

The Prognostic Value of Angiogenesis Factor Expression for Predicting Recurrence and Metastasis of Bladder Cancer after Neoadjuvant Chemotherapy and Radical Cystectomy¹

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ABSTRACT

To determine the prognostic value of angiogenesis factor expression for patients with muscle-invasive transitional cell carcinoma (TCC) of the bladder treated with neoadjuvant methotrexate, vinblastine, doxorubicin, and cisplatin (M-VAC) chemotherapy and radical cystectomy, we evaluated the expression of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and interleukin 8 (IL-8) by *in situ* hybridization, and we determined microvessel density (MVD) by immunohistochemistry. These factors were evaluated in 55 biopsy specimens prior to therapy and in the cystectomy specimens of 51 patients after completion of therapy. By univariate analysis, VEGF expression and MVD in the biopsy specimens were significant predictors of disease recurrence. By multivariate analysis, only VEGF expression was an independent prognostic factor. Pathological stage, bFGF expression, and MVD in the cystectomy specimens after therapy were all independent prognostic factors for disease recurrence. The results of this exploratory study indicate that the expression levels of VEGF and bFGF as indicated by *in situ* hybridization and MVD as indicated by immunohistochemistry identify patients with muscle-invasive TCC who are at high risk of developing metastasis after aggressive therapy with systemic M-VAC chemotherapy and radical cystectomy.

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INTRODUCTION

TCC⁴ of the bladder is the fifth most common solid malignancy in the United States (1). In the United States every year, this malignancy is diagnosed in ~54,000 patients and results in 12,000 deaths. The standard treatment for operable invasive bladder cancer is surgery, whereas systemic chemotherapy is the only viable therapeutic option for patients with distant metastasis (2–5). Although radical cystectomy will cure a substantial fraction of patients with minimally invasive TCC, many patients with deeply muscle-invasive or extravesical disease treated by radical cystectomy alone die of metastatic TCC (6). For this reason, patients at the Kochi Medical School with muscle-invasive TCC are currently uniformly treated with neoadjuvant M-VAC chemotherapy followed by radical cystectomy. Despite this aggressive approach, some patients still relapse, and nearly all of these will die of metastatic disease that is resistant to conventional chemotherapy. Therefore, it is important to identify prognostic markers that predict for disease recurrence so that we can design and implement more effective therapeutic strategies.

It is well established that tumor growth and metastasis depend upon the induction of a blood supply (7). This process of angiogenesis is regulated within a complex homeostasis by the balance between proangiogenic and antiangiogenic signals. Some proangiogenic factors including bFGF (8, 9), VEGF (10, 11), and IL-8 (12) are produced by tumors growing in their relevant microenvironment. Previous studies of bladder cancer have shown that high MVD is predictive of early disease progression for patients with muscle-invasive TCC (13, 14) but not superficial papillary bladder cancer (15). Overexpression of bFGF (8, 9) and VEGF (10, 11) has been identified in tissue, serum, and urine of patients with bladder cancer and correlates with disease recurrence. IL-8 (16) has also been shown to be proangiogenic and to promote invasion and metastasis of human TCC (17).

We evaluated MVD and the expression of bFGF, VEGF, and IL-8 in patients with muscle-invasive TCC before and after treatment with neoadjuvant M-VAC and radical cystectomy and then correlated clinical outcome with expression of these factors. We found that MVD and overexpression of VEGF prior to therapy correlated with an increased risk of metastatic disease. After M-VAC, MVD and overexpression of bFGF identified

⁴ The abbreviations used are: TCC, transitional cell carcinoma; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial cell growth factor; IL, interleukin; ISH, *in situ* hybridization; IHC, immunohistochemistry; MVD, microvessel density; M-VAC, methotrexate, vinblastine, doxorubicin, and cisplatin.

