

## Expression of VEGF and Its receptors VEGFR1/VEGFR2 Is Associated with Invasiveness of Bladder Cancer

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**Abstract.** Aim: Vascular endothelial growth factor (VEGF) signaling is frequently altered in invasive tumor cells and is associated with patient outcome. In the present study, we examined VEGF, VEGFR1, and VEGFR2 expression in non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC), and evaluated the association between VEGF and its receptors with disease characteristics and bladder cancer recurrence. Materials and Methods: Tissue microarrays containing bladder cancer specimens (n=212) and adjacent normal bladder mucosa (n=131) were immunostained using antibodies against VEGF, VEGFR1, and VEGFR2. The association between the expression of these proteins and clinical parameters including stage, lymph node metastasis, and recurrence-free survival were statistically evaluated. VEGF mRNA expression data were extracted from the public Oncomine database. Results: VEGF and VEGFR1 mRNA levels were significantly higher in bladder cancer specimens than that of normal mucosa (for VEGF,  $p<0.001$ ; for VEGFR1,  $p=0.02$ ). Analysis of their expression at protein levels showed that levels of VEGF and VEGFR1 were significantly higher in NMIBC than in MIBC ( $p<0.001$ ), while that of VEGFR2 was significantly higher in all cancer specimens compared to benign urothelial mucosa ( $p=0.001$ ). Further-more, the

expression of VEGFR2 was significantly higher in MIBC, as compared to NMIBC ( $p<0.001$ ). Patients with higher levels of VEGF, VEGFR1, and VEGFR2 tended to have poorer recurrence-free survival than those with lower levels, but this was not statistically significant. Conclusion: Our results suggest that alterations in the expression of VEGF and VEGF receptors are associated with disease stage and recurrence.

Urothelial carcinoma of the bladder (UCB) is one of the most prevalent types of cancer with high recurrence rates in the non-muscle invasive (NMIBC) type (1-3). NMIBC (pTa/pTis/pT1) accounts for 80% of bladder cancer cases, with the remaining 20% being muscle-invasive bladder cancer (MIBC) (pT2 or more) (4). Approximately 70% of patients with NMIBC will develop disease recurrence within two years of initial diagnosis (5, 6). Patients with NMIBC that are left untreated will ultimately progress to MIBC with a significantly worse prognosis (5, 7, 8). Identification and development of novel biomarkers to monitor and manage tumor recurrence is, therefore, essential.

Vascular endothelial growth factor (VEGF) is one of the key angiogenic factors that stimulates the formation of new blood vessels and tumor growth (9). Altered expression of VEGF has been observed in UCB cells (10). Elevated levels of VEGF expression were also detected in urine samples from UCB patients and correlated with disease-recurrence and progression (11). In agreement with this study, a high level of VEGF expression in tumors and in serum samples from patients with UCB, also predicted poorer prognosis and increased frequency of disease recurrence (12). Altered VEGF expression was associated with advanced pathological stage and lymph node metastasis (13).

Anti-angiogenic drugs, mainly bevacizumab, sorafenib and sunitinib, are currently approved for use in a wide number of

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tumor types, such as breast, colorectal, liver and kidney cancer, and present with significantly improved treatment of cancer (14). However, in UCB, anti-angiogenic agents are still in the preliminary phase of clinical studies (14). Furthermore, the prognostic values of angiogenic factors in treatment response of UCB remains largely unknown (15). Several studies have suggested that VEGF plays an important role in the growth of bladder cancer cells (16), and inhibition of VEGF transcripts significantly reduces the proliferation rate of the bladder cancer cells (17). VEGF signaling is mediated through its binding to the receptors VEGFR1 (fms-like tyrosine kinase-1) and VEGFR2 (Flk-1, fetal liver kinase) (4, 12, 18, 19, 20). Similar to VEGF, an elevated level of VEGFR2 was also observed in bladder cancer specimens from patients (21). Finally, several studies have shown that inhibition of the expression of VEGF receptors reduced growth and invasion of bladder cancer cells, similar what was observed in cells with the depletion of VEGF expression (22, 23).

It is, thus, suggested that VEGF, in combination with other angiogenic factors such as angiogenin and matrix metalloproteinases (MMPs), may serve as a biomarker for the diagnosis and prognosis of patients with UCB (24). However, limited studies have been conducted to evaluate the expression of VEGF in combination with its receptors VEGFR1 and VEGFR2, in bladder tumors and their clinical importance in disease recurrence. In the present study, we examined the expression of VEGF and its receptors in benign and malignant bladder cancer specimens and assessed the association between the expression of VEGF signaling proteins and disease recurrence in patients with bladder cancer.

