensity (B) and strong intensity (C) in prostate tumor cells. D and E: nuclear HIF1α staining of more than 10% of prostate tumor cells (A to D, ×400; E, ×1000).*

different cores, the retained score was the most representative score. Interobserver variability occurred for fewer than 5% of the patients, in which cases slides were re-scored by both pathologists until a consensus was reached.

Statistical analysis. We tested the association of different markers to each other and with clinicopathological characteristics (serum PSA concentration, Gleason score, pT stage, percentage of positive biopsies) and occurrence of relapse. Comparison of quantitative

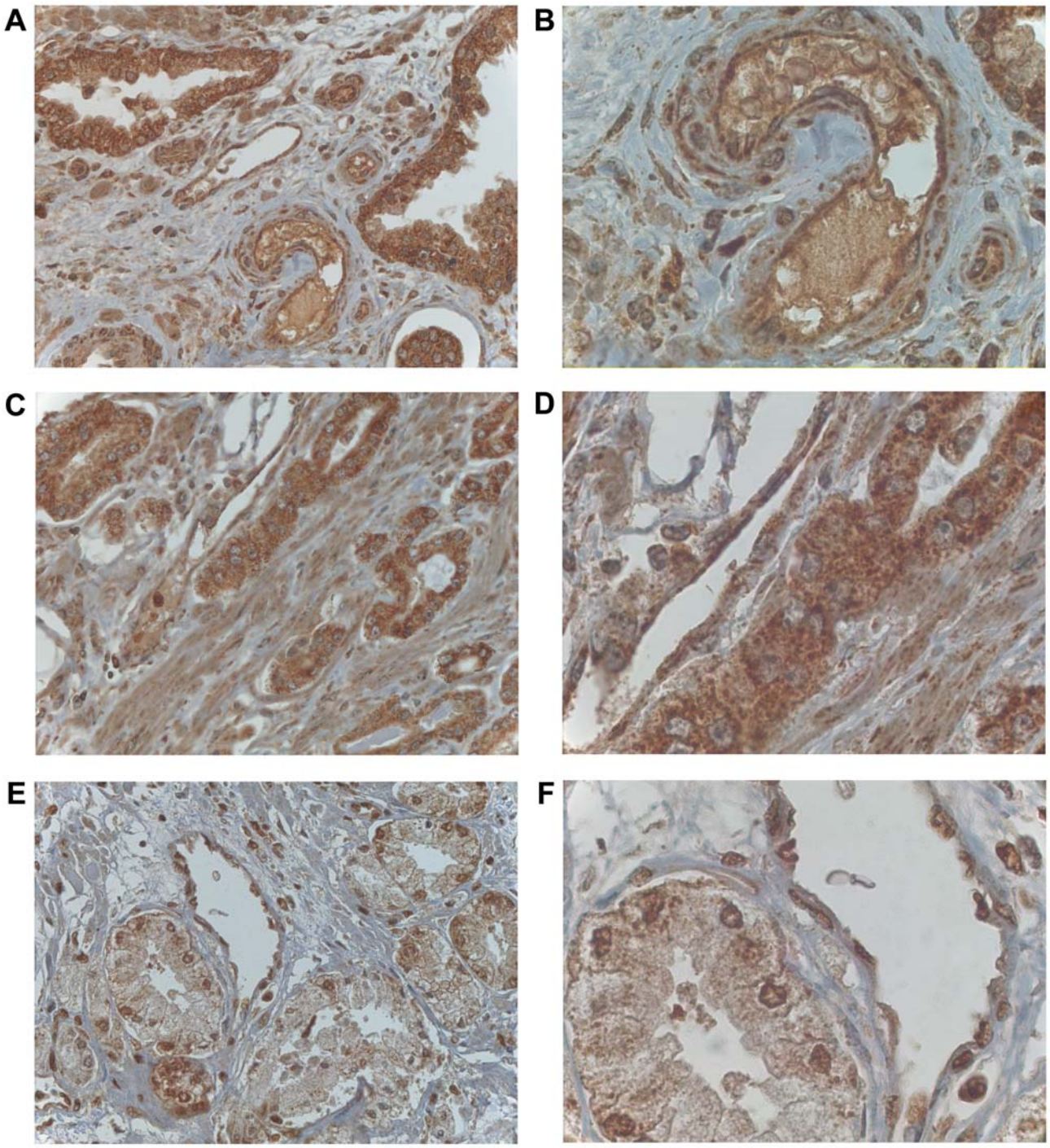

Figure 2. *Scoring of (VEGFR1) and (VEGFR2) immunohistochemical staining. Cytoplasmic and membranous VEGFR1 staining of strong intensity (A, B) and moderate intensity (C, D) and of VEGFR2 of weak intensity (E, F) in prostate tumor and endothelial cells (A, C, E ×400; B, D, F ×1000).*

