The Prognostic Value of Angiogenesis and Metastasis-related Genes for Progression of Transitional Cell Carcinoma of the Renal Pelvis and Ureter

Keiji Inoue,1,2 Masayuki Kamada,1 Joel W. Slaton, Satoshi Fukata, Chiaki Yoshikawa, Pheroze Tamboli, Colin P. N. Dinney, and Taro Shuin

Department of Urology, Kochi Medical School, Nankoku, Kochi, 783-8505 Japan [K. I., M. K., S. F., C. Y., T. S.], and Departments of Cancer Biology [J. W. S., C. P. N. D.] and Pathology [P. T.], The University of Texas M. D. Cancer Center, Houston, Texas 77030

ABSTRACT

Purpose: We reported previously that angiogenesis evaluated by intratumor microvessel density (MVD), expression of such angiogenic factors as vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF), and the matrix metalloproteinase-9:E-cadherin ratio (M:E ratio) could identify patients with advanced transitional cell carcinoma (TCC) of the bladder for whom chemotherapy and cystectomy will be unsuccessful. In the present study, we evaluated the significance of the M:E ratio as a predictor for prognosis for patients with TCC in the upper urinary tract (TCC-UUT).

Experimental Design: We evaluated MVD by immunohistochemistry and the expression of angiogenic and metastasis-related factors by in situ hybridization in 55 nephroureterectomy specimens from patients who received no neoadjuvant therapy. The expression of angiogenesis, angiogenic and metastasis-related factors, and clinicopathological characteristics were evaluated for their correlation with metastasis, recurrence, and disease prognosis.

Results: We found that tumor grade and pathological stage were important predictors for metastasis and survival in these patients. The expression level of matrix metalloproteinase type 9 (MMP-9) and type 2 (MMP-2) and the M:E ratio correlated with MVD. Increased MVD, elevated expression levels of MMP-9 and MMP-2, and a higher M:E ratio were associated with poor prognosis. Moreover, lower expression levels of E-cadherin were associated with fewer recurrences in the urinary bladder. Multivariate analysis indicated that the M:E ratio and E-cadherin expression were independent prognostic factors for disease progression and intravesical recurrence, respectively.

Conclusion: We suggest that the M:E ratio and E-cadherin expression may be targets for novel therapeutic strategies.

INTRODUCTION

TCC3 of the upper urinary tract (TCC-UUT) is a relatively rare cancer, occurring in only 2–8% of all patients with urothelial cancers (1). The standard treatment for localized renal pelvic and ureteral cancer is surgical resection. Recurrences occur in two forms, superficial bladder cancer and distant metastases. Although stage and grade are associated with distant metastases (2, 3), there are few predictors for superficial bladder recurrences after nephroureterectomy. Identification of such prognostic markers for subsequent intravesical recurrence would allow us to tailor cystoscopic surveillance and potentially offer novel chemoprevention strategies.

For patients with metastatic disease, chemotherapy offers the only viable therapeutic option. A combination of methotrexate, vinblastine, doxorubicin, and cisplatin (M-VAC) can induce complete pathological responses in primary renal pelvic/ureteral (4), nodal (5), or metastatic (1) TCC, resulting in long-term survival (6). However, most deaths from TCC-UUT are caused by metastases that resist conventional therapy. Therefore, it is important to identify prognostic markers beyond stage and grade that predict for disease recurrence so that we can design and implement more effective therapeutic strategies.

The progression of solid urothelial tumors has been shown to correlate directly with the expression level of several independent genes that regulate a number of steps responsible for metastasis. Intratumor MVD, a surrogate for angiogenesis (7), has been shown to predict for early progression in muscle-invasive bladder cancer (8–11). Overexpression of bFGF (11–13) and VEGF (11, 14, 15) has been identified in tissue, serum, and urine of patients with bladder cancer and have also been associated with disease progression. We have demonstrated that expression of IL-8, a putative angiogenic factor, regulates angiogenesis, tumorigenicity, and metastasis in an orthotopic murine model for human TCC of the urinary bladder (16). Expression levels of MMP-9 (17, 18) and MMP-2 (19) have been shown to be significantly increased with higher tumor grade and
greater invasiveness of bladder cancer. Reduced levels of E-cadherin have also been shown to be associated with poor prognosis in invasive bladder cancer (11, 20–22).