**Materials and Methods**

*Tissue samples and tissue microarrays.* Local Institutional Review Board (Fox Chase Cancer Center, Philadelphia, PA, USA) approval was granted to obtain bladder cancer specimens from patients treated with radical cystectomy. The cohort represents the available subset of patients reported in Mahklin *et al.* (25). The study was approved by the local Ethical Committees of Fox Chase Cancer Center and Lund University (25), and the Helsinki Declaration of Human Rights was strictly observed. Tissue microarrays (TMAs) were constructed using 1 mm cores (two per sample) and sampled both benign urothelial mucosa and malignant tissue, where possible. The TMAs contained benign urothelium (N=131), NIMBC (N=34), and MIBC (N=170). Patient demographics are summarized in Table I. Out of the 170 patients with MIBC, 68 had lymph node metastasis (40%). Tumors were staged according to the TNM classification and graded using the 1998 WHO classification (26). The patients were followed up from 0 to 120 months from the time of surgery, with mean follow-up of 80.77 months. Clinical information, such as surgical margin status, stage, grade, multi-focality and number of previous bladder tumors, was obtained for all patients.

*Source of antibodies and immunohistochemistry (IHC).* The following antibodies and dilutions were used for IHC: Rabbit polyclonal to VEGF (1:300), rabbit polyclonal to VEGFR1 (1:300), mouse monoclonal to

Table I. *Clinicopathological demographics for the 212 patients with bladder cancer.*

Clinical properties	No. of patients (%)
Total	212
Age (yr) (n=173)	
<or equal 65	58 (33.5)
>65	115 (66.5)
Median	70
Range	37-90
Gender (n=171)	
Male	128 (74.85)
Female	43 (25.1)
Race (n=168)	
White	147 (87.5)
Black	17 (10.1)
Asians	4 (2.4)
Primary tumor stage (n=204)	
pTis	9 (4.4)
pTa	10 (4.8)
pT1	15 (7.4)
pT2	34 (16.7)
pT3	79 (42.9)
pT4	49 (26.3)
Histology (n=184)	
Urothelial carcinoma (pure)	172 (93.5)
Urothelial carcinoma with mixed histology	12 (6.5)
Lymph node status (n=188)	
pN0	120 (63.83)
pN+	68 (36.17)

VEGFR2 (1:200); For western blot analysis: rabbit polyclonal to VEGF (1:200), rabbit polyclonal to VEGFR1 (1:200), rabbit polyclonal to VEGFR2 (1:400) all from Santa Cruz Biotechnology, Santa Cruz, CA, USA and mouse monoclonal to actin (1:15,000; MP Biomedicals, Solon, OH, USA). Formalin-fixed, paraffin-embedded tissue samples were de-paraffinized in xylene for 10 min, followed by washing in decreasing concentrations of ethanol (95%, 75%, 50%), each for 2 min. After de-paraffinization, antigen retrieval was performed by boiling the slides in 0.01 M citrate buffer, pH 6.0, in a microwave for 10 min. The slides were then applied on a semi-automatic IHC diagnostic system (Ventana ES, Ventana Inc., Tucson, AZ, USA) and IHC staining was performed using antigen-specific antibodies, as indicated above. The scoring of all the samples was performed by a urological pathologist (BDR) who was blinded to the clinical information. An intensity score was assigned that represented the average intensity of positive cells (0=no staining, 1=weak, 2=intermediate, and 3=strong staining).

*Analysis of VEGF, VEGFR1 and VEGFR2 mRNA expression from gene expression profiles.* We analyzed mRNA expression profiles of VEGF, VEGFR1 and VEGFR2 based on the published cDNA microarray database described in (27-29), which are available in a public database (www.oncomine.org). The raw signal intensities of the arrays were pre-processed by setting the intensity at the minimum value if the intensity was below a minimum value of 10 and normalized by subtracting the median log ratio of an array by all the log ratios on that array. *p*-Value <0.05 was considered significant.

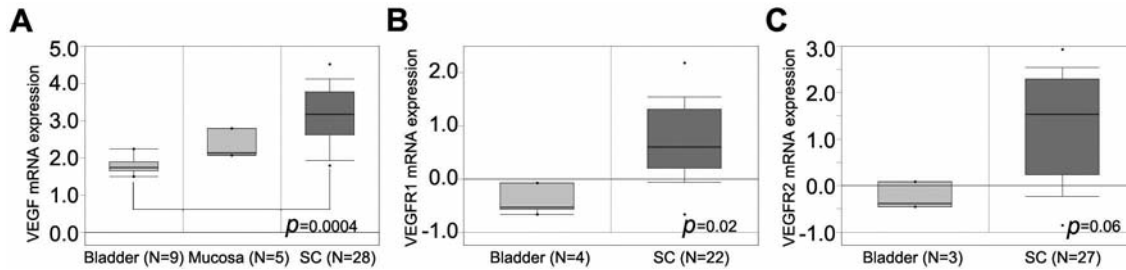


Figure 1. mRNA expression of VEGF and its receptors VEGFR1 and VEGFR2 in normal bladder tissues and bladder cancer specimens from patients. Normal bladder tissues and bladder cancer specimens from patients. Normal bladder cancer tissues are indicated as Normal and Mucosa. The bladder cancer specimens are non-muscle invasive subtype as indicated as superficial cancer SC. Data was obtained from cDNA microarray analysis published in Oncomine data-bases.