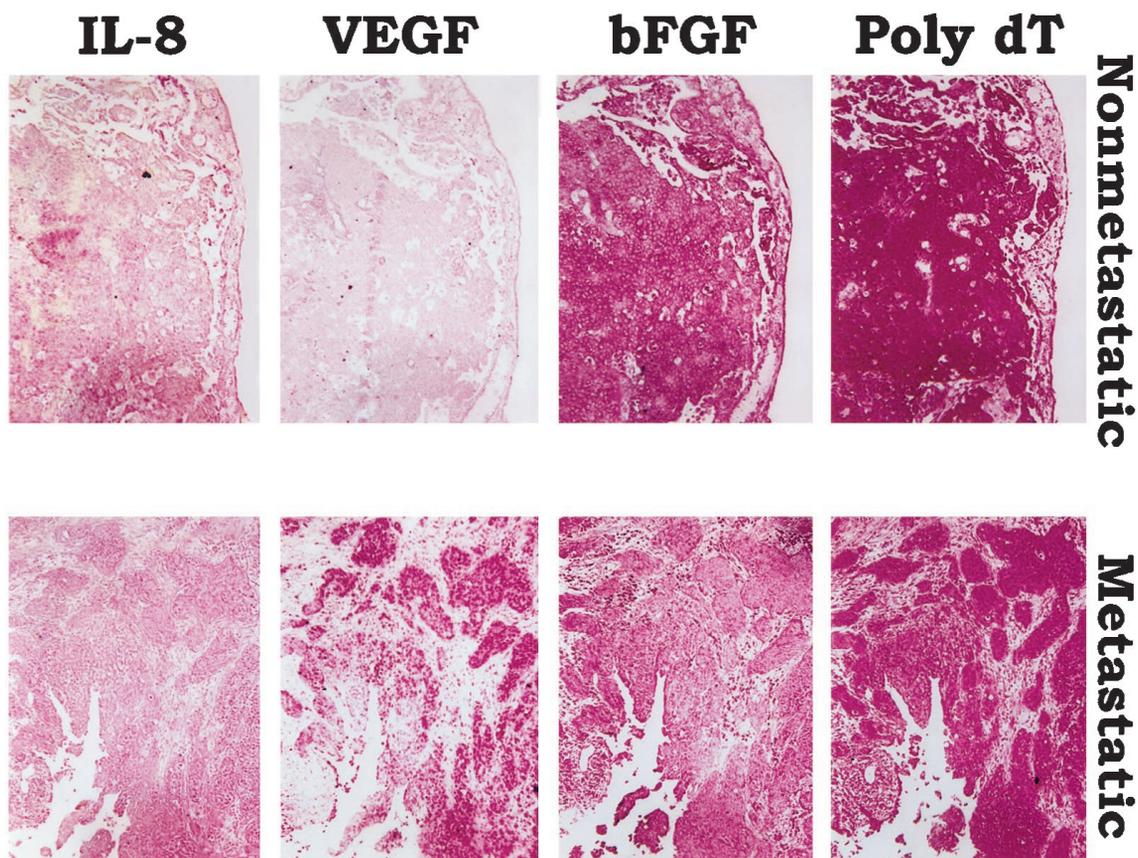


Fig. 1 Examples of ISH. Note the higher expression of VEGF in metastatic versus nonmetastatic biopsy specimens (187% versus 93%).

high-risk patients. In this exploratory study, expression of these proangiogenic factors was more significant than stage (either clinical stage before therapy or the pathological stage after M-VAC) for predicting disease recurrence.

