TABLE I. *Demographic data for 103 patients*

Characteristic	
Average age (yr)	72.20 ± 10.62
Sex	
Male	74 (71.8)
Female	29 (28.2)
Carcinoma site	
Upper urinary tract	12 (11.7)
Bladder	91 (88.3)
Operation	
TURBT	63 (61.2)
Total cystectomy	19 (18.4)
Nephroureterectomy ± cystectomy	14 (13.6)
Partial cystectomy or ureterectomy	2 (1.9)
Biopsy alone (no surgery)	5 (4.8)
Tumor stage	
pTis	15 (14.8)
pTa	42 (41.6)
pT1	17 (16.9)
pT2–T4	27 (26.7)
Tumor grade	
1	14 (13.6)
2	34 (33.0)
3	55 (53.4)
Carcinoma in situ	
Positive	31 (30.1)
Negative	72 (69.9)
Tumor growth pattern	
Papillary	65 (63.1)
Nodular	23 (22.3)
Flat (pTis)	15 (14.6)
Recurrence	
Initial presentation (0)	60 (58.3)
First recurrence (1)	16 (15.5)
Two or more recurrences (≥2)	27 (26.2)

Key: TURBT = transurethral resection of bladder tumor.
Data presented as number of patients, with the percentage in parentheses, unless otherwise indicated.

grade, tumor stage, and the patient's outcome.[7,9,10] These studies, however, evaluated isolated nuclei from frozen tissues[8,11] or paraffin-embedded tissue sections.[7,9,10,12] Urine-exfoliated cells would be usable in FISH, which might be able to evaluate *HER-2* alterations directly and independently.

To determine the clinical significance and applicability of *HER-2* alterations in urine-exfoliated cells, we examined the numeric aberrations of *HER-2* in samples from patients with urothelial carcinomas using FISH.

MATERIAL AND METHODS

Patients

A total of 103 patients, who were pathologically diagnosed with urothelial transitional cell carcinoma at the Akita University Medical Center and Hiraka General Hospital from 1999 to 2002, were entered in this study. The demographic data for the 103 patients are shown in Table I. Of the 103 patients, 91 had bladder carcinoma and 12 renal pelvic or ureteral carcinoma; 60 had tumors of initial presentation and 43 recurrent tumor; and 68 had papillary tumor and 23 nodular tumor. Of the 31 patients with pathologically confirmed carcinoma in situ (CIS; pTis), 15 did not have exophytic tumors (primary CIS; pTis) and 6 had tumor-associated CIS (concomitant CIS). Of the 103 patients, 98 underwent surgical intervention and 5 underwent random biopsy of the bladder alone. The tumor grade was available for all 103 patients, and the pathologic stage was available for 101 patients. The remaining 2 cases were excluded from pathologic staging because of inadequate specimens. The tumor grade and pathologic stage were determined by the general guidelines issued by the Japanese Urological Association, which are based mainly on the World Health Organization criteria.[14,15] In this study, grade 1-2 was considered low grade and grade 3 high grade, and pTa was described as superficial and pT1-T4 as invasive. The Internal Ethical Board of Akita University approved this study, and all patients provided written informed consent.

Urine Preparation for FISH

About 20 to 100 mL of a spontaneously voided urine sample was obtained before surgery and separated into two tubes, one for FISH and one for urinary cytology. Within 3 hours after sampling, the urine cells were centrifuged at 3000 rpm for 10 minutes. The cell pellet was rinsed with phosphate-buffered saline, fixed with a methanol/acetic acid 3:1 solution (Carnoy's solution), and re-suspended in about 300 μL of Carnoy's solution. An aliquot of the cell suspension was placed on a 12-well slide (Shandon, Pittsburgh, Pa) and dried overnight.

DNA Probes for FISH

FISH was performed using dual-color DNA probes that hybridize to the band region of 17q11.2-q12 (LSI *HER-2/neu* SpectrumOrange, Vysis, Downers Grove, Ill) and 17p11.1-q11.1, locus D17Z1 (Chromosome Enumeration Probe 17 [CEP17], SpectrumGreen, Vysis).

FISH of Urine-Exfoliated Cells

The cells in the 12-well slide were incubated in 2 × saline/sodium citrate (SSC) at 37°C for 10 minutes, 0.5 mg/mL pepsin at 37°C for 13 minutes, phosphate-buffered saline for 10 minutes, 1% formaldehyde for 5 minutes, and phosphate-buffered saline for 10 minutes. They were then dehydrated in ethanol, air-dried, denatured in 2 × SSC/70% formamide at 73°C for 5 minutes, and again dehydrated in ethanol. The cells were incubated with 3 μL of the denatured FISH probe mixture (probe mixture to hybridization buffer to water 1:7:2) at 37°C overnight in a humidified chamber and then rinsed in 0.4% SSC/0.3% NP-40 at 73°C for 2 minutes and 2 × SSC/0.1% NP-40 at room temperature for 1 minute. The cell nuclei were counterstained with 3 μL of 4,6-diamino-2-phenylindole.

Urinary Cytology

Urinary specimens were stained using the Papanicolaou method and evaluated by cytoscreeners at the Akita University Medical Center according to the five-grade classification. Class V was defined as positive cytology findings.

FISH of Paraffin-Embedded Tissue Sections

FISH of the tissue sections was performed as described previously.[16] In brief, 5-μm-thick tissue sections from each paraffin-embedded tissue block were put onto slides, deparaffinized, and dehydrated. The slides were microwaved in 10 mM citric acid (pH 6.0) for 10 minutes and incubated in 4 mg/mL of pepsin at 37°C for 13 minutes. Hybridization was done at 80°C for 2 minutes, 50°C for 30 minutes, and 37°C overnight in a humidified chamber with 3 μL of the denatured

TABLE II. *Number of copies for* HER-2 *and chromosome 17 centromere among 11 urine samples from patients with nonmalignant disease (control)*

Probe	Copy No.				
	0	1	2	3	≥4
HER-2	0.00 ± 0.00 (0–0)	0.55 ± 3.87 (0–4)	199.36 ± 3.84 (196–200)	0.09 ± 0.91 (0–1)	0.00 ± 0.00 (0–0)
CEP17	0.00 ± 0.00 (0–0)	0.64 ± 4.50 (0–5)	199.18 ± 4.62 (195–200)	0.18 ± 1.22 (0–1)	0.00 ± 0.00 (0–0)

Key: CEP17 = Chromosome Enumeration Probe 17; No. = number.
Data presented as mean ± 3 standard deviations, with the range in parentheses.

FISH probe mixture. The slides were washed in 1.5 M urea/0.1 × SCC at 45°C for 10 minutes three times. Finally, the cell nuclei were stained with 3 μL of 4,6-diamino-2-phenylindole.