**Tissue culture and immunoblot analysis.** Three human UCB cell lines [HTB1 (J82), HTB5 (TCCSUP), and HT1376] and one bladder squamous cell carcinoma cell line [HTB3 (ScaBER)] were a generous gift from Dr. Douglas Scherr, Weill Cornell Medical College, and cultured as previously described (30). The cells were harvested and lysed in ice-cold RIPA buffer (120 mM NaCl, 50 mM Tris-HCl pH 7.6, 50 mM NaF, 0.1mM Na<sub>3</sub>VO<sub>4</sub>, 1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) and 15% protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland). The bladder cancer cell lines: HTB1, HTB3, HTB5, HT1376 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as previously described (30). SDS-PAGE gels were loaded with 18 µg of the protein lysates for both VEGF and VEGFR1, and 30 µg of the protein lysate for VEGFR2, and bands were separated and were transferred onto nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad, Solna, Sweden). Signals were visualized using the Enhanced Chemiluminescence detection system (Millipore Corp. Solna, Sweden) and documented with an Alpha Imager CCD system (Bio-Rad).

**Statistical analysis.** Statistical analysis was performed using the SPSS Version 18 & 20 (SPSS Inc., Chicago, USA). Spearman Rho two-tailed correlation analysis was performed between VEGF, VEGFR1 and VEGFR2 to determine the correlation of the expression of these proteins in patient samples. The strength of association between the continuous variables is represented in the percentage form *r*. Non-parametric analysis using the Mann Whitney test was performed for comparison between benign and malignant tissues, as well as between lymph node-negative and lymph node-positive groups. Pearson chi-square analysis was performed to assess different clinical parameters including age, gender, race, TNM staging and lymph node status. Kaplan Meier survival analysis was performed for the assessment of disease-free survival, and log-rank tests were used to compare the differences in recurrence in subgroups.

## Results

**mRNA expression of VEGF, VEGFR1 and VEGFR2 in normal bladder tissues and in bladder cancer specimens.** We evaluated mRNA expression of VEGF, VEGFR1 and VEGFR2 in normal bladder/mucosa tissues and in cancer

specimens from patients with UCB. We found that VEGF mRNA level was significantly higher in UCB specimens than in normal bladder tissues ( $p < 0.001$ , 2-fold increase). VEGFR1 mRNA was also increased 2-fold in UCB specimens compared with the normal specimens ( $p < 0.02$ ). There was no statistically significant difference in VEGFR2 mRNA levels in these patient samples ( $p < 0.06$ ) (Figure 1).

**Expression of VEGF, VEGFR1 and VEGFR2 in benign bladder tissues and bladder cancer specimens by IHC.** We next examined by IHC the expression of VEGF, VEGFR1 and VEGFR2 on tissue microarrays containing primary bladder cancer specimens ( $n = 212$  cases) and adjacent normal bladder tissues ( $n = 131$ ). It was possible to evaluate 168 cases for VEGF expression, 117 cases for VEGFR1 expression and 171 cases for VEGFR2 expression. Expression of VEGF, VEGFR1, and VEGFR2 was observed to varying degrees in tumor cells, as shown in the four representative tumors from patients with UCB (Figure 2). The intensity of VEGFR2 staining was significantly higher in cancer specimens than in the non-malignant tissues ( $p = 0.001$ ) (Figure 3A, B and C). Rank-correlation analysis revealed a significantly-positive correlation between VEGF and VEGFR1 ( $r = 0.476$ ,  $p < 0.001$ ), and between VEGFR1 and VEGFR2 in bladder cancer specimens ( $r = 0.347$ ,  $p < 0.001$ ) (Table II).

**Association of VEGF, VEGFR1 and VEGFR2 and tumor stage.** We next assessed whether expression of VEGF and its receptors may be associated with bladder cancer stage. We divided the patients into two groups based on the invasiveness of tumors: the NMIBC group vs. the MIBC group. We compared the expression intensity of VEGF, VEGFR1 and VEGFR2 between the NMIBC group and the MIBC group. VEGF staining intensity was significantly higher in the NMIBC group compared to the MIBC group ( $p < 0.001$ ) and the group with lymph node metastasis ( $p = 0.007$ ) (Figure 4A and B). The VEGF expression pattern in tumors in NMIBC and MIBC is shown in Figure 4C and D. The intensity of