variables between patients with and without relapse was performed using the Wilcoxon test because of the small numbers involved. Comparison of unordered qualitative variables was performed using the chi-square test or Fisher's exact test depending on the conditions of validity (small numbers). Finally, in order to compare ordered qualitative categorical variables (markers, Gleason score, pT stage, percentage of positive biopsies) to each other or with quantitative variables, a Spearman rank correlation test was performed. These qualitative variables ordered in three or four classes were coded 1, 2, 3 and 4.

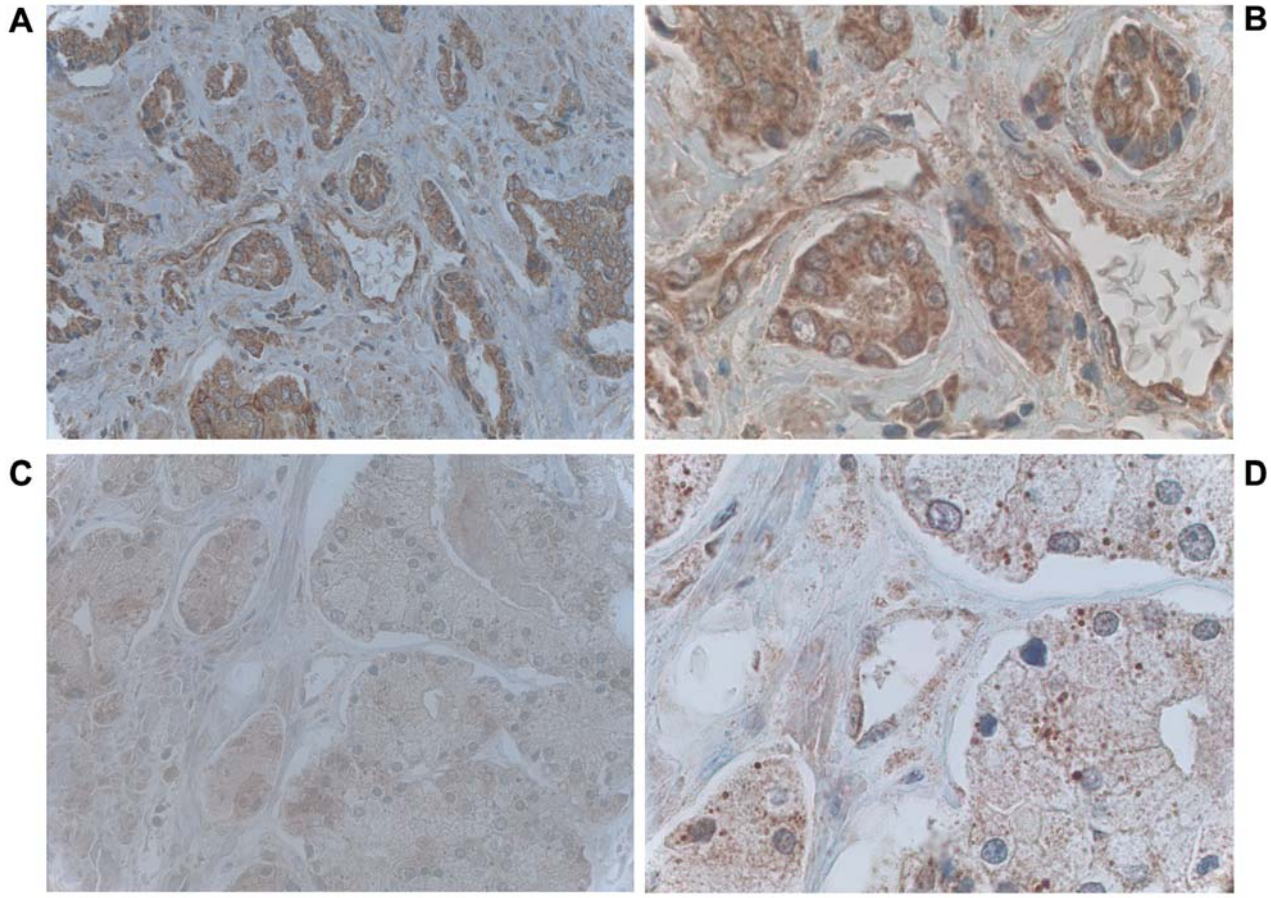

Figure 3. *Scoring of (NRP1) immunohistochemical staining. Cytoplasmic and membranous NRP1 staining of moderate intensity (A, B) and of weak intensity (C, D) in prostate tumor and endothelial cells (A, C ×400; B, D ×1000).*

