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Expression of bFGF, VEGF and c-met and their Correlation with Microvessel Density and Progression in Prostate Carcinoma

DAGMAR STROHMEYER[1], FRANZ STRAUß[2], CHRISTIAN RÖSSING[2], CHRIS ROBERTS[3], OLAF KAUFMANN[4], GEORG BARTSCH[1] and PETER EFFERT[5]

[1]Department of Urology, University of Innsbruck, Austria;
Departments of [2]Urology and
[4]Pathology, Charité, Humboldt University, Berlin, Germany;
[3]Division of Epidemiology and Biostatistics, Milan, Italy;
[5]Department of Urology, Franziskus Hospital and RWTH University of Aachen, Germany

Abstract. *Background: Previously, we found angiogenesis measured as microvessel density (MVD) to be associated with both pathological stage and clinical outcome after radical prostatectomy (RP). In addition, we have shown that Vascular Endothelial Growth Factor (VEGF) is one of the important inducers of angiogenesis in prostate cancer (PC). The aim of this study was to investigate the expression of additional angiogenic factors, namely basic Fibroblast Growth Factor (bFGF) and the c-met receptor of Hepatocyte Growth Factor/Scatter Factor (HGF/SF) in PC. Materials and Methods: Ninety-eight paraffin-embedded RP specimens and 20 adjacent normal prostatic tissues were evaluated for factor VIII staining and microvessel counting. Expression of VEGF (n=55), bFGF (n=65) and c-met (n=66) was studied by immunohistochemistry. Results were correlated with pathological grade and stage, MVD and clinical outcome. Results: While adjacent benign tissue in RP specimens generally showed low MVD, VEGF-, bFGF- and c-met-expression, this was different in PC. All angiogenesis inducers were associated with stage while c-met as well as VEGF expression were associated with grade. Tumor progression was associated with grade and MVD. There was a clear correlation between VEGF and c-met expression and MVD. Conclusion: VEGF and c-met expression increase with tumor stage and grade, while bFGF expression increases only with tumor stage. In addition to VEGF, c-met seems to be important and clinically relevant to the induction of angiogenesis in PC. Both VEGF and c-met appear to influence tumor progression, mainly through their effect on MVD.*

Correspondence to: Dagmar Strohmeyer, University of Innsbruck, Department of Urology, Anichstr. 35, A-6020 Innsbruck, Austria. Tel: +43-512-504-4810, Fax: +43-512-504-4073, e-mail: strohmeyer-kiefersfelden@t-online.de

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Identification of prognostic factors in prostate cancer (PC) is of particular scientific and clinical interest. A focus of attention has been the role of neoangiogenesis and its underlying biological mechanisms. In different human neoplasms quantification of angiogenesis has been demonstrated to provide prognostic information (1-4). A number of studies have demonstrated the relevance of angiogenesis for tumor growth, progression and metastasis in PC (5-7). Previously, we have shown that determination of microvessel density (MVD) in primary prostate carcinoma is an independent predictor of tumor progression (8).

Different growth factors and growth factor receptors have been implicated in the pathogenesis of PC, both by stimulation of angiogenesis and direct action as potent mitogens (9). Vascular endothelial growth factor (VEGF) has been identified as one of the important inducers of angiogenesis in PC (10-13). In addition to its angiogenic activities, VEGF has been shown to stimulate expression of hepatocyte growth factor/scatter factor (HGF/SF) (14). Apart from VEGF, bFGF and the c-met receptor of HGF/SF have been associated with the induction of angiogenesis and tumor progression in PC (9, 15, 16). bFGF is mitogenic to prostate epithelial and stromal cells (17) and bFGF serum levels were found to be 1.6-fold higher in advanced compared to localized PC (18). Expression of c-met, the receptor for HGF/scatter factor, has been associated with tumor progression in breast and prostate carcinoma (19, 20). According to recent evidence, HGF/SF is synthesized by stromal cells and activates the MET receptor in malignant prostate epithelial cells (20). The aim of the study presented here was to investigate the expression of VEGF, bFGF and c-met in PC. Results were correlated with pathological stage, MVD and progression after radical prostatectomy.