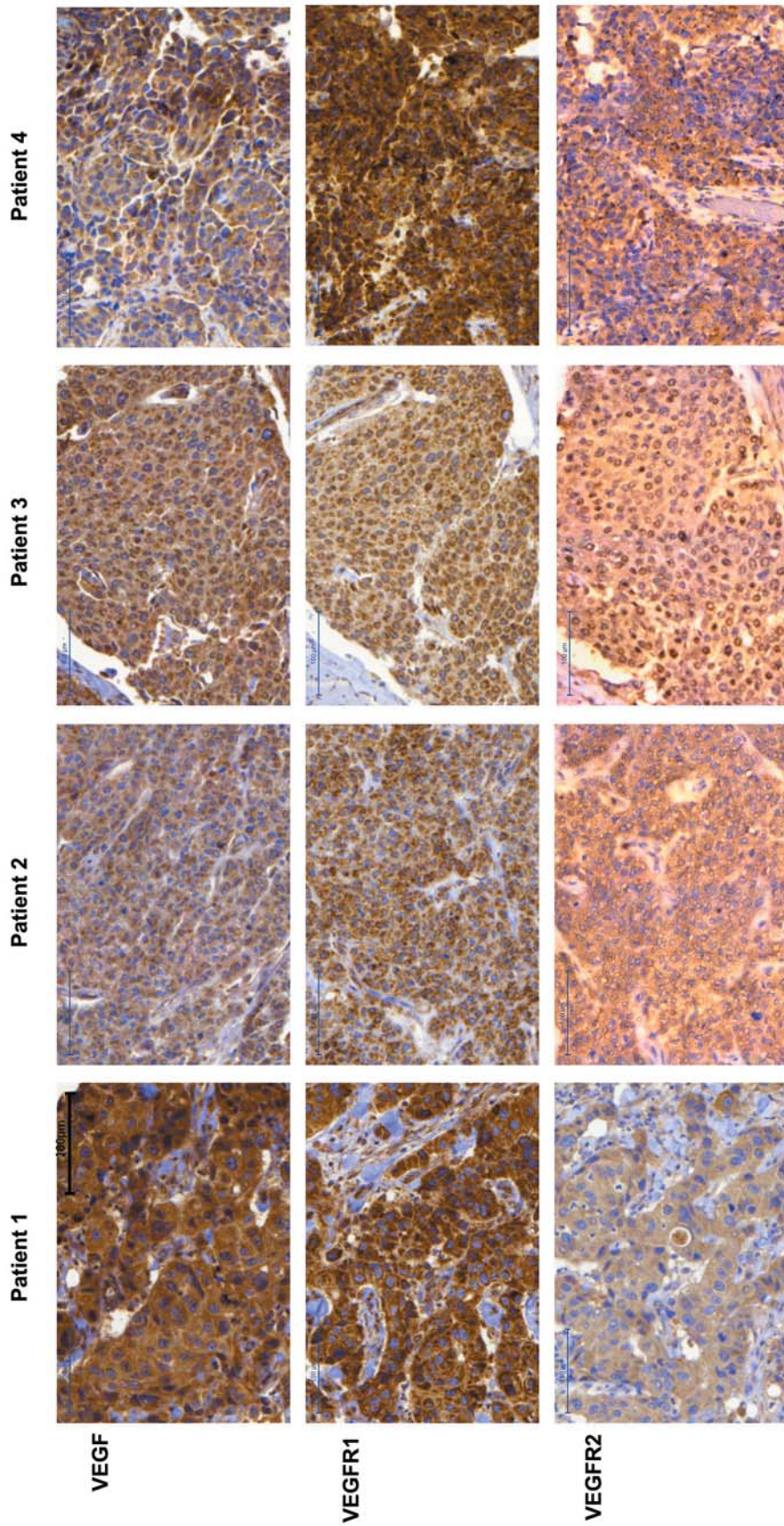


Figure 2. Expression of VEGF and its receptors VEGFR1 and VEGFR2 in cancer specimens from four patients with urothelial bladder cancer (UCB). An overview of VEGF, VEGFR1 and VEGFR2 expression in tumor areas from four patients with UCB. Bar=100 µM.

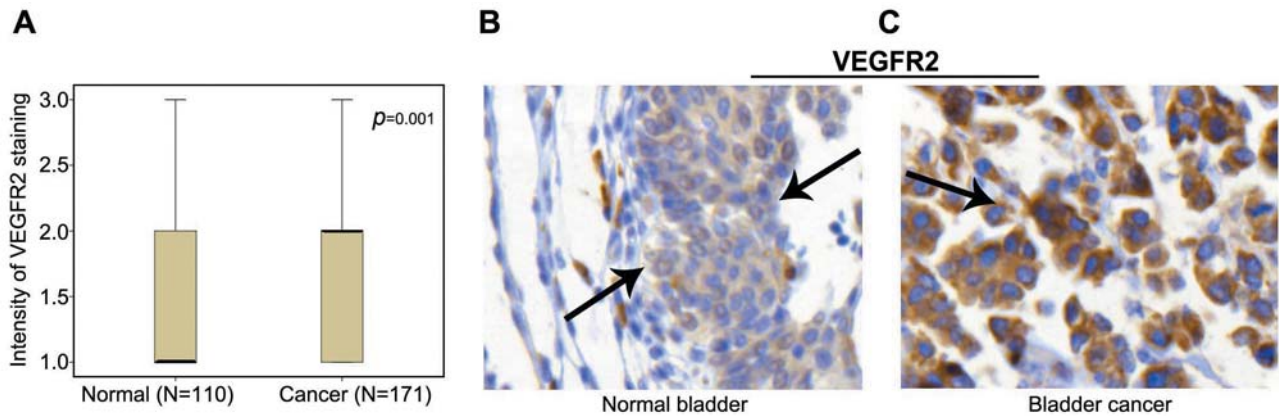


Figure 3. Protein expression of VEGFR2 in normal bladder tissues and bladder cancer specimens using immunohistochemical analysis. A: The Mann-Whitney test is shown in the Box-plot which indicates VEGFR2 expression in non-malignant adjacent bladder tissues (n=110), compared with that of bladder cancer specimens (n=171).  $p=0.001$  is indicated. B and C: Representative microphotographs of normal and bladder cancer specimens stained with antibody against VEGFR2 are shown. Arrows point to the membranes and cytoplasmic localization of VEGFR2 in normal mucosa and cancer cells. The microphotographs were taken under  $\times 40$  magnification.