A multivariate analysis using logistic regression was performed to assess the relationship between the expression of markers and relapse. Given the small numbers, we made groups and adjusted for different variables: serum concentration PSA: ≤10 ng/ml *versus* >10 ng/ml; status of surgical margins: positive or negative; percentage of positive biopsies: ≤55% *versus* >55%. The association is presented here as the risk of relapse using odds ratios (OR) and 95% confidence intervals (CI).

Statistical analysis was performed using Statistical Analysis System, version 9.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Clinicopathological characteristics of the patients. The group of 45 patients with distant relapse included two patients with distant metastasis (pM1), 23 patients with regional lymph node metastasis (pN1) (including one with distant metastasis), 16 patients with at least two predictors of distant relapse without associated metastasis (pNx/N0, pMx/M0), four patients with a single predictor of distant relapse without associated metastasis and one patient with failure of salvage radiotherapy without a predictor of distant relapse or associated metastasis. Their clinicopathological characteristics are detailed in Table I.

Immunohistochemistry. Staining for VEGF was significantly correlated with that of VEGFR1 (r^2=0.27, p=0.005), VEGFR-2 (r^2=0.42, p<0.0001) and NRP1 (r^2=0.25, p=0.007) in tumor cells. Staining for VEGFR1 and VEGFR2, VEGFR1 and NRP1, VEGFR2 and NRP1 in tumor cells was significantly interrelated (r2=0.39, p<0.0001; r^2=0.29, p=0.002; r^2=0.45, p<0.0001, respectively). Staining for VEGFR1 in tumor cells and endothelial cells was significantly associated with that for HIF1α (r^2=0.24, p=0.01; r^2=0.30, p=0.002, respectively). In contrast, no significant association was found between VEGF and VEGFR1, VEGFR2 and NRP1 in endothelial cells. Staining for VEGF and HIF1α in tumor cells, and for VEGFR1 and NRP1 in vessels was not significantly associated. Staining for VEGFR1 in endothelial cells was significantly correlated with Gleason score (r^2=0.24, p=0.01),

Table I. *Clinicopathological characteristics of the patients.*

Characteristic	Relapse patients (n=45) (%)	Relapse-free patients (n=68) (%)	*p*-Value
Median age, years (range)	65 (51-76)	64 (54-76)	0.31
Median preoperative PSA ng/ml (range)	9 (2.5-66)	7 (2.7-30)	0.003
Gleason score			<0.0001[a]
≤6	2 (4.5)	32 (47)	
7 (3+4)	9 (20)	21 (31)	
7 (4+3)	19 (42)	12 (18)	
≥8	15 (33.5)	3 (4)	
pT stage			<0.0001[a]
T2a	1 (2)	5 (7.5)	
T2b	0	0	
T2c	9 (20)	51 (75)	
T3a	13 (29)	11 (16)	
T3b	22 (49)	1 (1.5)	
Positive biopsies			0.0011[a]
<33	14 (31)	43 (63)	
33-55	14 (31)	12 (18)	
>55	17 (38)	13 (19)	
Positive surgical margin	26 (58)	21 (31)	0.01
Lymph node metastasis	23 (51)	0	<0.0001
Distant metastasis	2 (4.5)	0	-
Rising PSA ≤1 year after prostatectomy	39 (87)	-	-
PSA doubling time ≤6 months	22 (49)	-	-
Median follow-up, months (range)	-	84.5 (77-95)	-
Median relapse, months (range)	6 (2-48)	-	-

[a]*p*-Value for trend.