MATERIALS AND METHODS

Surgical Specimens. Formalin-fixed, paraffin-embedded samples were available from 55 pretherapy biopsy specimens and from 51 cystectomy specimens from patients with muscle-invasive TCC who underwent neoadjuvant M-VAC chemotherapy (median of three courses) followed by radical cystectomy and pelvic lymph node dissection. All patients were treated at the Kochi Medical School (Kochi, Japan) between 1984 and 1998. In 31 cases, biopsy and cystectomy specimens were available from the same patient. Initial clinical stage was determined by pathology of the transurethral resection in conjunction with the findings of the examination under anesthesia (18). All patients underwent chest X-ray and computed tomography of the abdomen and pelvis, and any patient with lymph node or distant metastasis was excluded from this analysis. Each specimen was evaluated for MVD by IHC and for angiogenic factor expression (bFGF, VEGF, and IL-8) by ISH. After cystectomy, patients were monitored carefully with periodic hepatic transaminases, alkaline phosphatase, chest radiography, and abdominal and pelvic computed tomographic scans. Time to recurrence and overall survival was recorded.

In Situ mRNA Hybridization Analysis. For ISH analysis, specific antisense oligonucleotide DNA probes for IL-8 (16), bFGF (19), and VEGF (20) were prepared. These were designed to be complementary to the mRNA transcripts based on published reports of the cDNA sequence, as described previously. The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm; these sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each sequence was also confirmed by Northern blot analysis (21). A poly(dT)₂₀ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3' end via direct coupling, with the use of standard phosphormidite chemistry (Research Genetics, Huntsville, AL). The lyophilized probes were reconstituted using a stock solution to 1 $\mu\text{g}/\mu\text{l}$ in 10 mmol/L Tris (pH 7.6) and 1 mmol/L EDTA. Immediately before use, the stock solution was diluted with probe diluent (Research Genetics).

In situ mRNA hybridization was performed as described previously with minor modifications (22, 23). ISH was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 24). Tissue sections (4 μm) of

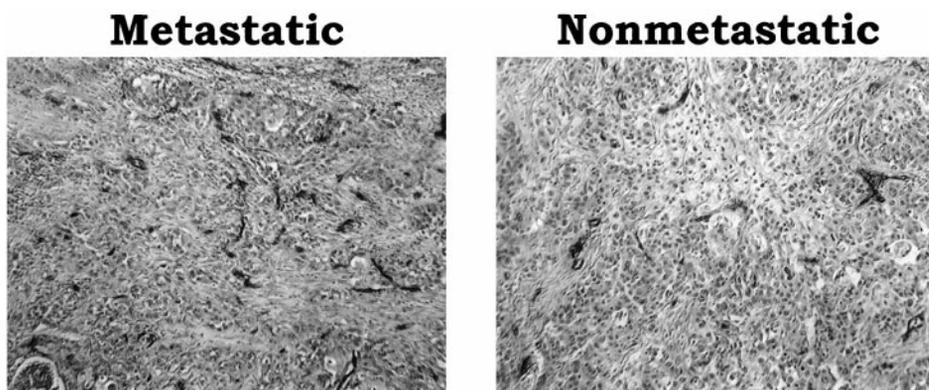


Fig. 2 Examples of CD34 immunohistochemistry. Note the higher MVD in the metastatic tumor compared with the nonmetastatic tumor.