Table II. Spearman's correlations shows VEGF to be significantly positively-correlated with VEGFR1, and VEGFR1 to be positively correlated with both VEGF and VEGFR2.

Protein	VEGF n=168	VEGFR1 n=117	VEGFR2 n=171
VEGF			
N		111	156
Correlation coefficient		0.476	0.142
p-Value		<0.001	0.076
VEGFR1			
N			114
Correlation coefficient			0.347
p-Value			<0.001

VEGFR1 staining was higher in tumors in NMIBC than in the MIBC ( $p<0.001$ ) (Figure 4E). But there was no significant difference in VEGFR1 expression in tumors between NMIBC and the MIBC from patients with lymph node metastasis (Figure 4F). The VEGFR1 expression pattern in tumors in NMIBC and MIBC is shown in Figure 4G and H. In contrast to what was observed for VEGF and VEGFR1, the intensity of VEGFR2 staining was significantly higher in the tumors in MIBC than in NMIBC ( $p<0.001$ ) (Figure 4I). But there was no significant difference in VEGFR2 expression in tumors between NMIBC and the MIBC from patients with lymph node metastasis (Figure 4J). The expression pattern of VEGFR2 in NMIBC and MIBC is shown in Figure 4K and L.

Association between VEGF, VEGFR1 and VEGFR2 expression and cancer recurrence. We next evaluated whether VEGF, VEGFR1 and VEGFR2 expression is associated with disease-recurrence. Patients were followed-up from 0 to 120 months, with a median follow up of 120 months, and the time to first recurrence was evaluated using Kaplan Meier survival analysis. Patients were stratified based on the staining intensities of VEGF, VEGFR1 and VEGFR2 in their tumors by IHC analysis. Patients who had higher intensities of VEGF and VEGFR1 staining (scored as 2 and 3) in their tumor cells tended to have poorer recurrence-free survival compared with those with lower staining intensities (scored as 0 and 1) (Figure 5A and B). In contrast, patients with lower VEGFR2 expression tended to have poorer recurrence-free survival than those with a higher expression. However, none of these trends were statistically significant (Figure 5C). To further examine whether VEGF, VEGFR1 and VEGFR2 expressions in bladder cancer specimens may be used to predict cancer recurrence in subgroups of patients with bladder cancer, we divided patients into two groups based on their lymph node status. We evaluated the association between VEGF, VEGFR1, VEGFR2 and cancer recurrence among patients with and those without lymph node metastasis. There was no statistically significant difference in recurrence-free survival between the two groups of patients by VEGF, VEGFR1, VEGFR2 expression (data not shown). Next we evaluated whether combined expression of VEGF and lymph node status may have any prognostic values in predicting cancer recurrence. We divided patients into four groups: group1: patients who had a low expression of VEGF and no lymph node metastasis; group 2: patients