Table II. *Univariate analysis. Staining for vascular endothelial growth factor (VEGF), fms-related tyrosine kinase 1 (VEGFR1), kinase insert domain receptor (VEGFR2), neuropilin 1 (NRP1) and hypoxia inducible factor 1, alpha subunit (HIF1α) depending on the presence or absence of distant relapse after radical prostatectomy.*

Studied marker (score)	Relapse patients (n=45) (%)	Relapse-free patients (n=68) (%)	*p*-Value for trend
VEGF			0.08 (–)
1	7 (16)	5 (7)	
2	33 (73)	49 (72)	
3	5 (11)	14 (21)	
VEGFR1 tumor			
1	2 (4)	3 (4)	0.08 (+)
2	17 (38)	38 (56)	
3	26 (58)	27 (40)	
VEGFR1 vessels			<0.0001 (+)
1	0	10 (15)	
2	16 (36)	44 (65)	
3	29 (64)	14 (20)	
VEGFR2 tumor			0.38 (–)
1	8 (18)	11 (16)	
2	35 (78)	49 (72)	
3	2 (4)	8 (12)	
VEGFR2 vessels			0.36 (+)
1	21 (47)	37 (54.5)	
2	22 (49)	30 (44)	
3	2 (4)	1 (1.5)	
NRP1 tumor			0.08 (–)
1	6 (13.5)	6 (9)	
2	29 (64.5)	36 (53)	
3	10 (22)	26 (38)	
NRP1 vessels			0.0002 (–)
0	0	1 (1.5)	
1	27 (63)	21 (31)	
2	16 (37)	31 (45.5)	
3	0	15 (22)	
HIF1α			0.79 (+)
0	0	3 (5)	
1	25 (60)	35 (54)	
2	17 (40)	26 (41)	
3	0	0	

(–) Negative correlation; (+) Positive correlation. Due to loss of cores, NRP1 was not assessed in two patients and HIF1α was not assessed in seven patients.

pT (r^2=0.29, p=0.002) and percentage of positive biopsies (r^2=0.28, p=0.003). There was also a significant correlation between staining for VEGFR1 in tumor cells and the percentage of positive biopsies (r^2=0.21, p=0.03). However, no significant correlation was found between staining for other markers studied (VEGF, VEGFR2, HIF1α and NRP1) and clinicopathological variables.

The distribution of VEGF, VEGFR1, VEGFR2, NRP1 and HIF1α expression according to the presence or absence of distant relapse after radical prostatectomy is shown in Table II. In univariate analysis, the occurrence of systemic relapse was significantly associated with more intense endothelial staining for VEGFR1 (p for trend <0.0001) and lower staining for NRP1 (p for trend=0.0002). This association was not found for the other markers studied.

The multivariate analysis showed that strong staining for VEGFR1 in endothelial cells was a predictor of distant relapse after radical prostatectomy, independently of serum PSA concentration, surgical margin status and percentage of positive biopsies (OR=7.17, 95% CI=2.79-18.43, p<0.0001). Moderate to strong staining for NRP1 in endothelial cells appeared to be predictor of absence of systemic relapse after radical prostatectomy (OR=0.32, 95% CI=0.14-0.73, p=0.01) (Table III).

Discussion

Despite hormonotherapy and chemotherapy, the prognosis of distant relapse, unlike that of local relapse, remains unfavorable. This justifies our choice to separate patients with distant relapse rather than considering patients with

Table III. *Multivariate analysis. Predictors of distant relapse after radical prostatectomy. The studied markers were each divided into two categories: (VEGFR1): strong intensity versus moderate and weak; (NRP1): strong or moderate intensity versus weak or none. The adjustment variables were grouped into two categories: rate of preoperative PSA ≤10 ng/ml versus >10 ng/ml; status of surgical margins: positive or negative; percentage of positive biopsies ≤55% versus >55%.*

Studied marker	Odds ratio	95% confidence interval	*p*-Value
VEGFR1, vessels	7.17	2.79-18.43	<0.0001
Preoperative PSA	1.47	0.58-3.72	0.42
Surgical margin	3.20	1.25-8.23	0.02
Positive biopsy (%)	1.02	0.35-2.96	0.97
NRP1, vessels	0.32	0.14-0.73	0.01
Preoperative PSA	1.94	0.80-4.67	0.14
Surgical margin	2.31	0.96-5.53	0.06
Positive biopsy (%)	0.61	0.23-1.61	0.32

relapse as a single entity. With the exception of two studies on small numbers of patients (21, 26), none of the publications regarding the prognostic value of markers of angiogenesis in clinically localized prostate cancer separated patients with distant relapse after radical prostatectomy.

The selection criteria for patients with distant relapse reported here could be a limitation of our study. Many patients were classified as distant relapsers only based on the presence of predictors (four patients with a single predictor and 16 with at least two). Indeed, distant relapse is not synonymous with the presence of a predictor. For more than half of the patients selected, there was histological evidence of distant tumor extension (N+ and/or M+) and over one-third had at least two predictors.