When MVD (23) and MMP-2 (24) and E-cadherin (25) protein levels were individually examined in specimens of TCC-UUT by a single group of investigators, they were shown to be of limited independent value when compared with stage in determining prognosis. These correlative studies reached the inevitable conclusion that the correlation of a given gene is necessary but insufficient to account for the multistep process of metastasis. Because each discrete step in the pathogenesis of metastasis is regulated by one or more independent genes, the identification of cells with metastatic potential in heterogeneous primary human tumors requires multiparametric, multivariate analysis of gene expression (26).

We previously developed a rapid colorimetric ISH technique for the evaluation of gene expression in formalin-fixed, paraffin-embedded surgical specimens. This technique has been used to study prognosis in a multivariate analysis in lung, colon, prostate, bladder, and renal cancers (11, 27–30). We hypothesize that multivariate analysis of the expression levels of genes that regulate angiogenesis/metastasis would identify not only patients with TCC-UUT who will need adjuvant therapy but also those who are at risk for intravesical recurrence.

MATERIALS AND METHODS

Samples. We evaluated MVD by IHC and the expression of angiogenic and metastasis-related factors by ISH in 55 nephroureterectomy specimens (28 specimens from patients treated in Kochi Medical School between 1984 and 1998 and 27 specimens from patients treated in at The University of Texas M. D. Anderson Cancer Center between 1985 and 1997). The mean age of the patients (37 men and 18 women; 29 Asians, 22 Caucasians, and 4 African-Americans) was 64 years (range, 41–88 years). The follow-up period was 60.8 months (range, 4.9–227.8 months). Seventeen patients with pathologic tumor stage T1 (grade 3), T2, T3, or T4 received two courses of M-VAC chemotherapy after the surgery (Table 1). None of these patients had TCC of the bladder before their nephroureterectomy.

In Situ mRNA Hybridization Analysis. Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cDNA sequence: bFGF (CGG GAA GGC GCC GCT GCC GCC), 85.7% guanosine cytosine (GC) content (31); VEGF/VPF (TGG TGA TGT TGG ACT CTT CAG TGG GCU), 57.7% GC content (32); IL-8 (CTC CAC ACC CCT CTG CAC CC), 66% GC content (10); MMP-9 (CCG GTC CAC CTC GCT GCC GCT CGG GU), 80.0% GC content (33); MMP-2 (GGC CAC ATC TGG GTC CGC GCC), 70% GC content (28); and E-cadherin (mixture) (TGG AGC GGC CGT GAG TCT GAA CGT), 62.5% GC content and (GAC GCC GGC GCC TTC ACA GTC), 75.0% GC content (34). 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 (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 of the sequences was also confirmed by Northern blot analysis (34). A poly d(T)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 phosphoramine chemistry (Research Genetics, Huntsville, AL). The lypophilized probes were reconstituted to a stock solution at 1 g/l in 10 mM Tris (pH 7.6) and 1 mM 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 (35, 36). ISH was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 37). Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on glass slides and were deparaffinized, rehydrated, and subjected to autoclaving. Following the treatment, the sections were digested with proteinase K and subjected to autoclaving at a pH of 9.0. A 30-μl stock solution containing 100 μl of 1× standard hybridization buffer (Research Genetics) and 10 μl of a 10 μg/ml solution of biotinylated probe stock solution was diluted with probe diluent (Research Genetics). The specificity of each of the sequences was also confirmed by Northern blot analysis (34). A poly d(T)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 phosphoramine chemistry (Research Genetics, Huntsville, AL). The lypophilized probes were reconstituted to a stock solution at 1 g/l in 10 mM Tris (pH 7.6) and 1 mM 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 (35, 36). ISH was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 37). Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on glass slides and were deparaffinized, rehydrated, and subjected to autoclaving. Following the treatment, the sections were digested with proteinase K and subjected to autoclaving at a pH of 9.0. A 30-μl stock solution containing 100 μl of 1× standard hybridization buffer (Research Genetics) and 10 μl of a 10 μg/ml solution of biotinylated probe stock solution was diluted with probe diluent (Research Genetics).
silane-treated ProbeOn slides (Fisher Scientific; Refs. 35, 36). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 45 min at 45°C. The samples were then washed with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mM Tris buffer (pH 7.6), and rinsed with alkaline phosphatase enhancer for 1 min. Samples were incubated with fresh chromogen substrate if necessary to enhance a weak reaction. A red stain indicated a positive reaction. Control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of chromogen alone.