Materials and Methods

Tissues and patient characteristics. A series of 98 paraffin-embedded radical prostatectomy specimens, recently evaluated for factor VIII staining and microvessel density, was available (8). All tumors were lymph node-negative. None of these patients had received any form of neoadjuvant or adjuvant treatment.

The specimens were obtained at the University of Berlin (Charité), University of Aachen and the Klinikum of Schwerin, Germany. From each specimen, 2 paraffin blocks containing predominantly tumor tissue were selected on the basis of hematoxylin and eosin-stained sections.

Histopathological assessment of tumor differentiation and stage was performed according to the WHO International Histological Classification of tumors and the TNM classification of prostatic carcinoma (21). For normal controls, benign adjacent tissues from radical prostatectomy specimens (n=20) were evaluated.

Tumor progression was defined as increasing postoperative PSA level (cut-off: 0.2 ng/ml) or clinical evidence of tumor progression. Progression analysis was performed with a mean follow-up of 42.3 months.

Determination of microvessel density. Factor VIII staining and scoring were performed as described previously (8). Staining of endothelial cells in 4-mm sections from tumor blocks with a polyclonal primary factor VIII antibody (AB) (Dako Polyclonal, Dako, Inc., Santa Barbara, CA, USA) highlighted the microvessels. Microvessel density was determined according to the procedure described by Weidner *et al.* in a 0.74-mm area at 200-fold magnification (22). Scoring was performed by two investigators blinded for the clinical and histopathological status of the patients. Tumor classification was confirmed by the same pathologist (O.K.).

bFGF staining and scoring. Four-μm sections from tumor blocks from 65 radical prostatectomy specimens were deparaffinized with xylene, rehydrated with ethanol and subjected to a 20-minute pre-treatment with pronase. In order to block endogenous peroxidase activity, 3% H_2O_2 in distilled water was applied for 5 minutes. Unspecific binding sites were blocked with 10% normal rabbit serum and sections were afterwards incubated with the primary bFGF AB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:500 for an incubation period of 2 hours. Immunoreaction was visualized by application of biotinylated secondary rabbit anti-goat AB (Dianova, Hamburg, Germany) followed by peroxidase-conjugated streptavidin. Amino-ethyl-carbazol (AEC) was used as chromogen. For negative controls the primary antibody was replaced by non-immune goat serum (Sigma, Deideshofen, Germany).

bFGF expression was evaluated twice in the area corresponding to the "hot spot" on the slide employed for evaluation of microvessel density. Scoring was done in a 0.74 mm field at 100-fold magnification. bFGF expression in up to 50% of tumor cells was classified as "low" (+), in 51-75% as "medium" (++) and bFGF expression in more than 75% of tumor cells was defined as "high" (+++) expression.

VEGF staining and scoring. VEGF staining was also performed on 4-mm sections of a total of 55 radical prostatectomy specimens prepared as described above. However, pre-treatment was done with trypsin 0.1% in Tris-HCl buffer (pH 7.6). A polyclonal goat AB to VEGF (Santa Cruz Biotechnology) was applied overnight at 4˚C at 1:100 dilution. Immunoreactions were visualized employing the avidin-biotin complex method described above. Vascularized placental tissue, previously shown to react with the primary antibody, served as positive control. The scoring system again differentiated between "low" (+), "medium" (++) and "high" (+++) expression.

c-met staining and scoring. For immunhistochemical detection of c-met expression, 4-μm sections from 66 radical prostatectomy specimens were pretreated with citrate. The primary polyclonal c-met AB (Santa Cruz Biotechnology) was applied overnight at a 1:500 dilution at 4˚C. Localization of primary immunoreactions was again visualized with biotinylated secondary antibodies and streptavidin-biotin-peroxidase complexes. As described above, expression was classified as "low" (+), "medium" (++) or "high" (+++).

Statistical analysis. Associations between expression of angiogenesis markers and pathological stage and grade of the tumors were assessed using log linear models and χ^2 test of association.