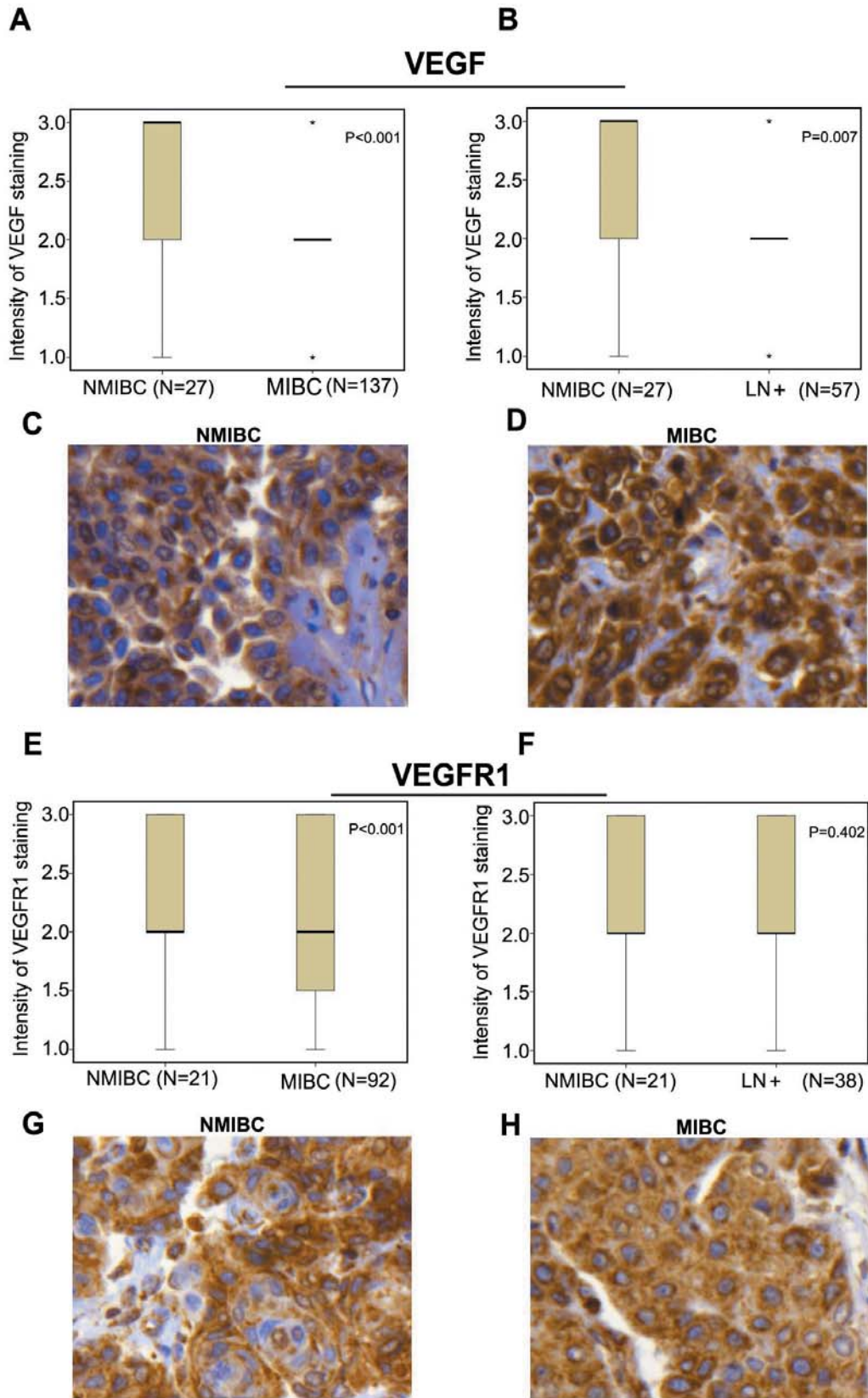


Figure 4. continued

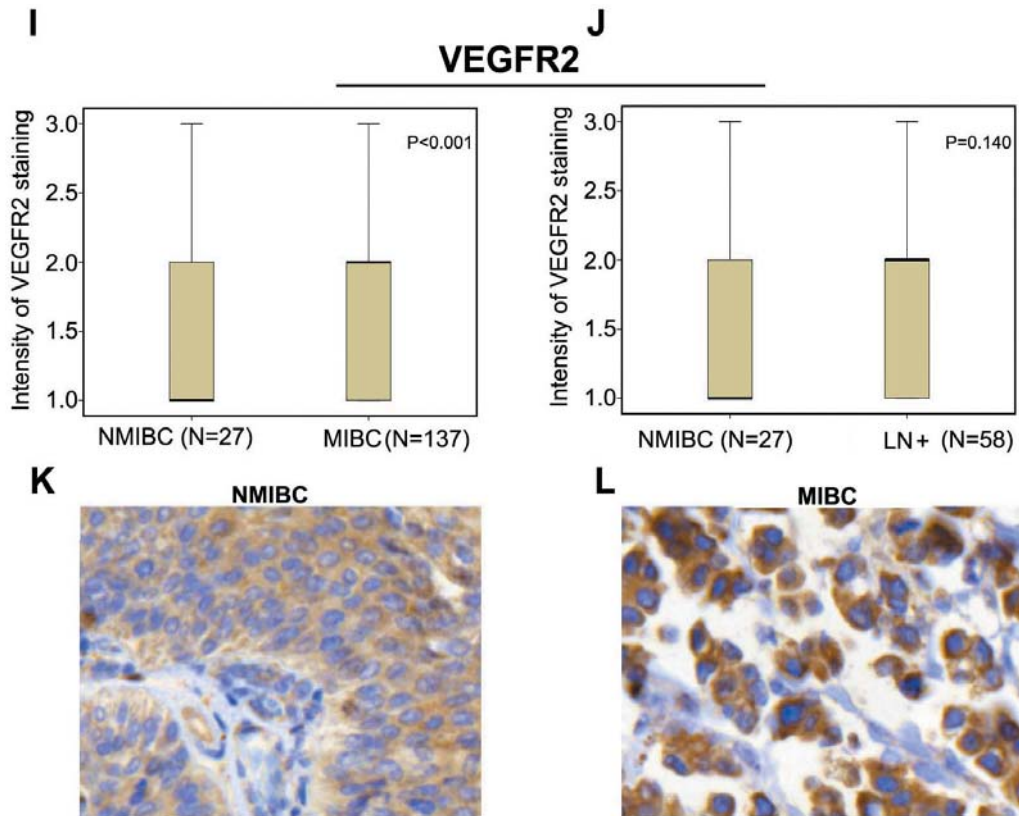


Figure 4. Expression of VEGF and its receptors VEGFR1 and VEGFR2 in the NMIBC, MIBC and MIBC sub-group with lymph node metastasis. A: Mann-Whitney analysis in Box-plot show that VEGF expression in NMIBC (n=27) and MIBC (n=137). The difference in VEGF expression between NMIBC and MIBC groups was assessed using.  $p < 0.001$  is indicated. B: Box-plot analysis shows the difference between NMIBC and MIBC with lymph node metastasis (LN+). C and D: Representative microphotographs showing the immunostaining with VEGF antibody in NMIBC and MIBC. E and F: Box-plot shows the VEGFR1 expression in NMIBC (n=21), MIBC (n=92) and LN+MIBC (n=36). G and H: Representative microphotographs of VEGFR1 protein expression in NMIBC and MIBC are shown. I-J: Box-plot analysis of the VEGFR2 expression in NMIBC (n=27) and MIBC (n=137), and LN+MIBC (n=58). K and J: Representative microphotographs of VEGFR2 protein expression in tumors in NMIBC and MIBC are shown.

who had a high expression of VEGF and no lymph node metastasis; group 3: patients who had a low expression of VEGF and lymph node metastasis; group 4: patients who had a high expression of VEGF and lymph node metastasis. Recurrence-free survival analysis showed that patients with high expression of VEGF and lymph node metastasis tended to have worse recurrence-free survival and shorter time-to-recurrence, compared with the other three groups (Figure 6A). However, the difference was not statistically significant. Similar analysis was performed to evaluate whether VEGFR1 and VEGFR2 expression may be associated with recurrence-free survival. Patients who had a low expression