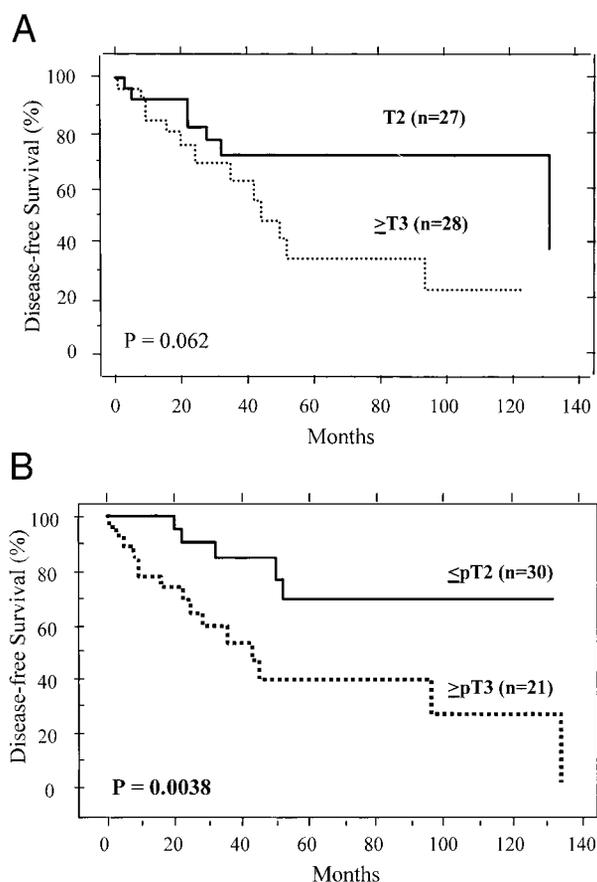


Fig. 3 The disease-free survival curves of these patients [Kaplan-Meier estimates, subdivided according to different stages (T_2 , $\geq T_3$ in biopsy specimens (A); $\leq pT_2$, $\geq pT_3$ in cystectomy specimens (B)). Pathological stage ($P < 0.0038$), but not clinical stage ($P = 0.062$), was an important prognostic factor for stage disease progression.

formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific; Refs. 22 and 23). The slides were placed in the Microprobe slide holder, dewaxed, and rehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 45 min at

45°C, and the samples were then washed three times with $2 \times$ SSC for 2 min at 45°C. The samples were incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mM Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 min, and incubated with fresh chromogen substrate for 15 min at 45°C. If necessary, samples were incubated a second time with fresh chromogen to enhance a weak reaction. Complementary mRNA in the sample leads to a red stain in this assay. Control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of chromogen alone. Four cases of normal urothelium were used as reference material to which all of the tumor samples were referenced.

Quantification of Color Reaction. Stained sections were examined in a Zeiss photomicroscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a three-chip charged-coupled device color camera (model DXC-960 MD; Sony Corp., Tokyo, Japan). The images were analyzed using the Optimas image analysis software (version 6.2; Media Cybernetics, Silver Spring, MD). The slides were prescreened by one of us to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue modes of the color camera. All subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined by the mean log inverse gray scale values multiplied by the area of the field. The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the ISH reaction. For each section, we determined the absorbance in several 2×2 -mm zones located at the periphery of the tumor. Five different fields within each 2×2 -mm zone were quantified, and an average value was determined (24, 25). The intensity of staining was recorded as a ratio of the observed intensity to the intensity of the integrated absorbance of poly(dT)₂₀ in the same sections, and this ratio was then normalized by comparison with the integrated absorbance of a reference set of normal urothelium stained simultaneously with the tumor section according to the following equation: $(A/B)/C/D \times 100$, where A is the absorbance of the gene expression in the tumor specimen, B is the absorbance of poly(dT)₂₀ expression in the tumor specimen, C is the absorbance of the gene expression in the normal urothelium, and D is