A linear regression analysis was carried out to investigate if the angiogenesis markers c-met, bFGF or VEGF are associated with MVD. The distribution of MVD was skew so a log transformation was used to obtain a normal distribution.

The influence of the angiogenesis markers on progression was assessed using a Cox proportional hazards model. Progression curves were calculated using the Kaplan Meier estimates.

Results

Histopathological stage and grade of the tumors examined (Figure 1). Of 98 lymph node- negative prostate carcinomas there were 3 pT1-tumors, 54 pT2-tumors, 40 pT3-tumors and 1 pT4-tumor. Seventy-one tumors were classified as grade 2 (G2), 27 tumors were grade 3 (G3).

Microvessel density. As described previously, immuno-histochemical staining for factor VIII yielded reproducible results and there was no background staining (Figure 2). MVD increases significantly with tumor stage and grade ($p<0.001$).

VEGF expression. Four out of 20 adjacent normal prostate tissues showed low VEGF expression while the other normal adjacent tissues were without VEGF expression. Areas of cytoplasmic staining were detectable in all prostate cancer specimens examined. As reported previously, different tumor areas varied in their degree of VEGF expression (8). However, within one tumor focus staining was homogenous (Figure 3). Areas with positive and negative staining were interspersed and focal areas of peritumoral stromal cells also stained positive for VEGF. Staining was reproducible without background staining. 29.1% of tumors had low intensity (+) VEGF staining, 38.2% had medium (++) and 32.7% displayed high intensity (+++) staining. VEGF-expression was significantly associated with both grade ($p<0.001$) and stage ($p<0.001$).

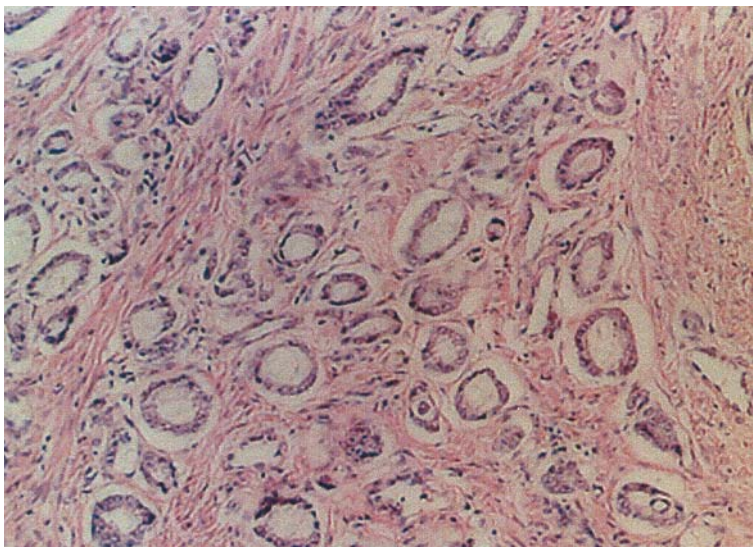

Figure 1. *H&E-stained tissue section from prostate carcinoma (x 20).*

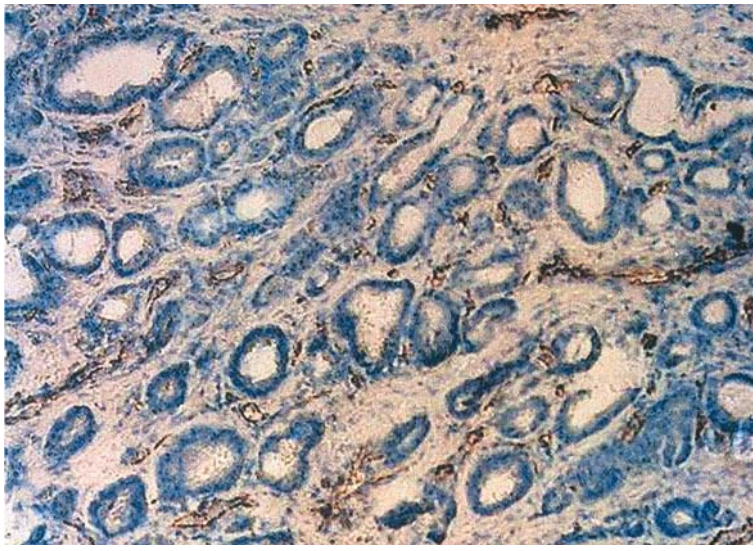

Figure 2. *Microvessel staining (Factor VIII antibody) in prostate carcinoma (x 40).*