Scoring of FISH Signals

FISH signals were evaluated under an Olympus BX10 microscope (Olympus, Tokyo, Japan) equipped with filters for Texas Red, FITC, and 4,6-diamino-2-phenylindole and a digital camera (DXM1200, Nikon, Tokyo, Japan). The normal value study was performed by enumerating the *HER-2* and CEP17 signals in urine-exfoliated cells from 11 patients who proved not to have any pathologic findings in the urinary tract. Of these age-matched 11 patients, 6 were men and 5 were women; their disease condition was benign prostatic hyperplasia in 4, chronic renal failure in 4, urolithiasis in 2, and erectile dysfunction in 1. On the basis of the normal value study (Table II), the numeric aberration of the FISH signals in the urine-exfoliated cells was categorized as a gain of chromosome 17 (G-17) and a relative increase in *HER-2* (RI-HER2). The RI-HER2 category, which contains overrepresentation (eg, duplication, triplication) and amplification of *HER-2*, was defined as having at least four nuclei with a *HER-2*/CEP17 ratio of 2.0 or more and two or more signals for CEP17. The G-17 category was defined as having at least four nuclei with three or more signals for CEP17. The samples with RI-HER2 and/or G-17 were described as FISH positive. Up to 100 nuclei of cytologically atypical features, such as nuclear enlargement or irregular nuclear contour for the urine specimens and pathologic carcinoma cells for the tissue sections, were scored in each case. When at least four nuclei were classified as RI-HER2 or G-17 positive, no more cells were counted. However, in cases that were FISH negative, we routinely counted at least 100 cells to exclude false-negative results.

In the evaluation of FISH of the tissue sections, more than 10% of the nuclei with three or more same-color signals were required for the categorical classification of a numeric aberration and thus to be considered FISH positive.

Statistical Analysis

Fisher's exact test or the chi-square test was used to examine the relationship between RI-HER2, G-17, FISH-positive findings, and the cytologic parameters. A P value less than 0.05 was considered statistically significant.

RESULTS

The FISH and cytologic examinations of the exfoliated cells were performed for all 103 patients. The average number of counted cells per FISH specimen was 50.0 (range 8 to 100). The average *HER-2*/CEP17 ratio in the RI-HER2 and G-17 categories was 2.13 and 1.13, with an average *HER-2* signal number per nucleus of 6.04 and 4.39, respectively. The frequencies of RI-HER2 and G-17 are listed in Table III.

RI-HER2 and G-17 was found in 23 (22.3%) and 46 (44.6%) of 103 urine specimens, respectively. RI-HER2 tended to be more frequently found in recurrent tumors than in tumors of initial presentation (30.2% versus 16.6%, $P = 0.149$). RI-HER2 was significantly more frequent in the tumors with two or more recurrences compared with those of initial presentation or a first recurrence (40.7% versus 15.8%, $P = 0.010$) and in those with CIS than in those without CIS (35.4% versus 15.9%, $P = 0.029$; Table III). However, RI-HER2 was not associated with tumor grade, stage, or growth pattern. G-17 was significantly more frequent in high-grade than low-grade (69.1% versus 16.7%, $P = 0.032$), invasive than superficial (63.6% versus 14.3%, $P < 0.001$), CIS-positive than CIS-negative (77.1% versus 29.0%, $P < 0.001$), and nodular than papillary (65.2% versus 30.8%) tumors ($P = 0.004$; Table III). However, G-17 was not associated with the number of recurrences.

When "FISH positive" was defined as the presence of RI-HER2 and/or G-17, FISH was positive in 59 (57.3%) of the 103 cases. The positive rate was significantly greater in the high-grade tumors (78.2% for G3 versus 31.2% for G1-G2, $P < 0.001$), invasive tumors (70.5% for pT1-T4 versus 33.3% for pTa, $P = 0.001$), and in the presence of CIS (90.3% for tumors with CIS versus 42.0% for those without CIS, $P = 0.010$; Table III). When compared with urinary cytology, the positive results tended to be more frequently found using FISH than using cytology (57.3% versus 45.6%, $P = 0.094$; Table III).

FISH of the corresponding tissue sections was successfully performed in 45 cases. When the positive or negative FISH category was applied and compared between the results from the urine-exfoliated cells and those from the tissue sections, 36 (80.0%) of the 45 cases had the same result.

COMMENT

We selected *HER-2* as a genetic marker of urothelial carcinoma cells because this gene is potentially

TABLE III. *Cytology and FISH results of urine-exfoliated cells and tumor characteristics*

Tumor Characteristic	n	RI-HER2 (%)	G-17 (%)	RI-HER2 and/or G-17 (%) (FISH Positive)	Cytology (%)	*P* Value (FISH Positive vs. Cytology)
Total	103	23 (22.3)	46 (44.6)	59 (57.3)	47 (45.6)	0.094
Grade						
1	14	3 (21.4)	1 (7.1)	4 (28.6)	0 (0.0)	0.256
2	34	6 (17.6)	7 (20.6)	12 (35.3)	11 (32.3)	
3	55	14 (25.5)	38 (69.1)	43 (78.2)	36 (65.5)	0.138
P value (G1–G2 vs. G3)		0.482	0.032	<0.001	<0.001	
Stage*						
pTa	42	8 (19.0)	6 (14.3)	14 (33.3)	6 (14.3)	0.173
pT1–T4	44	9 (20.5)	28 (63.6)	31 (70.5)	28 (63.6)	0.496
P value (pTa vs. pT1–T4)		0.999	<0.001	0.001	<0.001	
Carcinoma in situ*						
Positive	31	11 (35.4)	24 (77.1)	28 (90.3)	24 (77.4)	0.167
Negative	69	11 (15.9)	20 (29.0)	29 (42.0)	22 (31.9)	0.217
P value (positive vs. negative)		0.029	<0.001	0.010	<0.001	
Growth pattern						
Papillary	65	14 (21.5)	20 (30.8)	31 (47.7)	21 (32.3)	0.073
Nodular	23	4 (17.3)	15 (65.2)	16 (69.6)	14 (60.9)	0.400
P value (papillary vs. nodular)		0.668	0.004	0.071	0.016	
Recurrence						
Initial presentation (0)	60	10 (16.6)	31 (51.7)	36 (60.0)	33 (55.0)	0.416
First recurrence (1)	16	2 (12.5)	6 (37.5)	7 (43.8)	5 (31.2)	
Two or more recurrences (≥2)	27	11 (40.7)	9 (33.3)	16 (59.3)	9 (33.3)	0.056
P value (0–1 vs. ≥2)		0.010	0.168	0.810	0.135	