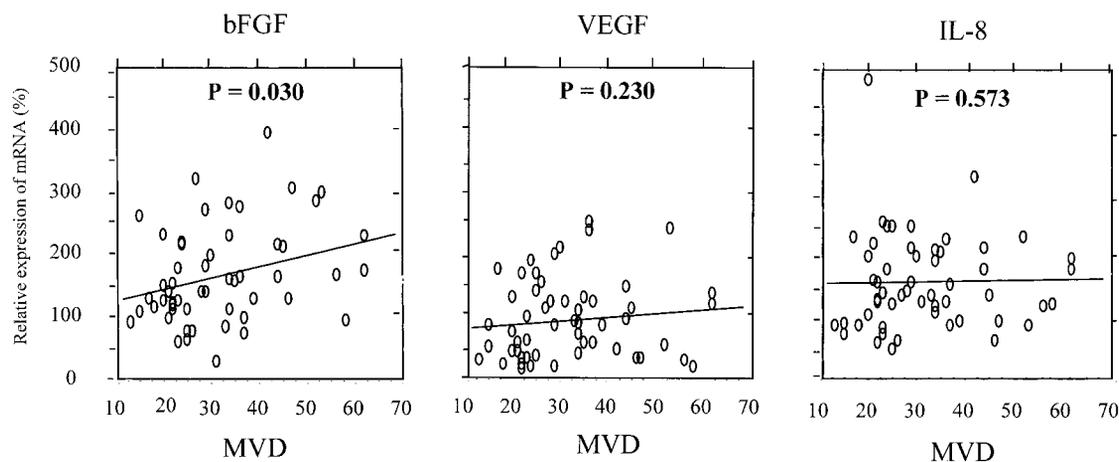


Fig. 4 The expression of bFGF, VEGF, and IL-8 mRNA and CD34 were analyzed by ISH and IHC, respectively. The expression of bFGF in the biopsy specimen correlated with MVD ($P = 0.03$).

the absorbance of poly(dT)₂₀ expression in normal urothelium. An example of ISH and relative gene expression is shown in Fig. 1.

Immunohistochemistry. Tissue sections (5 μ m thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with PBS, and antigen retrieval was performed with pepsin for 12 min. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution containing 5% normal horse serum, 1% normal goat serum, and PBS (pH 7.5). Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with the appropriate dilution (1:100) of rat monoclonal anti-CD34 antibody (PharMingen, San Diego, CA; Ref. 26). The samples were then rinsed four times with (1:400) PBS, incubated for 60 min at room temperature, and then developed using a multilink system (Biogenex Laboratories, San Ramon, CA). The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill's hematoxylin (Biogenex Laboratories), and washed three times with PBS. The slides were mounted using a Universal mount medium (Research Genetics).

Quantification of MVD. MVD was determined by light microscopy after sections were immunostained with anti-CD34 antibodies according to the procedure described by Weidner *et al.* (27). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. Tissue images were recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corp.). The MVD was expressed as the average count of the five areas with the highest MVD identified within a single $\times 100$ field. An example of CD34 immunostaining in a representative metastatic tumor and nonmetastatic tumor is shown in Fig. 2.

Statistical Analysis. Although the staining intensity by ISH is a continuous variable by the method outlined above, these data were made dichotomous for the correlative studies by taking the median value of each factor as a cutoff point for designating "high" or "low" expression. Clinical outcome by expression status was analyzed by the Mann-Whitney test. Multivariate analyses were conducted using the Cox proportional hazards model. Statistical significance was defined as $P < 0.05$.

RESULTS

Patient Characteristics. The cohort we studied consisted of 46 men and 9 women; the median age was 69 years (range, 44–75 years). The median follow-up was 38 months (range, 6–132 months). All tumors were grade 2 or 3 by WHO criteria.

Pretreatment Biopsy Specimens. The Kaplan-Meier disease-free survival curve by clinical stage is shown in Fig. 3A. Clinical stage (organ confined *versus* extravesical, two-sided, $P = 0.062$) approached significance as a prognostic factor for disease-free survival, whereas disease-free survival was independent of grade (data not shown). The expression of bFGF, VEGF, and IL-8 mRNA as revealed by ISH was evaluated in relation to anti-CD34 immunostaining results. The expression of bFGF in the biopsy specimens was correlated with MVD ($P = 0.03$), whereas there was no significant correlation between the expression of VEGF or IL-8 and MVD (Fig. 4). Nine of 51 patients (19%) were rendered pT₀ after M-VAC and transurethral resection. VEGF expression was significantly lower in the biopsy specimens of these 9 patients than in those with persistent tumor ($P < 0.05$).