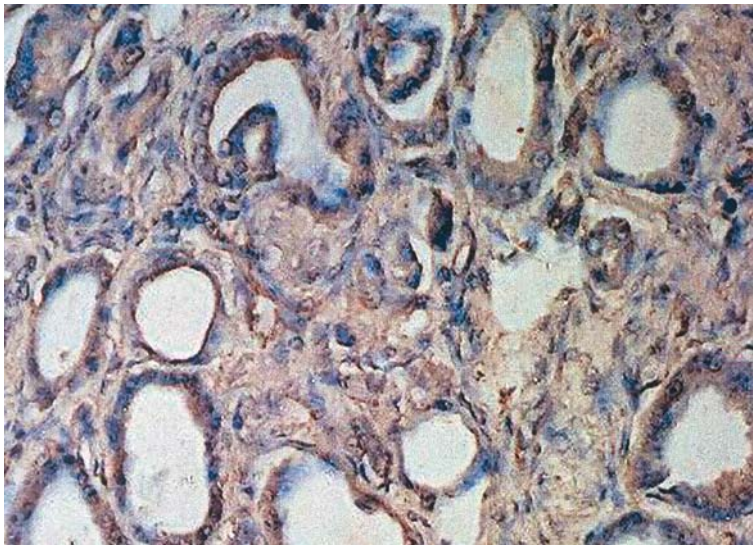

Figure 3. *Immunohistochemistry detects VEGF expression in prostate carcinoma (x 50).*