The inclusion of patients with lymph node metastasis has a theoretical limit: tumor dissemination *via* the lymphatic system is facilitated by lymphangiogenesis and not angiogenesis. However, there are many interconnections between angiogenesis and lymphangiogenesis. Recent studies emphasized the involvement of VEGF and VEGFR1 in lymphangiogenesis (35-37). Similarly, anti-PGF inhibits tumor lymphangiogenesis whereas PGF binds only VEGFR1 (38).

The choice of strict selection criteria limited the number of patients with distant relapse included in this study. This number, although higher than the number of patients described in most of the published series, forced us to pool values for multivariate analysis. Finally, using the Gleason score and pathological stage for the selection of patients with distant relapse did not allow us to adjust the markers studied to these variables in multivariate analysis.

However, the strict selection criteria allowed us to work on homogeneous populations. Indeed, predictors of distant relapse were each associated with a positive predictive value exceeding 80%: elevated PSA ≤1 year after prostatectomy, seminal vesicle invasion (pT3b) and Gleason score ≥8 (34). Relapse-free patients were followed for at least six years after radical prostatectomy. It is known that the majority of relapses occur within three years after surgery and that 99% of patients with an undetectable serum PSA level after six years are considered cured (39).

The simultaneous study of five proteins involved in a common signaling pathway, the VEGF pathway, allowed us not only to evaluate the association of their expression with distant relapse, but also to establish (or not) correlations between their respective staining and to better understand the roles played by each in prostate tumor growth and metastasis.

To our knowledge, this work is the first to assess the prognostic value of the expression of VEGFRs (VEGFR1, VEGFR2 and NRP1) in endothelial cells in prostate cancer. Until now, immunohistochemical studies have only considered expression of VEGFR1 in prostate tumor cells (autocrine action), without considering endothelial cells (paracrine pro-angiogenic action) (23, 26).

Strong staining for VEGFR1 in endothelial cells is a predictor of distant relapse after radical prostatectomy, independent of initial PSA concentration, surgical margin status and percentage of positive biopsies. Our results compare with those of Ferrer *et al*. who noted that, unlike other types of cancer, vessels of prostate cancer exhibited less intense labeling for VEGFR2 than for VEGFR1, suggesting that VEGFR1 could play an important role (10). In a comparable way, Dales *et al*. showed in breast cancer, that expression of VEGFR1, but not of VEGFR2, in endothelial cells was an independent predictor of local and distant relapse (40). The identification of endothelial expression of VEGFR1 as an independent predictor of distant relapse after radical prostatectomy, without correlation with VEGF expression, echoes several publications that suggested a possible link between VEGFR1, PGF and metastasis.

PGF, which binds only to VEGFR1, activates the receptor differently from VEGF, allowing the activation of intracellular signaling pathway of mitogen-activated protein (MAP) kinase (9, 41, 42). The potentially important role of PGF in angiogenesis and tumor growth is underscored by the fact that PGF inhibitors block the growth of tumors resistant to anti-VEGF and anti-VEGFR, reflecting the probable synergistic action between PGF and VEGF (38, 43, 44). Similarly, most of the pro-tumor actions mediated by VEGFR1 depend, at least in part, on PGF: vascular permeability and endothelial cell survival (43, 45), recruitment of endothelial progenitor cells (46), and recruitment of hematopoietic stem cells (47). PGF is expressed in prostate cancer (48) and is associated with a more aggressive disease (49). Could PGF be the main ligand of VEGFR1 in endothelial cells and thus contribute to the formation of metastasis in synergy with its actions on different cells expressing VEGFR1?

Several experiments on mouse models emphasize the pro-metastatic role of VEGFR1. The activation of growth of metastases is mediated by VEGFR1 (43). Expression of metalloproteinase 9 (MMP9), induced by VEGFR1 in pulmonary endothelial cells, allows their migration and the release of pro-angiogenic factors sequestered in the extracellular matrix suitable for the development of lung metastases. The lung parenchyma of patients with various types of cancer (esophageal, colonic, ovarian, *etc.*) is also richer in MMP9 than is that of healthy individuals (50). Under the influence of PGF released by tumor cells, hematopoietic stem cells, which express VEGFR1, are able to migrate to form pre-metastatic niches from which they recruit tumor cells and endothelial progenitor cells. As proof of this, anti-VEGFR1 prevents the formation of these niches, while the anti-VEGFR2 only slows the growth of metastases (51).

In our work, there was no significant association between the expression of NRP1 in tumor cells and the occurrence of distant relapse. According to the two studies that have analyzed NRP1 expression in prostate cancer, NRP1 is most intensely expressed in hormone-refractory metastatic prostate cancer rather than in clinically localized cancer (13, 27).