Quantification of Color Reaction. Stained sections were examined under a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip, charge-coupled device color camera (model DXC-969 MD; Sony Corp., Tokyo, Japan). The images were analyzed using the Optimas image analysis software (version 4.10; Optimas, Bothell, WA). The slides were prescreened by one investigator to determine that the range in staining intensities were captured electronically; a color bar (montage) was created, and a threshold value was set in the red, green, and blue mode of the color camera. All subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined based on its equivalence to the mean log inverse gray value multiplied by the absorbance of the selected fields. The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. The intensity of determined by comparison with the integrated absorbance of poly d(T)20. The results were presented as a number of each cell line compared with the control, which was set to 100 (11).

IHC. For immunohistochemical analysis, frozen tissue sections (8-μm thick) were fixed with cold acetone. Tissue sections (5-μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, dehydrated 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. 38). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody-peroxidase-conjugated antirat IgG (H+L) (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA). 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, San Ramon, CA), and washed three times with PBS. The slides were mounted using Universal mount (Research Genetics).

Quantification of Microvessel Density. Microvessel density was determined by light microscopy after immuno-staining of the sections with anti-CD34 antibodies according to the procedure of Weidner et al. (39). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The tissue image was recorded using a cooled charge-coupled device Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corp.). The MVD was expressed at the average value for the five highest areas identified within a single ×200 field (11).

Statistical Analysis. The average number of vessels was correlated with sex, age, race, adjuvant chemotherapy, pathological stage, and tumor grade using Student’s t test. Differences in the number of vessels and in staining intensity for bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin within the renal pelvis and ureter tumors were analyzed by regression analysis. Overall, disease-free, and recurrence-free curves were plotted by using the Kaplan-Meier method. Significant differences were calculated using the log-rank test. A value of P < 0.05 was considered significant. Significant factors related with survival or recurrence were determined by multivariate analysis. Multivariate analysis was performed using the Cox proportional hazards model with stepwise forward selection. All Ps were two sided and are presented with hazard ratios and 95% CIs.

RESULTS

Correlation among the Expression Levels of Angiogenic and Metastasis-related Factors and MVD. The expression levels of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin mRNA and CD34 was analyzed by ISH and IHC, respectively (Fig. 1). We examined the relationship among the expression of angiogenesis factors and MVD in the nephroureterectomy specimens. A significant correlation was recognized between MVD and both MMP-9 (correlative coefficient, 0.375; P < 0.01) and MMP-2 (correlative coefficient, 0.455; P < 0.01).

Stratification of Survival by Clinicopathological Characteristics. Kaplan-Meier estimates showed an overall 5-year survival of 57.8%, a 5-year cancer-specific survival of 67.8%, and 5-year intravesical recurrence-free rate of 83.5% (Fig. 2). An analysis of patient outcomes found 30 patients with no evidence of disease, 2 patients who are alive with disease (distant organ metastasis), 14 patients who died of TCC, and 9 patients who died of other diseases (Table 1). We evaluated the overall and cancer-specific survival in these patients (Kaplan-Meier estimates), subdivided according to sex, age, race, grade (G1 and G2 versus G3), pathological stage (pT1, pT2, and pT3), and adjuvant chemotherapy (Figs. 3 and 4). Although sex, age, race, and adjuvant chemotherapy were significantly correlated with overall survival, both grade and pathological stage were important prognostic factors for overall (P = 0.0008 and P = 0.005, respectively) and cancer-specific (P = 0.03 and P = 0.05, respectively) survival (Fig. 3).

Stratification of Survival by MVD and Angiogenesis and Metastasis-related Gene Expression. The prognostic significance of angiogenesis and metastasis-related gene expression and MVD was evaluated in the nephroureterectomy specimens using log-rank analysis. We chose to use the median value of each factor in the entire cohort as the cutoff point for high or low expression. We demonstrated that MVD (P < 0.0001) and
high expression levels of MMP-9 (P = 0.015 and P = 0.0194), MMP-2 (P = 0.003 and P = 0.0003), and a high M:E ratio (P = 0.0001) by ISH in the nephroureterectomy specimens predicted for decreased overall and cancer-specific survival (Fig. 5).

**Multivariate Analysis of Risk Factors Predicting for Overall and Cancer-specific Survival.** Kaplan-Meier univariate analysis revealed that grade and pathological stage and the high expression levels of MMP-9 and MMP-2, MVD, and the M:E ratio in nephroureterectomy were significant predictors for both overall and cancer-specific survival (Figs. 3–6). Multivariate analysis was performed to identify independent prognostic factors using the Cox proportional hazards model with stepwise forward selection (Tables 2 and 3). By multivariate analysis, both grade (P = 0.031) and a high M:E ratio (P = 0.016) were independent prognostic factors for overall survival (Table 2), whereas only a high M:E ratio (P = 0.031) was an independent prognostic factor for cancer-specific survival (Table 3).