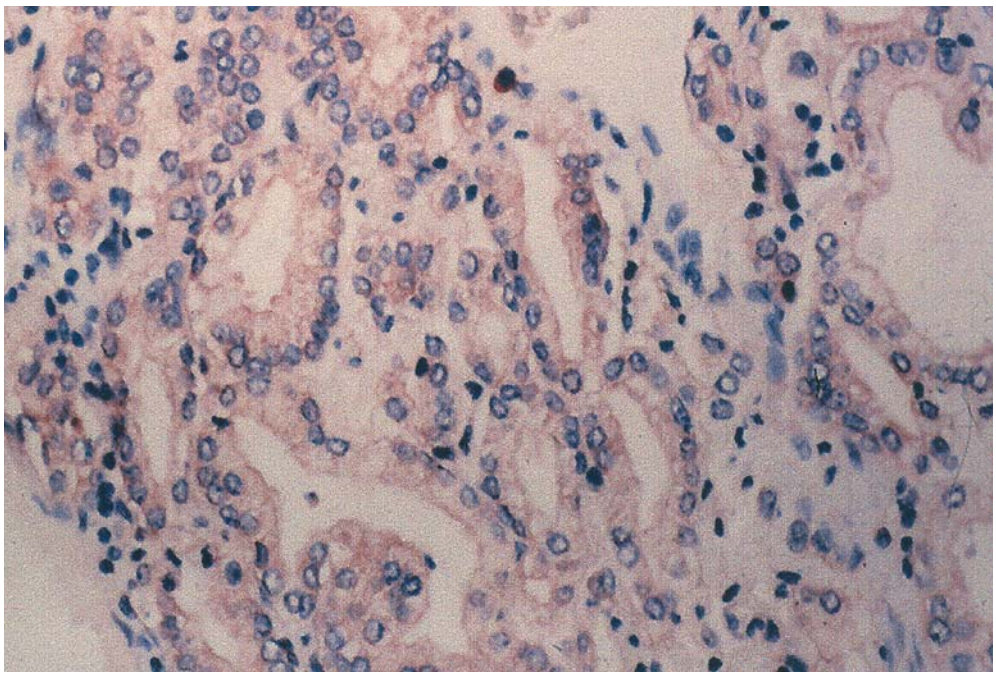

Figure 4. *bFGF expression in prostate carcinoma (x 50).*

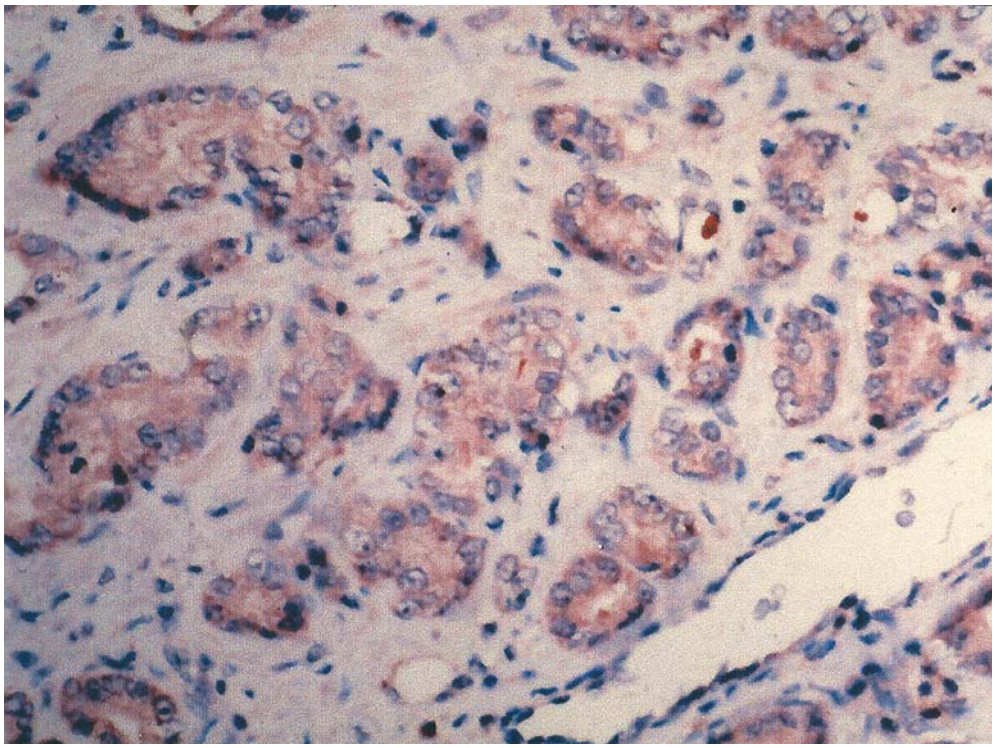

Figure 5. *Immunohistochemistry for c-met expression in prostate carcinoma (x 50).*