KEY: RI-HER2 = relative increase of HER-2 *gene; G-17 = gain of chromosome 17; FISH = fluorescence in situ hybridization.*
** Statistical analysis performed for 101 patients.*

implicated in urothelial carcinogenesis.[6–10] Using FISH, previous studies have demonstrated *HER-2* "amplification" in 3.4% to 7.0% of urothelial carcinomas and a "gain or relative increase" in 8.5% to 41.4%.[11–13] However, none of these studies evaluated the copy number of *HER-2* and chromosome 17 on urine-exfoliated cells using FISH. In the present study, an RI-HER2 was found in 22.3% of the cases. Although the FISH criteria were considerably different among the individual studies, the frequency of the RI-HER2 for the urine-exfoliated cells as shown by our study was comparable to that for the tissue specimens reported by the others.[11–13]

In 36 (80%) of 45 cases, the FISH findings were the same in the urine-exfoliated cells as in the tissue sections. This may indicate the validity of using urine-exfoliated cells, as well as the good reproducibility of the FISH analysis. Of the 9 cases in which the results were not the same, 8 were low-stage (pTa-T1) tumors, and 7 of the 9 cases were positive using the urine samples and negative using paraffin-embedded tissue, suggesting a greater sensitivity for the urine-exfoliated samples. The discrepancy might have stemmed from a low tumor burden, poor exfoliation of tumor cells, genetic heterogeneity among different cell clusters, or combinations thereof.[17] The present findings suggested that the abnormal genetic characteristics of urothelial tumors can be determined in most cases by examining urine-exfoliated cells.

Of the alterations detected with the FISH analysis using exfoliated cells, RI-HER2 tended to be more frequently found in recurrent tumors than in tumors of initial presentation and was significantly more frequent in the tumors with two or more recurrences. Interestingly, although RI-HER2 was infrequently found in patients with grade 1, all three grade 1 tumors with RI-HER2 were those with two or more recurrences. An RI-HER2 in urine specimens, therefore, may reflect a distinct potential for recurrence even in low-grade tumors.

In this study, both RI-HER2 and G-17 were more frequently observed in cases with CIS than in those without. However, the presence of RI-HER2 did not correlate with tumor grade or stage, and G-17 correlated significantly. In partial support of our findings, Ohta *et al.*[11] reported that no correlation was found between the relative increase in the *HER-2* copy number and tumor stage but that the gain in chromosome 17 correlated more significantly with tumor grade and stage. Sauter *et al.*[12] also showed that *HER-2* amplification, found more frequently in pT2-T4 tumors than in pTa-T1, did not correlate with tumor grade and was found only in tumors with aneusomy of chromosome 17. In

our study, G-17 was more strongly associated with the presence of CIS than was RI-HER2 (Table III). When grade 3 tumors with CIS were compared with grade 3 tumors without CIS, no difference in the RI-HER2 frequency was found ($P = 0.999$). Also, when tumors without CIS and those with primary CIS (pTis) were compared, no statistically significant difference was found in the RI-HER2 frequency ($P = 0.160$). Therefore, our statistically significant association between RI-HER2 and the presence of CIS might have derived from the strong association between G-17 and CIS. Furthermore, the results of our study and others[11,12] may indicate that other genes on chromosome 17 play an additional significant role in progression, although *HER-2* may be partly involved in the progression of urothelial cancer.

A recent study using Urovision, which includes chromosome 17 centromere probes, showed that numeric alterations of chromosome 17 correlated significantly with tumor recurrence.[18] In the present study, although the high positive rate of FISH was obviously derived from chromosome 17 centromere analysis alone, the addition of the relative increase in the number of *HER-2* copies increased the positive rate from 77% to 90% in the presence of CIS (Table III). Furthermore, although G-17 was not associated with the rate of recurrence, the RI-HER2 may be significantly associated with multiple recurrences (Table III). This suggests that the *HER-2* increase associated with a chromosome 17 gain may reflect an accumulation of genetic alterations involving the development of CIS, which has a high probability of progressing to invasive carcinoma.[19] Furthermore, RI-HER2, which presumably leads to overexpression of HER-2,[12] may be biologically related to recurrence in the heterotopic urothelium independent of a gain of chromosome 17.

CONCLUSIONS

Numeric alterations of the chromosome 17 centromere in urine-exfoliated cells detected by FISH may reflect the malignant potential of urothelial carcinoma. In addition, the relative increase of the *HER-2* copy number may be associated with the number of recurrences and the presence of CIS. Periodic FISH analysis of urine samples could be used to determine whether the *HER-2* alterations occurred sequentially or were enhanced in individual cases.

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Original Article

Impact of adjuvant systemic chemotherapy on postoperative survival in patients with high-risk urothelial cancer

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Abstract

Background: The objective of this study was to retrospectively investigate the effectiveness of adjuvant combination chemotherapy for locally advanced urothelial cancer.

Methods: Between 1987 and 1998, 56 patients with locally advanced bladder ($n = 27$) or upper urinary tract ($n = 29$) cancer (pathological stage T3, T4 or N1, N2 and M0) were treated by radical cystectomy or radical nephroureterectomy and regional lymphadenectomy. Thirty-one patients had lymph node-positive disease and 25 patients did not. Twenty patients underwent adjuvant chemotherapy and 36 patients were observed after surgery. Cox proportional hazards models were used to determine the impact of numerous clinicopathological findings on survival. A subgroup analysis of patients with lymph node-positive disease was conducted to evaluate disease-free survival and overall survival rates.

Results: In this series, the median follow-up period was 39 months (range, 4–163) after surgery. Disease-free and overall survival rates of all 56 patients were 45% and 58%, respectively, at 3 years. Only lymph node status was significantly associated with disease-free and overall survival in the multivariate analyses. In a subgroup analysis of patients with lymph node-positive disease, 16 patients who underwent adjuvant chemotherapy had superior disease-free survival compared to 15 patients with no adjuvant chemotherapy ($P = 0.0376$).

Conclusion: These findings show that the prognosis of advanced urothelial cancer is significantly associated with nodal status. Furthermore, adjuvant combination chemotherapy has a positive impact on survival in patients with lymph node-positive disease.

Key words adjuvant chemotherapy, pathological positive node, urothelial cancer.