Yacoub *et al*. also studied VEGF and semaphorin 3A (SEMA3A) expressions (27). SEMA3A, which competes with VEGF for binding to NRP1 has an anti-tumoral effect (52). Unlike VEGF, SEMA3A is less expressed in metastatic hormone-refractory metastatic cancer than in clinically localized cancer where it is also associated with a lower pT stage and correlated with the expression of NRP1. According to Yacoub *et al*., the role of NRP1 in prostate cancer could therefore depend on the type of ligand. NRP1 expression might be associated with a good prognosis when the main ligand is SEMA3A (anti-tumoral, as in clinically localized prostate cancer) but with an aggressive profile when the main ligand is VEGF (pro-tumoral, as in metastatic hormone-refractory cancer). In our study, moderate to strong expression of NRP1 in endothelial cells was an independent predictor of the absence of distant relapse after radical prostatectomy. These results bring us closer to the hypothesis formulated by Yacoub *et al*. (27). Indeed, although the endothelial expressions of NRP1 and VEGFR2 were correlated, NRP1 was more intensely expressed in relapse-free patients. NRP1 might therefore act independently of VEGFR2, with SEMA3A as possible main ligand, giving a favorable prognosis for patients with these tumors.

To date no study has conclusively demonstrated a significant association between VEGF expression and the occurrence of relapse after radical prostatectomy. Only three studies indicated such an association, one of them even considering it as an independent predictor of relapse (19-21). Unfortunately, these studies dealt with a small number of patients with relapse, without any distinction between local and distant relapses for two of them, and a short duration of follow-up, which does not exclude the presence of micro-metastases in patients considered as being relapse-free. The results of our work, focusing on distant relapse, suggest that VEGF, despite its participation in the growth of prostate cancer, probably does not play a discriminant prognostic role in terms of distant relapse. However, in accordance with the current understanding of the VEGF pathway, we found a significant correlation between the expression of VEGF, VEGFR1, VEGFR2 and NRP1 in tumor cells. Moreover, in agreement with literature data, the occurrence of distant relapse was not associated with VEGFR1 expression in prostate tumor cells (23, 26).

In this work, we confirmed the nuclear accumulation of HIF1α in prostate tumor cells, but did not demonstrate a significant association between HIF1α expression and occurrence of relapse. Vergis *et al*. found such an association, but considered only cytoplasmic staining (20). However, being a transcription factor, only the nuclear labeling for HIF1 should be considered (24, 25, 53). The discordant results urge standardization not only of the immunohistochemical techniques but also methods for their interpretation, bearing in mind the need to relate the location of the marker being studied to its biological activity. We found no correlation between VEGF and HIF1α expressions. This association was however noted by Boddy *et al*., but at the limit of significance (p=0.05) (24). Interestingly, the same authors showed a much stronger correlation between the expression of HIF2α, an isoform of HIF1α, and VEGF expression (p<0.001), suggesting that HIF2α might be the predominant isoform in prostate cancer. Taken together, these results could reinforce the idea, still being discussed, that HIF1α would preferentially regulate the transcription of genes involved in glycolysis, while HIF2α would preferentially activate genes such as VEGF or VEGFR2 (54, 55).

Conclusion

VEGF, which has long aroused the most attention, plays an undeniable but probably not discriminatory role in the dissemination of prostate cancer. However, two of its receptors, VEGFR1 and NRP1, from their endothelial expression, appear to be independent predictors of distant relapse after radical prostatectomy. Further studies on larger cohorts, including patients with local recurrence, are needed to confirm the relevance of these proteins as novel prognostic factors. Similarly, the simultaneous study of PGF and SEMA3A expressions might help to better understand the interactions and the respective roles of different actors of angiogenesis in prostate cancer growth.

Our study also illustrates the strength of an immunohistochemical approach in the search for prognostic markers. Indeed, this technique is probably the easiest way to highlight the expression of proteins in situ and establish links

with pathophysiology and cell biology. The evaluation by immunohistochemistry of endothelial expression of NRP1 and VEGFR1 could be an additional tool in the assessment of tumor aggressiveness of clinically localized prostate cancer to better identify patients at high risk of distant relapse after radical prostatectomy, as candidates for additional treatments. However, it should be kept in mind that if NRP1 and VEGFR1 are predictors of distant relapse after prostatectomy, they are not necessarily predictors of therapeutic response.

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