of VEGFR1 and lymph node metastasis tended to have poor recurrence-free survival and shorter time-to-recurrence compared with the other groups (Figure 6B). In addition, patients who had a high expression of VEGFR2 and lymph node metastasis tended to have worse recurrence-free survival compared with the other groups (Figure 6C). Again, however, the difference

was not statistically significant. We also evaluated whether expression of VEGF, VEGFR1, VEGFR2 and lymph node status may predict cancer recurrence within the MIBC group. We did not find a significant association between the expression of these proteins, absence or presence of lymph node metastasis and cancer recurrence.

*Expression of VEGF, VEGFR1 and VEGFR2 in bladder cancer cell lines.* Next, we examined the expression levels of VEGF, VEGFR1 and VEGFR2 proteins in a series of bladder cancer cell lines including: HTB1 (transitional cell carcinoma), HTB3 (squamous cell carcinoma), HTB5 (grade III transitional cell carcinoma from a female), HT1376 (grade III transitional cell carcinoma) cells. High expression of VEGF and VEGFR2 was observed in HTB1 and HT1376 cells, while low level of these proteins was detected in HTB5 (Figure 7). VEGFR1 was observed in all the four bladder cancer cell lines appearing to be most intense in HTB5 cells (Figure 7).

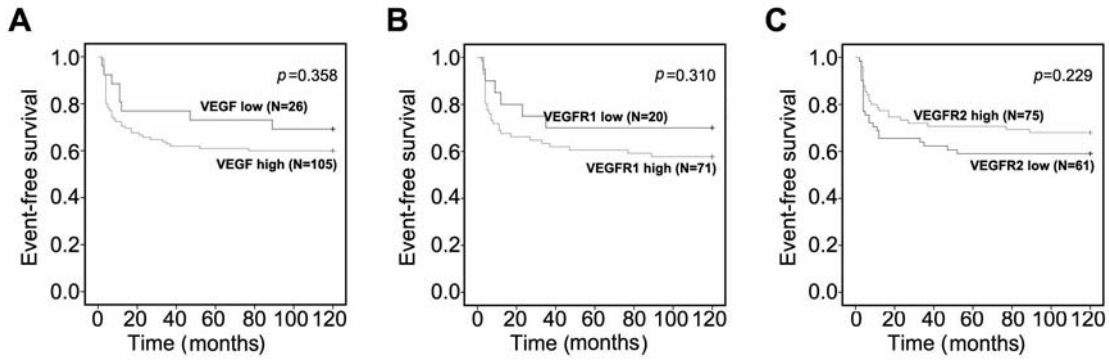


Figure 5. Evaluation of the correlations between expression of VEGF and its receptors VEGFR1 and VEGFR2 and disease-recurrence. A: Recurrence-free survival analysis shows two different patient groups stratified based on the levels of protein expression. Patients with high protein expression were compared with those with low expression. A: VEGF; B: VEGFR1; C: VEGFR2.