Introduction

The prognosis of patients with locally advanced urothelial cancer is poor. The aggressive nature of this cancer, and the associated high morbidity and mortality, warrant appropriate therapeutic intervention. Although the optimal treatment for the individual patient with advanced urothelial cancer is still unclear, radical surgery (e.g. radical cystectomy for locally advanced bladder cancer or radical nephroureterectomy for advanced upper urinary tract cancer) is considered to be the standard treatment option for selected patients with a long life expectancy. In fact, the 5-year overall survival rate in patients who undergo radical surgery is approximately 50–60%. However, clinical outcomes of these patients are not uniform. To improve the prognosis of these urothelial cancer patients, some additional treatment along with radical surgery is needed. Since the report by Sternberg *et al.*, systemic chemotherapy consisting of methotrexate, vinblastine, adriamycin and cisplatin (M-VAC), has been shown to be effective for advanced urothelial cancer.[1] Several reports have described the

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positive clinical impact of adjuvant systemic chemotherapy after radical surgery.[2–4] However, it is still unknown whether adjuvant systemic chemotherapy improves the prognosis of locally advanced urothelial cancer. Clearly, if it does, we have to make clear feasible selection criteria to determine which patients should undergo adjuvant chemotherapy.

This retrospective analysis of our experience with adjuvant systemic chemotherapy was conducted using the medical records of patients who underwent radical surgery for locally advanced and/or node-positive urothelial cancer of the bladder and upper urinary tract. Our aims were to gain further insight into the positive survival impact of adjuvant systemic chemotherapy on this disease and to guide selection of patients who might benefit most from this multimodal approach.

Patients and methods

Of the patients with advanced urothelial cancer who underwent either radical cystectomy and bilateral pelvic lymphadenectomy or radical nephroureterectomy and lymphoadenectomy at our institute from 1987 to 1998, 56 fulfilled the eligibility criteria for the present study. The eligibility criteria included: (i) pathological stage T3 or more, based on the 1997 tumor, nodes, and metastasis (TNM) system for histopathological tumor staging of bladder and upper urinary tract cancer; (ii) pathologically defined nodal disease regardless of the pathological stage of the primary tumor; (iii) lack of evidence of macroscopic residual disease and distant metastases; and (iv) good performance status (PS 0 or 1). The routine lymphadenectomy included the bilateral obturator and iliac lymph nodes in patients with cancer of the bladder or lower part of the ureter, and the unilateral retroperitoneal lymph nodes in patients with cancer of the renal pelvic or upper and middle part of the ureter. We excluded patients with pathological stage T2 or less who had both bladder cancer and upper urinary tract cancer without nodal metastasis, patients with distant metastasis, patients who received neoadjuvant chemotherapy or preoperative radiation therapy, and patients with renal-, hepatic- or myelo-dysfunction.

Although the indications were not defined precisely, the decision to administer adjuvant chemotherapy to these high-risk patients was based on patient acceptance after informed consent. Adjuvant chemotherapy consisted of methotrexate, vinblastine, adriamycin and cisplatin (M-VAC) or methotrexate, epirubicin and cisplatin (MEC).[1,5] Follow-up examinations were performed four times in the first 2 years, semiannually for the next 3 years, and annually thereafter, or as clinically indicated. Follow-up examinations of both bladder cancer patients and upper urinary tract cancer patients included physical examination with laboratory tests, chest X-ray, computed tomography (CT) of the abdomen and the pelvis, and cytological examination of urine. Bone scintigraphy and chest CT were performed if indicated clinically. For the patients with upper urinary tract cancer, cystoscopy was performed in addition to the other follow-up examinations at these visits. Although the decision for additional treatment was open when a tumor recurred, most affected patients received several courses of systemic chemotherapy (M-VAC or MEC).

Disease-free survival and overall survival times were recorded from the date of radical surgery to the date of documented recurrence or death, as were all causes of death. Patients who had not relapsed, or were alive with/without cancer were censored. Cox proportional hazards models were used to determine the prognostic significance of numerous clinical and pathological findings using disease-free survival and overall survival as the end points. Significant tests were based on the test score for a Cox proportional model. Stepwise variable selection was used, with a P-value of 0.05 or less required to enter the model. Survival curves were obtained using the Kaplan–Meier method and were compared with the use of the log-rank test. A P-value less than 0.05 was considered statistically significant and all P-values were two-sided.

Results

Patient and tumor characteristics are detailed in Table 1. The mean age at operation was 65.1 years (median, 66 years; range, 41–85 years). Forty-six of the patients were men and 10 were women. The primary cancer site was the urinary bladder in 27 patients and the upper urinary tract in 29 (renal pelvis in eight, ureter in 16, and both in five patients). Performance status was 0 or 1 in these patients. The mean follow-up period was 49.4 months (median, 39.5 months; range, 4–163 months). Histological examination of these patients demonstrated urothelial (transitional cell) carcinoma in 50 patients, urothelial carcinoma plus adenocarcinoma in three, urothelial carcinoma plus squamous cell carcinoma in one, and urothelial carcinoma plus adenocarcinoma plus squamous cell carcinoma in two. The pathological grade of operated specimens was Grade 2 in 17 patients and Grade 3 in 39 patients. Although the pathological stage (pT) of bladder cancer was T1 in one patient, T2 in two, T3a in one, T3b in 16 and T4 in seven patients, that of upper urinary tract cancer was T1 in

one, T2 in one, T3 in 22 and T4 in five patients. Thirty-one patients had node-positive disease (N1 in 12 and N2 or more in 19) and 25 patients had node-negative disease. Twenty patients underwent adjuvant chemotherapy after surgery and the remaining 36 patients did not. M-VAC was given to 17 patients (1–3 cycles, median two cycles) and MEC was given to three patients (three cycles in each patient). The median interval from surgery to adjuvant chemotherapy was 1 month (range, 1–3 months).

Disease-free and overall survival rates of all 56 patients were, respectively, 45% and 58% at 3 years, and 41% and 46% at 5 years.

Table 1 Baseline patient and tumor feature

	n
Gender	
Male	46
Female	10
Age (years)	
Range	41–85
Median	66
Primary site	
Upper urinary tract	29
Bladder	27
Tumor histology	
Pure urothelial cancer	50
Others	6
Tumor grade	
G2	17
G3	39
Pathological stage	
pT1†	2
pT2†	3
pT3	39
pT4	12
Nodal status	
N0	25
N1	12
N2	19

†These cases had nodal disease.

Gender, age, primary site, tumor histology, pathological grade, lymph node status, and adjuvant chemotherapy were included in the multivariate analysis. The most significant risk factor that predicted both disease-free and overall survival was lymph node status. Adjuvant chemotherapy reached a marginally significant level for disease-free survival ($P = 0.051$) (Table 2).

In 31 patients with lymph node-positive disease, which was the most significant risk factor, we analyzed whether adjuvant chemotherapy had a positive survival benefit or not. Baseline characteristics of the 31 patients are presented in Table 3. There was a similar distribution of gender, age, P and N stage and tumor histology in both groups. Disease-free and overall survival rates in 16 patients who underwent adjuvant chemotherapy were 56% and 63% at 3 years, respectively, whereas disease-free and overall survival rates for 15 patients with no adjuvant chemotherapy were 10% and 27%, respectively (Fig. 1). A significant difference in disease-free survival was shown between patients with adjuvant chemotherapy and those without it ($P = 0.0376$). There was a slight, non-significant, difference in overall survival between the two groups.