**Predictors of Bladder Cancer Recurrence after Nephroureterectomy.** We assessed the prognostic value of histopathological features (grade and pT classification) of the TCC-UUT, and MVD and angiogenesis and metastasis gene expression (bFGF, VEGF, IL-8, MMP-9, MMP-2, E-cadherin, and the M:E ratio) by the TCC-UUT for predicting intravesical recurrence of TCC after nephroureterectomy. None of the histopathological factors were prognostic for recurrence bladder tumor (data not shown). Among the genes analyzed, E-cadherin expression was the only significant prognostic factor for intravesical recurrence by both univariate (P = 0.009) and multivariate (P = 0.038) analysis. The M:E ratio failed to demonstrate significance as a predictor for bladder cancer recurrence.

**DISCUSSION**

The majority of TCC-UUT and TCC of the urinary bladder share a common urothelial origin. However, differences not only in anatomy but also in biological behavior of the tumor result in differences in survival. The prognostic significance of tumor stage and grade has already been established (2–8). Epidermal growth factor receptor (40), c-erbB-2 (40), p53 (41), MMP-2 (23), and E-cadherin (24) have been associated with early progression of TCC-UUT. In the present study, we evaluated several clinicopathological factors in concert with the expression of several angiogenic/metastatic genes as prognostic factors in a multiparametric setting. We demonstrated that the M:E ratio was an independent predictor for metastasis and survival and that E-cadherin was a significant predictor for the intravesical recurrence.

Nephroureterectomy is the standard treatment for localized
TCC-UUT, whereas combination chemotherapy offers the only viable therapeutic option for prevention of initial distant metastasis or local recurrence. Although TCC is a chemosensitive tumor, most deaths from TCC-UUT are caused by metastases that resist conventional therapy. Moreover, TCC-UUT often recurs in the urinary bladder after surgery independently of conventional therapy. Therefore, it is important to identify prognostic markers that will predict which patients are likely to have disease progression, both grade and pathological stage were important prognostic factors for these patients ($P < 0.00008$ and $P < 0.005$).

The prognosis of bladder cancer has been shown to correlate directly with the expression level of several independent genes that regulate angiogenesis [bFGF (11–13), VEGF (11, 14, 15), and IL-8 (16)], invasion [type IV collagenase genes (17, 18, 19)], and metastasis [E-cadherin (20–22)]. MVD, a surrogate marker of angiogenesis, has also been shown to predict for early progression in muscle-invasive disease (11, 16, 17) in bladder cancer. In this study, we also showed that MVD was a good predictor for survival after nephroureterectomy for TCC-UUT. Overexpression of bFGF and VEGF has been identified in tissue, serum, and urine of patients with bladder cancer and has also been associated with disease progression (11–15). Previously, we reported that the expression of VEGF in biopsy specimens was correlated with the prognosis of patients with advanced bladder cancer undergoing neoadjuvant chemotherapy and cystectomy; on the other hand, bFGF was a predictor for recurrence if residual disease was present in the cystectomy specimen (11). In this study, overexpression of VEGF in TCC-UUT after nephroureterectomy had a marginal correlation with
the development of metastasis and no correlation with cancerspecific survival. IL-8 is a strong proangiogenic factor in TCC of the bladder (16), but it does not have prognostic significance in TCC-UUT.

E-cadherin was reported previously to predict for progression in TCC-UUT (20–22). Furthermore, we have reported that the M:E ratio could identify patients with TCC of advanced bladder cancer for whom chemotherapy and cystectomy will be unsuccessful (42). In the current study, the M:E ratio was a strong, independent predictor for both the development of metastases and death. This information may not only allow us to better predict who needs adjuvant therapy but may also provide targets for novel therapeutic strategies.

Patients who undergo nephroureterectomy for TCC-UUT...
remain at significant risk for developing intravesical TCC recurrences necessitating the use of routine cystoscopic surveillance. Currently, there are no predictors for bladder recurrences after nephroureterectomy. In this study, we demonstrated that decreased expression level of E-cadherin was the only independent predictor for intravesical recurrence. This knowledge may enable us to modify currently surveillance protocols to provide optimal cost-effective follow-up for intravesical recurrence and to develop novel adjuvant strategies to reduce the rate of recurrence in the bladder.

REFERENCES