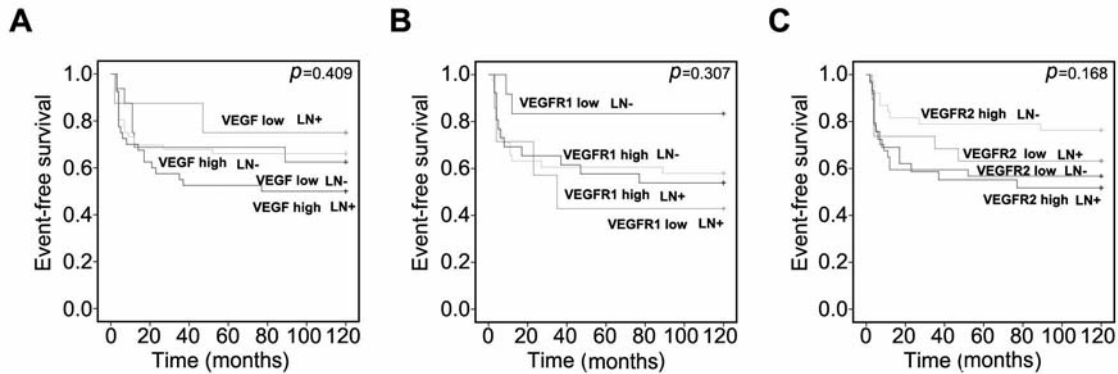


Figure 6. Evaluation of the correlation between expression of VEGF and its receptors VEGFR1 and VEGFR2 in combination with lymph node metastasis and disease-recurrence. A: Recurrence-free survival analysis shows four different patient groups stratified based on the level of protein expression in combination with the lymph node status i.e. low expression and no lymph node metastasis (LN-); high expression and LN-; low expression and no lymph node metastasis (LN-); low expression and lymph node metastasis (LN+); and high expression and LN+. A: VEGF; B: VEGFR1; C: VEGFR2.

**Discussion**

In the present study, we analyzed the expression of angiogenic factor VEGF and its receptors VEGFR1 and VEGFR2 in a large number of benign and malignant bladder tissues on TMAs containing samples from a cohort of 212 cystectomy cases. We showed that mRNA levels of VEGF and VEGFR1 were significantly higher in bladder cancer specimens compared to normal bladder specimens. This suggests that VEGF and VEGFR1 expression is altered in bladder cancer. In the present study, we attempted to investigate whether VEGF and expression of its receptors may aid in defining the stage or prognosis of bladder cancer. Bladder cancer has a very low survival rate once the cancer has reached advanced stages. Our findings show that VEGF and VEGFR1 expression was significantly higher in NMIBC compared to that in invasive cancer. VEGF binds to its receptor, VEGFR1 to induce cell proliferation. It might be

anticipated that the rate of tumor growth is higher at the early stage of disease and thus NMIBC may be correlated with the abundance of VEGF and VEGFR1. Our study on VEGF expression in UCB is in agreement with previous studies in which VEGF expression failed to correlate with clinical variables (21). Targeted therapy using anti-VEGF was effective in inhibiting tumor progression in breast and colon cancer, however, single-agent VEGF-targeted therapy produces low response rates in patients with aggressive and late-stage cancer (31). This raises the question relating to the identification of patients most likely to benefit from VEGF targeted therapy. Only patients with high VEGF expression are most likely to benefit from therapy. In addition, VEGF receptor expression may also interfere with response to VEGF-targeted therapy. VEGFR2, which is required for the mitogenic response to VEGF, was expressed in half of the cases, and its expression was significantly higher in bladder cancer specimens compared with the normal bladder tissues.



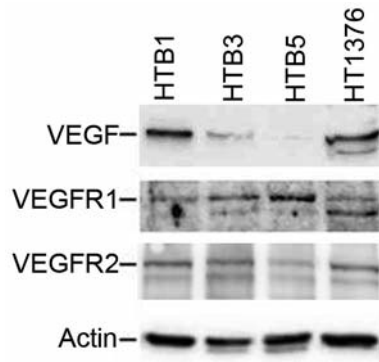


Figure 7. Western blot analysis using antibodies against VEGF, VEGFR1 and VEGFR2 in bladder cancer cell lines: HTB1J82, transitional cell carcinoma (TCC); HTB3, Scaber, squamous cell carcinoma; HTB5, grade IV TCC from a female; HT1376, grade 3 TCC.

VEGFR2 expression was significantly higher in MIBC than that in NMIBC, which suggests that VEGFR2 expression increases with tumor invasion. Our study is consistent with a previously reported study in which VEGFR2 was associated with tumor progression and poor prognosis (21). *VEGFR2* mRNA expression was also elevated in invasive bladder cancer (32). The use of genetic programming in the analysis of quantitative gene expression profiles may be useful for prediction of nodal status in bladder cancer. We found a significant correlation between VEGF and VEGFR1, and VEGFR1 and VEGFR2. It is likely that when co-expressed, the VEGF/VEGFR pathways are activated, and VEGF and its receptors may cooperatively promote proliferation, survival and invasion of tumor cells. Identification of patients with disease recurrence can lead to optimization of follow-up of disease progression and personalization of adjuvant treatment strategies. Our results suggest that VEGF and its receptor pathways may be responsible for the biological behaviour of bladder cancer. It has been shown that different angiogenic pathways are involved at different stages of this type of cancer (33), and it has been shown that blockade of VEGF receptors inhibits proliferation and invasion of bladder cancer cells (22). There are several limitations to our present study. Our patient cohort consists in the majority of invasive UCB. We did not achieve statistical significance comparing high and low expression of VEGF, VEGFR1, and VEGFR2 in recurrence-free survival, this may be due to the small number of patients with NMIBC.

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