Discussion

In the present study, we showed that the prognosis of advanced urothelial cancer was significantly associated with nodal status in patients with these tumors. Adjuvant chemotherapy did not affect the survival of all patients together. However, in subgroup analysis of patients with node-positive disease, adjuvant chemotherapy had a positive survival benefit.

Locally advanced bladder cancer is defined as muscle-invasive tumor growth beyond the bladder wall and/or involving regional lymph nodes, which includes pelvic lymph nodes below the aortic bifurcation (tumor stages pT3a, pT3b, pT4 and/or pN+ and M0, 1997 TNM classification). Locally advanced upper urinary tract

Table 2 Multivariate analysis of factors associated with survival in 56 patients

Factors	Variables	Disease-free survival		Overall survival	
		P-value	Risk ratio (95% CI)	*P*-value	Risk ratio (95% CI)
Gender	Male/female	0.068	0.437 (0.180–1.063)	0.335	0.626 (0.242–1.620)
Age	≥65/<65	0.192	1.685 (0.770–3.691)	0.059	2.281 (0.968–5.368)
Primary site	UUT/B	0.239	0.625 (0.286–1.366)	0.170	0.577 (0.263–1.266)
Tumor histology	Others/pure UC	0.104	0.288 (0.064–1.291)	0.206	0.381 (0.085–1.700)
Tumor grade	G3/G2	0.528	1.295 (0.581–2.885)	0.992	1.044 (0.435–2.510)
Nodal disease	N+/N0	0.015	2.843 (1.226–6.592)	0.012	3.077 (1.281–7.390)
Adjuvant chemotherapy	No/Yes	0.051	2.367 (0.996–5.631)	0.257	1.660 (0.691–3.987)

B, bladder; CI, confidence interval; UC, urothelial cancer; UUT, upper urinary tract.

Table 3 Baseline characteristics of patients with lymph-node positive disease

	Adjuvant chemotherapy ($n = 16$)	Surgery alone ($n = 15$)	*P*-value
Gender			
Male	14	13	
Female	2	2	>0.9999
Age (years)			
Range	41–78	47–84	
Median	56	67	0.1198
Primary site			
UUT	8	5	
B	8	10	0.4725
Tumor histology			
Pure UC	14	13	
Others	2	2	>0.9999
Tumor grade			
G2	2	4	
G3	14	11	0.3944
Pathological stage			
pT1	0	2	
pT2	2	1	
pT3	11	8	
pT4	3	4	0.307
Nodal status			
N1	6	6	
N2	10	9	>0.9999

B, bladder; UC, urothelial cancer; UUT, upper urinary tract.

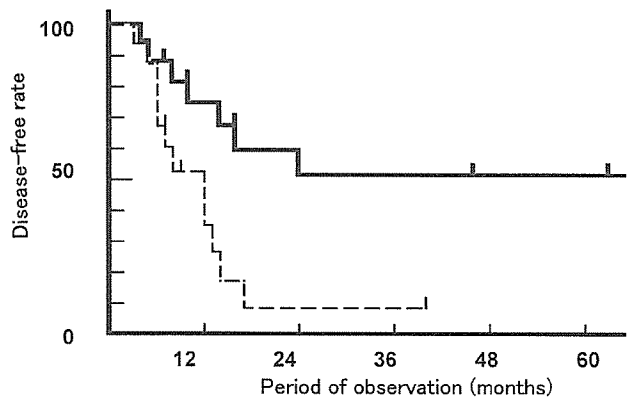

Fig. 1 Disease-free rate curves for 16 patients who underwent adjuvant chemotherapy (–, $n = 16$) and for 15 patients who did not undergo adjuvant chemotherapy (--, $n = 15$) in patients with lymph node metastases ($P = 0.0376$).

cancer is similarly defined as muscle-invasive tumor growth beyond the renal pelvic or ureteral wall and/or regional lymph node metastases (tumor stages pT3, pT4 and/or N+ and M0, 1997 TNM classification).[6] The 5-year relative survival rate for patients with locally advanced bladder cancer has been known to be approximately 50%, and that for patients with advanced upper urinary tract cancer has been known to be at less than 40%.

After the efficacy of combination chemotherapy for metastatic urothelial cancer using MVAC was first described in 1985,[1] several cisplatin-based systemic regimens have been investigated as adjunctive treatments after therapy for locally advanced urothelial cancer by radical surgery.

The German genitourinary oncology group compared cystectomy alone and cystectomy followed by three adjuvant cycles of MVEC (methotrexate, vinblastine, epirubicin and cisplatin) combination therapy in patients with locally advanced bladder cancer.[7] This study demonstrated that there was no disease-specific survival difference, according to the log-rank test. Subgroup analyses also revealed no significant differences for patients with stage pT3pN0, stage pN1 and stage pN2 cancer.[7] Lerner *et al.* retrospectively analyzed long-term progression and survival rates in patients who underwent pelvic lymphadenectomy with en bloc radical cystectomy for bladder cancer and had pathologically proved nodal metastases.[8] Their study showed that there was no significant difference in survival or the interval to progression among patients who received adjuvant chemotherapy compared to those treated with surgery alone.[8]

Skinner and co-workers randomly compared cystectomy plus four cycles of adjuvant chemotherapy (CISCA: cisplatin, cyclophosphamide and adriamycin) and radical cystectomy alone in locally advanced transitional cell carcinoma.[2] The trial demonstrated a significant disease-free survival advantage for the adjuvant treatment arm in the 5 years after cystectomy, whereas overall survival was not significantly prolonged. In a subgroup analysis of patients with only one positive lymph node, patients in the adjuvant treatment arm had superior disease-free and overall survival rates compared to patients in the cystectomy alone arm.[2] Freiha and colleagues compared cystectomy alone and cystectomy followed by adjuvant CMV (cisplatin, methotrexate, vinblastine) combination chemotherapy in patients with locally advanced bladder cancer.[4] The study reported a significant difference in disease-free survival in favor of patients receiving adjuvant chemotherapy, whereas overall survival was not significantly different. Another randomized study by Stockle and colleagues reported adjuvant MVAC or MVEC in 26 of 49 patients with locally advanced bladder cancer.[3] A large, disease-free survival difference at 3.5 years in favor of the 26 patients receiving adjuvant treatment was noted. This study also noted a distinctly improved survival rate for lymph node-positive patients. Of the 13 patients with lymph node-positive status who underwent surgery without adjuvant treatment, 12 developed progressive disease (92%), whereas only three of 11 patients with adjuvant combination therapy developed progressive disease (27%). All three studies have been criticized for their small patient numbers and statistical shortcomings. However, Stockle reported that the addition of 117 non-randomized patients to the original 49 randomized patients (80 patients with three cycles of adjuvant MVAC or MVEC, and 86 patients with cystectomy alone) yielded a highly significant difference in disease-free survival for patients who received adjuvant treatment. This study also demonstrated that adjuvant chemotherapy achieved the highest therapeutic benefit in patients suffering from pN1 disease.[9]