The prognostic significance of angiogenesis factor expression and MVD was evaluated in biopsy specimens collected prior to therapy using log-rank analysis (Fig. 5). Median MVD was 30 (range, 16–62) vessels per $\times 100$ field. VEGF expression (relative expression $\geq 140\%$ of the expression in normal urothelium) and MVD (≥ 30 per $\times 100$ field) in the biopsy specimens were correlated with recurrence ($P = 0.032$ and $P =$

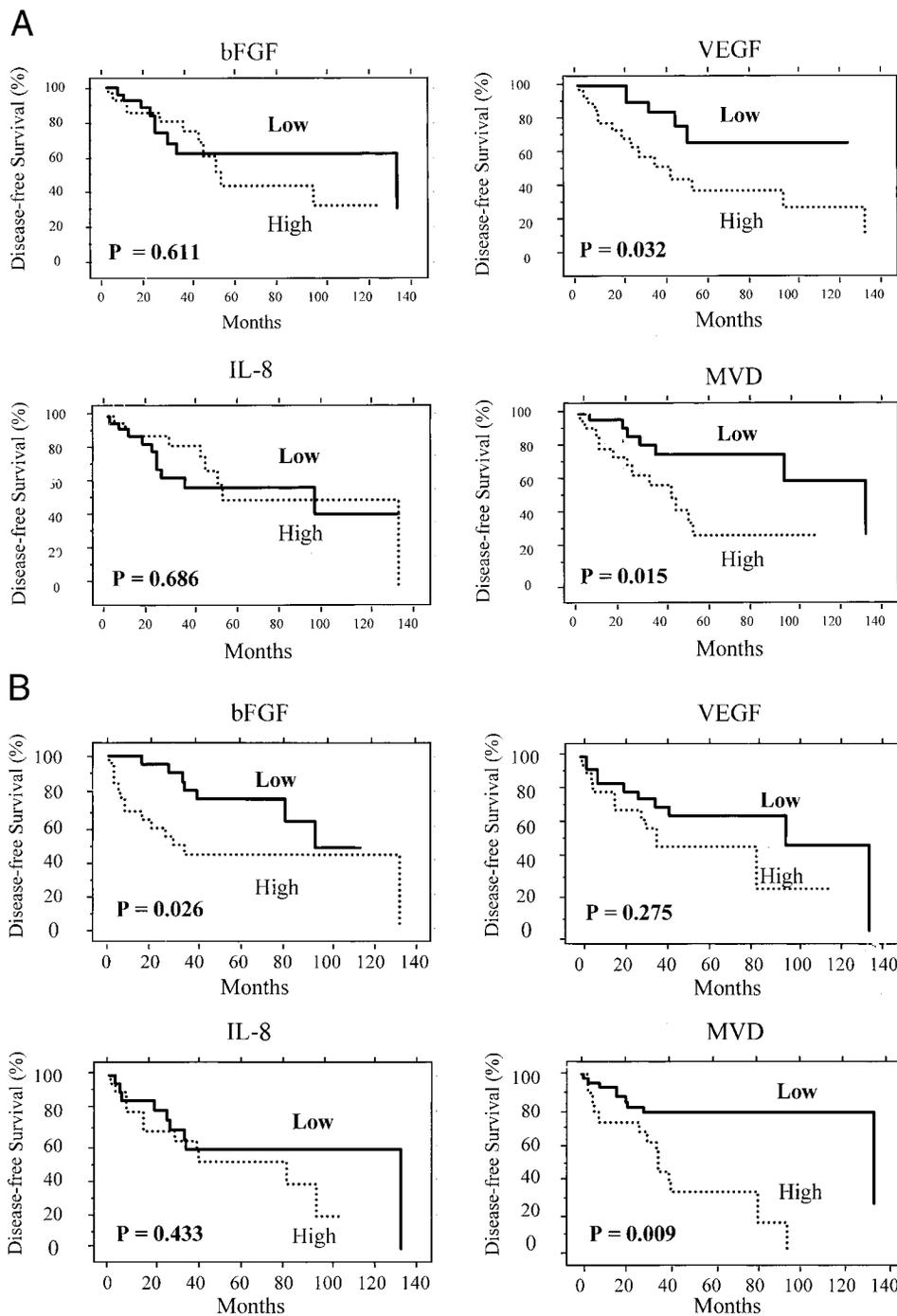


Fig. 5 The relationship between prognosis and the expression of each angiogenic factor and MVD in biopsy in specimens (A) and cystectomy specimens (B). VEGF expression and high MVD in biopsy specimen and bFGF expression and high MVD in cystectomy specimens predicted for disease progression.