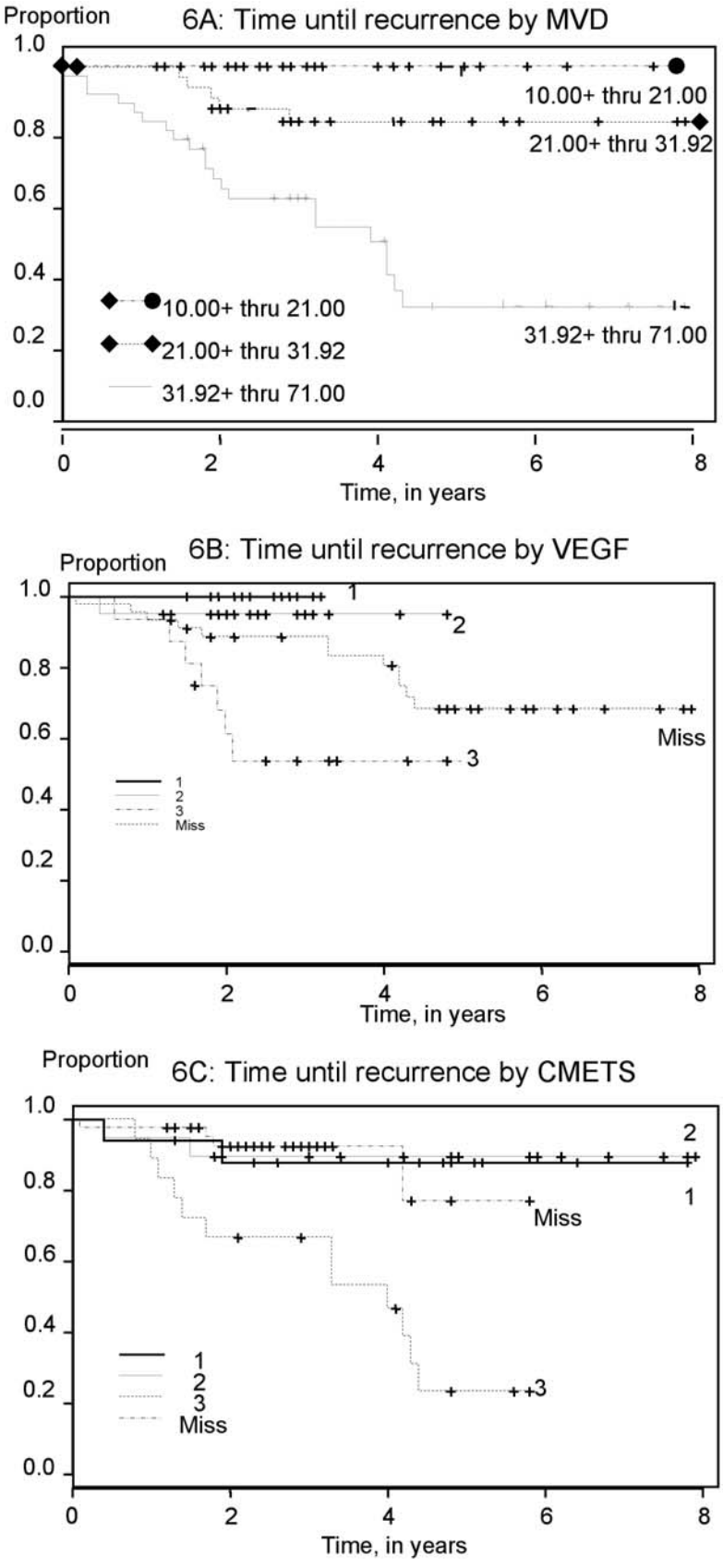

Figure 6. *Kaplan-Meier curves associating time to recurrence with the various molecular prognostic factors: A - microvessel density (MVD); B – expression of VEGF; C – c-met expression.*

bFGF expression. Staining for bFGF was mainly cytoplasmic (Figure 4). In benign adjacent tissue low staining intensity was noted. Twenty-three % of the tumors showed low intensity (+) staining, 52% medium (++) and 25 % high intensity (+++) staining. While staining intensity varied between different tumor areas, homogenous staining was present within a given tumor focus. Minor background staining was noted and staining was reproducible. Increasing bFGF expression was associated with higher tumor stage ($p=0.006$) while there was no significant association with grade.

c-met expression. Weak c-met immunostaining was consistently observed in basal epithelial cells of benign prostate glands. Distribution of c-met staining intensity was as follows: 32% low intensity (+), 35% medium intensity (++) and 33% high intensity (+++) (Figure 5). Increasing c-met expression was associated with both higher tumor grade ($p=0.02$) and stage ($p=0.02$).

Statistical analysis. All angiogenesis inducers were associated with stage while c-met as well as VEGF expression were associated with tumor grade. Grade was an important predictor of progression ($p<0.001$). In addition to grade, progression after radical prostatectomy was associated with MVD as well. There was a significant difference ($p=0.002$) in MVD seen between patients with tumor progression and those without. Adjusting for grade, high values of log(MVD) were associated with increased progression ($p<0.001$).

The linear regression model with log(MVD) as the dependent variable included terms for T-stage and grade. The effects of T-stage ($p=0.02$) and grade ($p=0.03$) were significant. Addition of the angiogenesis markers to the model including grade and stage revealed that bFGF was not associated with MVD ($p=0.34$). However, there was a significant correlation between VEGF or c-met expression and MVD. From the regression analysis on log(MVD) we conclude that c-met and VEGF both have independent effects on MVD over and above the contribution of grade and stage.