Adjuvant chemotherapy has been a matter of a controversial and serious debate. Improvement of tumor-free and overall survival rates by adjuvant chemotherapy in patients with locally advanced urothelial cancer strongly depends on meticulous selection by the tumor stage. Bono *et al.* randomized only lymph node-negative patients for four courses of adjuvant cisplatin/methotrexate versus cystectomy alone and noted no significant difference in disease-free survival.[10] In the present study, similar to the data reported by Skinner *et al.* and Stockle *et al.*, adjuvant chemotherapy showed a positive impact on disease-free survival in patients with lymph node-positive disease.

In conclusion, our data suggest that adjuvant chemotherapy with MVAC/MEC after radical cystectomy or nephroureterectomy improves disease-free survival rates in patients with pathological lymph node-positive urothelial cancer.

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Original Article

Decreased expression of *KAI1* metastasis suppressor gene is a recurrence predictor in primary pTa and pT1 urothelial bladder carcinoma

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Abstract

Objective: To examine the expression of the *KAI1* metastasis suppressor gene and to evaluate its relationship with tumor recurrence in primary pTa and pT1 urothelial bladder carcinoma.

Methods: Samples were obtained from 87 patients after transurethral resection (TUR). Tumor stage and grade were reviewed in 33 patients with pTa and in 54 patients with pT1, with a mean follow-up time of 47.4 ± 30.1 months. The *KAI1* protein immunohistochemical assay was performed. Prognosis was analyzed using the Kaplan–Meier method and Cox's proportional hazards model. Correlation between *KAI1* expression and recurrence according to each clinicopathological factor was comparatively evaluated using the chi-squared test.

Results: Decreased expression of *KAI1* protein failed to reach statistical significance for stage ($P = 0.25$) or morphology of tumor stem ($P = 0.19$), but it was significantly related to tumor size ($P = 0.016$). The recurrence-free 5-year survival rates of the group with decreased *KAI1* expression was 69.7%, which was significantly higher than the 22.2% for the *KAI1*-positive group ($P < 0.0001$). In univariate and multivariate analyses, decreased expression of *KAI1* protein, stage pT1, tumor size >3 cm and sessile tumors were independent prognosis factors of recurrence. Despite the lower recurrence rate expected by considering only the clinicopathological factors, decreased *KAI1* expression was able to identify the group with a high risk of recurrence.

Conclusions: Downregulated *KAI1* expression in bladder tumors tends to relate to stage and morphology of the tumor stem and was significantly correlated to tumor size. Decreased expression of *KAI1* was associated with the degree of invasiveness and progression of the cancer and was an independent prognostic factor of recurrence in primary pTa and pT1 urothelial bladder carcinoma.

Key words clinicopathological factors, *KAI1* expression, pTa and pT1 urothelial bladder carcinoma, recurrence.

Introduction

Bladder carcinoma is the second most common malignancy encountered by urologists; approximately 70–80% of patients with bladder carcinoma present with pTa and pT1 urothelial bladder carcinomas,[1] which can be managed with transurethral resection (TUR) alone or with intravesical therapy. However, recurrence occurs in more than half of all cases of pTa and pT1 urothelial bladder carcinoma within 5 years after TUR, and the majority of patients who progress eventually succumb to their disease.[2] Tumor recurrence is the most important problem in the treatment of pTa and pT1 urothelial bladder carcinoma. Different causes have been proposed to explain why tumor recurrence may appear, including the persistence of residual tumor due to incomplete resection, tumor may be present but not visible at the time of TUR, cancer cells from the primary tumor may

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have transplanted to other parts of the urothelium and/or it can be attributable to the continual insult of the carcinogenic process.[3] Several recurrence risk factors have been reported, such as grade, stage, tumor morphology, tumor size and tumor number.[4–6] Among these risk factors, the number of tumors and tumor size are considered the most important prognostic factors related to recurrence.[7,8] Access to molecular biology analysis has permitted as to understand those factors involved in bladder cancer, such as chromosome 9,[9] the *p53* suppressor gene[10] and *Ki-67* antigen.[11] Also, the detection of abnormal genes has become possible using clinicopathology. Recent studies have shown that multifocal bladder cancer and tumor recurrence have a monoclonal origin and suggest that tumor cells have either an intraepithelial spread or are transplanted directly to the bladder mucosa after TUR.[12–15]

The *KAI1* metastasis suppressor gene has been examined in many cancers, including bladder carcinoma specimens and cell lines,[16–20] and its expression has been related to tumor invasiveness, metastases, growth of metastatic tumors,[21] cell motility and adhesion.[22–24] It has been reported that decreased *KAI1* expression appears at an early phase of tumor progression[25–28] and is a predictor of recurrence in breast cancer and small cell lung cancer.[20,29] Thus, whether examination of *KAI1* gene expression could contribute to predicting the recurrence of cancer in primary pTa and pT1 bladder urothelial carcinoma is unknown. Using immunohistochemical staining, we examined *KAI1* gene expression, evaluated the correlation between *KAI1* expression and clinicopathological factors and also studied its effect on tumor recurrence.

Methods

Clinical characteristics of patients

Tumor specimens were obtained from 87 patients (72 males and 15 females) with primary pTa and pT1 bladder urothelial (transitional cell) carcinoma who were treated by TUR of the bladder tumor at Mie University Hospital. The mean age of patients was 64 ± 13 years (range 34–91 years). Patients were followed up for a mean of 47.4 ± 30.1 months (range 4–78 months). After TUR, five patients underwent intravesical Bacillus Calmette–Guérin (BCG) instillation treatment and 77 patients received intravesical anticancer drug (mitomycin C and cytosine arabinoside or adriamycin) treatment. Tumor stage and grade were reviewed and determined according to the *General Rule for Clinical and Pathologic Studies on Bladder Cancer* of the Japanese Urological Association and the Japanese Society of Pathology,[30] which is based on the TNM classification[31] and World Health Organization criteria.[32] Recurrent time was calculated from the time of the initial TUR-bladder tumor (Bt) until the first tumor recurrence had been confirmed by cystoscopy. These patients did not show progress defined as the presence of muscle invasion, metastasis.[33] The clinicopathological characteristics of the patients are given in Table 1.