0.015, respectively). Expression of bFGF and IL-8 were not significant prognostic factors for disease recurrence. Univariate analysis by Cox proportional hazards model confirmed that pretherapy VEGF expression and MVD were significant predictors for disease progression ($P = 0.010$ and $P = 0.023$, respectively; Table 1A), whereas on multivariate analysis only, VEGF expression was a predictor for disease recurrence ($P = 0.012$; Table 1A).

Posttreatment Cystectomy Specimens. The Kaplan-Meier disease-free survival by pathological stage is shown in

Fig. 3B. Patients with extravesical disease ($\geq pT_3$, N \times) after chemotherapy had a significantly worse survival rate than did patients with organ-confined or no residual disease ($P = 0.0038$). The survival of five patients with lymph node metastasis detected at pelvic lymph node dissection was not significantly different from the survival of patients with extravesical disease without metastasis, although the numbers are too small to make a meaningful comparison. To determine whether we could further stratify prognosis for patients with residual disease, the prognostic significance of angiogenesis factor expres-

Table 1 Multivariate analysis of predictors for tumor recurrence

A. Biopsy specimens		
	Univariate analysis ^a	Multivariate analysis ^a
Clinical stage	0.062	0.123
MVD	0.023	0.073
bFGF	0.618	0.524
VEGF	0.010	0.012
IL-8	0.688	0.670
B. Cystectomy specimens		
Pathological stage	0.035	0.054
MVD	0.010	0.048
bFGF	0.032	0.008
VEGF	0.289	0.100
IL-8	0.999	0.350

^a Cox proportional hazards model.

sion and MVD was evaluated in the cystectomy specimens of patients with residual cancer using log-rank analysis. The median MVD was 32 (range, 12–74) vessels per $\times 100$ field. Using median values as the cutoff for high and low expressions, high expression of bFGF ($>200\%$ of expression by normal urothelium) and high MVD (≥ 32 per $\times 100$ field) were both significant predictors for disease progression ($P = 0.025$ and $P = 0.0091$, respectively; Fig. 5B). Upon univariate analysis using the Cox proportional hazards model, pathological stage ($\geq pT_3$, N \times), bFGF, and MVD were all significantly correlated with disease recurrence, whereas upon multivariate analysis, bFGF expression in cystectomy specimens was the strongest predictor ($P = 0.008$) of recurrence after neoadjuvant chemotherapy and cystectomy, even more so than posttherapy pathological stage (Table 1B).

Effect of Systemic Chemotherapy on Angiogenesis Factor Expression. Corresponding pretherapy and postchemotherapy specimens were available from 31 patients. Table 2 shows that the median expression of both VEGF and bFGF was higher in the postchemotherapy cystectomy specimens than in the pretherapy biopsy, whereas IL-8 expression and MVD were similar in both.

DISCUSSION

Angiogenesis is critical for tumor growth and metastasis (7). High MVD, a histological surrogate for angiogenesis, has been shown to be correlated with aggressive clinical behavior for a number of different neoplasms (28–32), and recent studies have shown that high MVD is an independent prognostic factor for progression in advanced TCC (13, 14, 33). In this study, we confirmed that MVD is a significant predictor for disease outcome in patients with muscle-invasive TCC treated with neoadjuvant M-VAC chemotherapy and cystectomy, regardless of whether it was assessed in biopsy specimens or in residual disease after chemotherapy.