The association between the angiogenesis markers as well as MVD and progression are shown in the plots (Figure 6). When considered independently, all inducers, except bFGF, were associated with progression. Taking into account the effects of grade and log(MVD), there was no further influence of the angiogenesis markers over and above their effect on MVD. If the effect of log (MVD) was ignored and only grade was taken into account, then there was significantly increased progression if the tumor had a high c-met ($p=0.01$) but not bFGF ($p=0.06$) or VEGF expression ($p=0.14$).

Discussion

By looking at an unselected series of primary prostate carcinomas of different stages and grades, we and others have identified MVD as an independent prognosticator (8,9). The precise mechanisms leading to neoangiogenesis in PC are largely unknown. VEGF has been identified as one of the most potent mitogenic and highly tumor angiogenic factors (23). The pattern of VEGF-expression in our series, significantly associated with both grade and stage, appears to confirm this. An association of VEGF-expression and grade was reported by others as well and therefore proposed as a marker of angiogenic phenotype in PC (24). A role for VEGF in PC progression is further supported by the findings of Kwak and coworkers who reported significantly higher levels of VEGF expression in tissues from metastatic rather than localized PC (25). No significant correlation, however, was noted between VEGF expression and Gleason Score by these investigators.

Data from experimental studies have strongly implicated bFGF as an important mitogen and inducer of angiogenesis in PC (9, 17, 26). In addition, inhibition of bFGF activity has resulted in increased survival and inhibition of progression in a prostate cancer animal model (27). However, according to our results, expression of bFGF at the tissue level in primary human PC does not contribute to prognostic stratification. Although there was an association between bFGF expression and stage, bFGF expression did not correlate with either grade or MVD. Conflicting data have been obtained regarding localization of bFGF in prostate cancer tissue. While Giri and coworkers detected bFGF in stromal fibroblasts and endothelial cells, other investigators have reported bFGF expression in malignant prostatic epithelium (26, 28). Our results, as well as experimental data from prostate cancer cell lines, would confirm localization of bFGF expression to prostate cancer tumor cells (15).

Evidence from recent studies strongly suggests a role for c-met in prostate cancer progression. A paracrine loop consisting of hepatocyte growth factor/scatter factor (HGF/SF) produced by stromal cells and its receptor encoded by c-met has therefore been suggested to promote prostate cancer progression (29). The majority of primary tumors and nearly all metastases were found to express c-met (16, 30). However, c-met expression was not a prognostic marker for prostate-specific antigen recurrence in tumors with a Gleason sum of 6 or 7 (16). In contrast, our findings not only support a role for c-met in PC progression but also indicate that c-met expression is of prognostic significance after adjusting for grade. This conflicting information may be explained both by methodological differences as well as the use of different grading systems (WHO *vs.* Gleason). However, in our hands, staining for c-

met was consistent and reproducible. Nevertheless, the inherent limitations of expression studies done by immunohistochemistry include the semiquantitative scoring system as well as the dependence of staining results on both technical details as well as selection of primary antibodies.

In accordance with findings in other human neoplasms, we found c-met expression to be associated with grade and stage and a clear correlation between c-met expression and MVD as well as poor outcome was shown by linear regression analysis (31-33).

Considered independently, VEGF and c-met are associated with progression. However, no further influence of VEGF and c-met on progression was noted after adjusting for MVD. This indicates that both VEGF and c-met act on progression mainly through their angiogenic activity. Both MVD and c-met expression have been identified as independent prognosticators in malignancies other than PC. However, these studies have not established the prognostic value of c-met after adjusting for MVD (31-33).

In summary, particularly VEGF and c-met appear to be relevant to the induction of angiogenesis in PC. The association of VEGF and c-met with progression seems to be mediated primarily through their effect on MVD. No independent influence of any of the angiogenesis inducers appears to exist over and above their effect on MVD.

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