Immunohistochemical assays

Immunohistochemical staining was performed using the streptavidin–biotin (SAB) method. Tissue sections were obtained from formalin-fixed, paraffin-embedded samples and mounted on aminopropyltriethoxysilane-coated glass slides (DAKO, Kyoto, Japan). Tissue sections (3 μm) were deparaffinized and rehydrated. Endogenous peroxide was blocked by exposure to 0.3% hydrogen peroxide for 15 min and washed twice with phosphate-buffered saline (PBS). Subsequently, sections were placed in citric acid buffer (10 mmol/L, pH 6.0), followed by heating in a microwave oven (500 W) for five successive periods of 3 min, necessary for antigen activation. After heating, sections were allowed to cool in citrate buffer for 20 min at room temperature and were washed four times with PBS. To prevent non-specific binding, sections were blocked with a super-block (ScyTek stain kit; ScyTek Laboratories, West Logan, UT, USA) for 8 min at room temperature and washed with PBS. Sections were incubated overnight at 4°C with polyclonal serum to *KAI1* (C-16) from Santa Cruz Biotechnology (Santa Cruz, CA, USA; dilution 1 : 100), as described previously,[19] and washed four times with PBS. Subsequently, samples were incubated with biotinylated link antibody (ScyTek stain kit; ScyTek Laboratories) for 20 min at room temperature and washed four times with PBS. Sections were incubated with streptavidin/horseradish peroxidase (HRP) label (ScyTek stain kit; ScyTek Laboratories) for 20 min at room temperature and washed four times with PBS. After the above reaction ended, the peroxidase reaction was performed using a solution of 3,3′-diaminobenzidine (ScyTek stain kit; ScyTek Laboratories) as the chromogen substrate for 5 min. Finally, slides were lightly counterstained with hematoxylin and then observed.

In each specimen, endothelial and lymphocyte cells in tumor stroma were used as internal positive controls[27] and a normal serum at the same concentration of the primary antibody was used as a negative control. When staining intensity on the cell membrane and cytoplasm appeared to be similar to that of endothelial and

Table 1 Clinical characteristics of patients and association between decreased *KAI1* expression and clinicopathological factors in primary pTa and pT1 urothelial bladder carcinomas

Prognostic factors	Total (%)	Expression of *KAI1* (%)		*P*-value
		Positive	Decreased	
Histology				
Grade 1	23 (26.4)	16 (70)	7 (30)	0.78
Grade 2	56 (64.4)	33 (59)	23 (41)	
Grade 3	8 (9.2)	5 (62)	3 (38)	
Pathology				
Stage pTa	33 (37.9)	23 (70)	10 (30)	0.25
Stage pT1	54 (62.1)	31 (57)	23 (43)	
Morphology				
Papillary	75 (86.2)	48 (64)	27 (36)	0.35
Non-Papillary	12 (13.8)	6 (50)	6 (50)	
Pedunculated	60 (69.0)	40 (67)	20 (33)	0.19
Sessile	27 (31.0)	14 (52)	13 (48)	
Size (cm)				
<1	21 (24.1)	15 (71)	6 (29)	0.016
1–3	49 (56.3)	33 (67)	16 (33)	
>3	12 (13.8)	6 (50)	6 (50)	
Unidentified	5 (5.8)	0	5 (100)	
No. tumors				
1	46 (52.9)	29 (63)	17 (37)	0.12
2–4	25 (28.7)	14 (56)	11 (44)	
>5	10 (11.5)	9 (90)	1 (10)	
Unidentified	6 (6.9)	2 (33)	4 (67)	

lymphocyte cells, it was estimated to be strong positive (2+) or moderately positive (1+). When staining intensity appeared to be similar to the negative control, it was estimated to be negative (–), if staining intensity appeared to be between positive (1+) and negative (–), it was estimated to have a weaker staining (+/–).

The staining pattern of *KAI1* expression was classified as: (i) moderate (or positive) when tumor cell membranes and cytoplasm showed uniform and moderate staining or when tumor tissue staining was not uniform in intensity but more than 50% of tumor cells were stained; (ii) decreased if 5–50% of tumor cells were stained; and (iii) negative if less than 5% of tumor cells were stained for *KAI1* within the tumor tissue. As a staining contrast for the expression of *KAI1* protein in pTa and pT1 urothelial bladder carcinoma, tissue sections from 17 cases with non-malignant 'normal' adjacent urothelial and from 32 patients who underwent cystectomy with muscle-invasive bladder cancers (stage pT2–4) were evaluated at the same time.

Statistical analysis

Correlation between *KAI1* expression and clinicopathological factors (grade, stage, morphology, tumor size and number of tumors) and an association between decreased expression of *KAI1* protein and tumor recurrence according to each clinicopathological factor were evaluated comparatively by the chi-squared test. The recurrence rate for decreased *KAI1* expression and each clinicopathological factor was calculated based on the Kaplan–Meier method and log-rank test. To identify the risk factors for recurrence, we performed a multivariate analysis using Cox's proportion hazard model. These calculations were performed using StatView 5.0 software (SAS Institute, Cary, NC, USA) and $P < 0.05$ was considered statistically significant.

Results

Expression of KAI1 protein and clinicopathological factors

Staining was moderate or strong and uniformly spread in all 17 cases of 'normal' adjacent urothelial tissue of the bladder (Fig. 1a). In pTa and pT1 bladder urothelial carcinoma, the staining intensity was lower than for normal epithelial cells and ranged from uniform intense to negative staining or exhibited a heterogeneous staining pattern that has been described previously.[19] Fifty-four cases (62.1%) were classified as moderate expression (or positive; Fig. 1b) and 33 cases (37.9%) had decreased expression (Fig. 1c,d), including 29 cases