Angiogenesis is regulated by the balance between stimulatory and inhibitory factors released by the tumor and its microenvironment (34, 35). Overexpression of proangiogenic factors by tumor cells is one mechanism by which tumors promote neovascularity. Overexpression of angiogenic factors

Table 2 Differences in expression levels of angiogenesis factors in matched pairs of biopsy and cystectomy specimens from 31 patients

	Biopsy	Cystectomy	No. of patients with increased levels (%) in cystectomy versus biopsy specimens
MVD	30 (15–72)	36 (23–86)	14 (45%)
VEGF	142 (65–210) ^a	195 (115–320) ^a	26 ^b (84%)
bFGF	173 (105–285) ^a	231 (120–355) ^a	22 ^b (71%)
IL-8	157 (82–255)	165 (102–268)	18 (58%)

^a $P < 0.05$, Mann-Whitney.

^b $P < 0.05$, χ^2 .

bFGF (8, 9) and VEGF (10, 11, 35, 36) has been identified in the tissue, serum, and urine of patients with invasive bladder cancer and has also been associated with disease progression. Overexpression of VEGF is also associated with the altered expression of p53 (36), which is a significant predictor of progression in patients with both superficial and muscle-invasive bladder cancer (37–41). Our results showed that VEGF is a stronger predictor than MVD and clinical stage in predicting for recurrence in a group of patients with muscle-invasive TCC treated with neoadjuvant M-VAC prior to radical cystectomy. In a similar manner, bFGF overexpression within the residual tumors after neoadjuvant chemotherapy was predictive of disease progression and maintained independent prognostic significance when compared with advanced pathological stage (pT_3 , N \times). For this analysis, patients with pelvic lymph node metastasis ($n = 5$) were grouped with patients with extravesical disease, because their survival times were similar and there were too few patients with lymph node metastasis to analyze them independently. Given these limitations of this retrospective analysis, the expression of bFGF within the residual tumors from patients with muscle-invasive TCC after neoadjuvant M-VAC appears to be an independent prognostic factor for disease progression.

When we compared 31 matched sets of biopsy and cystectomy specimens, we found that bFGF and VEGF expression was higher in the residual tumor after M-VAC compared with the pretherapy biopsy, although only bFGF expression correlated with disease recurrence. Overexpression of bFGF by human bladder cancer cell lines is associated with resistance to cisplatin, possibly by protecting the tumor cells from cisplatin-induced apoptosis (42, 43). Our observation of enhanced bFGF expression within residual tumors after cisplatin-based chemotherapy suggests that bladder cancer cells that overexpress bFGF selectively survive systemic chemotherapy, possibly secondary to resistance to cisplatin-induced apoptosis.

VEGF was also overexpressed within post M-VAC residual tumors compared with the pretherapy tumor biopsies. VEGF is a survival factor that protects both tumor and endothelial cells from exposure to environmental stresses such as hypoxia or acidosis (44, 45). Recent reports indicate that VEGF also protects tumor cells against chemotherapy-induced apoptosis (46). Therefore, the relative overexpression of VEGF observed within the residual tumors after M-VAC may reflect the clonal selection resulting from the death of tumor cells expressing low levels of VEGF and the survival of tumor cells expressing relatively high levels of VEGF, which rendered them relatively resistant to chemotherapy-induced apoptosis.

IL-8 is a putative proangiogenic factor (16), and in preclinical studies, we found that IL-8 expression regulates angiogenesis, tumorigenicity, and metastasis of human TCC (17). However, in the current study, we did not detect any significance of IL-8 expression in the prognosis of these patients with TCC.

Herein, we report the results of an exploratory study evaluating the prognostic significance of proangiogenic markers for patients with muscle-invasive TCC treated with neoadjuvant M-VAC and radical cystectomy. The results are based upon a relatively small patient population, and yet we observed that increased expression of angiogenic factors and a high MVD were indicative of a poor prognosis for patients treated with chemotherapy and cystectomy. Clearly, these results from an exploratory study need to be confirmed in larger cohorts before these markers can be applied more generally for the clinical management of bladder cancer.

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