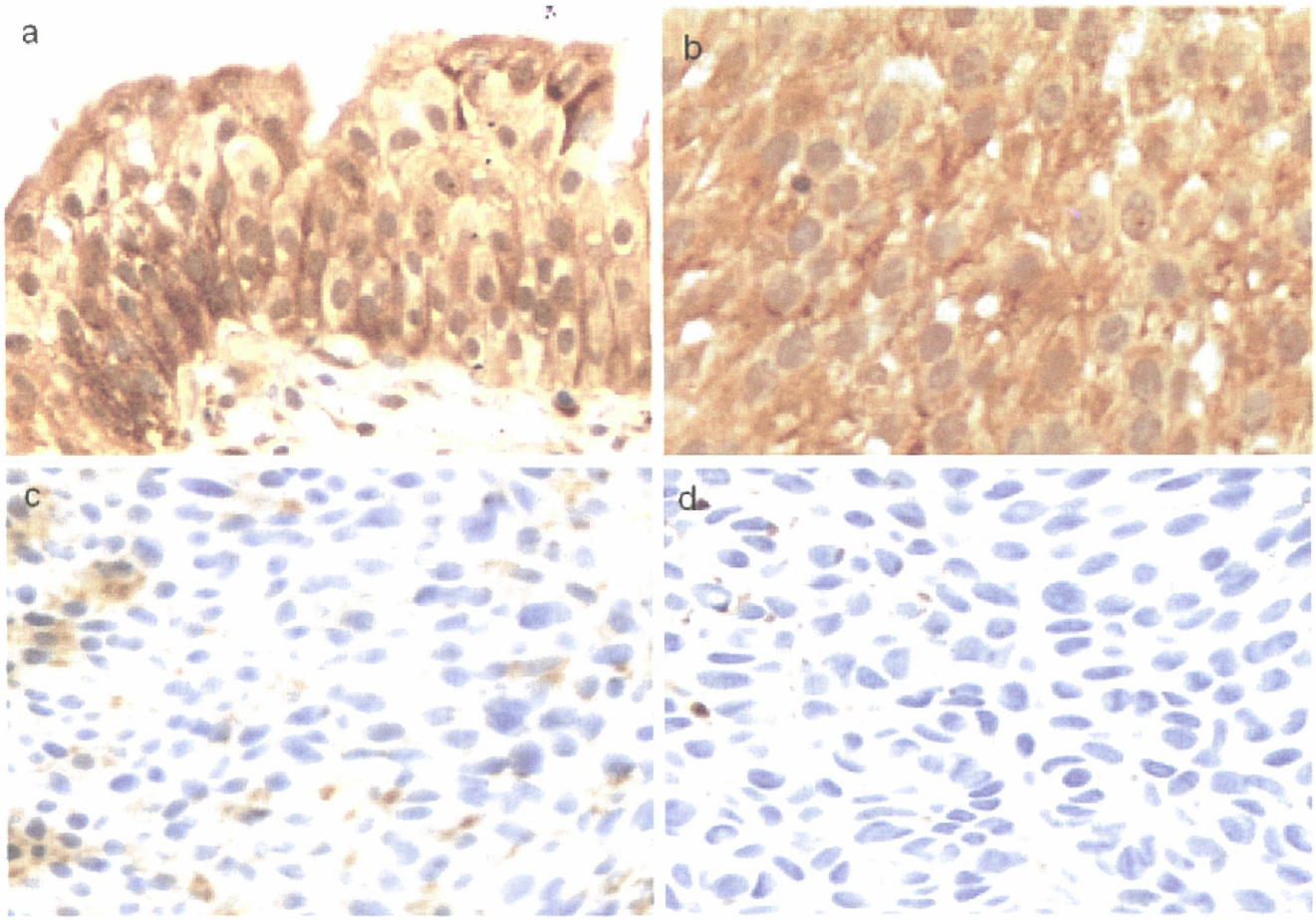

Fig. 1. (a) The 'normal' adjacent urothelial tissue of bladder carcinoma exhibited a uniform, moderate to strong staining of cell membranes and cytoplasm (original magnification ×400). (b) Moderate (or positive) *KAI1* expression. Tumor cells showed a uniform and moderately stained membrane and cytoplasm (original magnification ×400.) (c,d) Decreased (or negative) *KAI1* expression. Cells exhibited a non-uniform or heterogeneous staining (original magnification ×400.)

with less than 50% of stained tumor cells and four cases with no staining within the tumor tissue. In multiple tumors, a total of 116 tumors were investigated and 41 tumors (35.3%) were recorded as having the poorest staining pattern, exhibiting most staining at the surface of the tumor, secondly at fragments or heterogeneously in fragments (Fig. 1c) and at the base of tumors; these different staining patterns were also seen in large tumors (size >3 cm). In invasive bladder cancers, only three cases (9.4%) were classified as moderately stained and expression of *KAI1* protein was significantly lower ($P < 0.0001$) compared with the 62.1% found in pTa and pT1 tumors.

The correlation between the expression of *KAI1* protein and clinicopathological factors in pTa and pT1 urothelial bladder carcinoma is summarized in Table 1. There was no significant difference in the expression of *KAI1* protein in relation to tumor grade; seven of 23 grade 1 tumors (30%), 23 of 56 grade 2 tumors (41%) and three of eight grade 3 tumors (38%) showed decreased expression of *KAI1* protein. Decreased expression of *KAI1* protein was more common in stage pT1 tumors (43%) compared with stage pTa (30%). However, this difference failed to reach statistical significance ($P = 0.25$). Decreased expression of *KAI1* protein also showed a weak relationship with the morphology and number of tumors, but this correlation was not statistically significant ($P > 0.05$). Decreased expression of *KAI1* protein was significantly related to tumor size ($P = 0.016$).

Decreased KAI1 expression and tumor recurrence

In the present study, the recurrence rate after TUR was 25.3, 36.8 and 40.2% for 1, 3 and 5 years, respectively.

When recurrence factors were analyzed with *KAI1* and clinicopathological factors, the recurrence-free survival rates at 5 years were significantly related to the expression of *KAI1* protein (Fig. 2; $P < 0.0001$), stage, morphology of the tumor stem and tumor size (Table 2; $P = 0.0158$, $P = 0.007$ and $P = 0.0001$, respectively). Although there was a trend towards a higher recurrence rate in multiple tumors (two to four tumors), the difference was not statistically significant ($P = 0.3234$). Furthermore, in cases of more than five tumors, the recurrence-free survival rate at 5 years was lower (31.4%) than for cases with single tumors and, although the cause for this is uncertain, patients with multiple tumors also had lower *KAI1* expression (10%; Table 1). The results of multivariate analysis concur with those of univariate analysis, namely that decreased *KAI1* expression, stage pT1, sessile tumors and tumor size >3 cm are independent prognostic factors of tumor recurrence (Table 3; $P = 0.0013$, $P = 0.031$, $P = 0.0026$ and $P = 0.014$, respectively). Clinical material with a homogeneous tumor phenotype and consistent management are more relevant for testing new markers. We comparatively evaluated differences in expression of *KAI1* protein between the recurrence and non-recurrence groups according to each clinicopathological factor. Despite the low recurrence risk in univariate analysis for low grade, stage pTa, papillary tumors, tumor diameter <3 cm and single tumors (Table 2), when decreased *KAI1* expression was used, these factors also indicated an increased risk of recurrence (Table 4). These results show that decreased KAI1 expression can identify patients with a

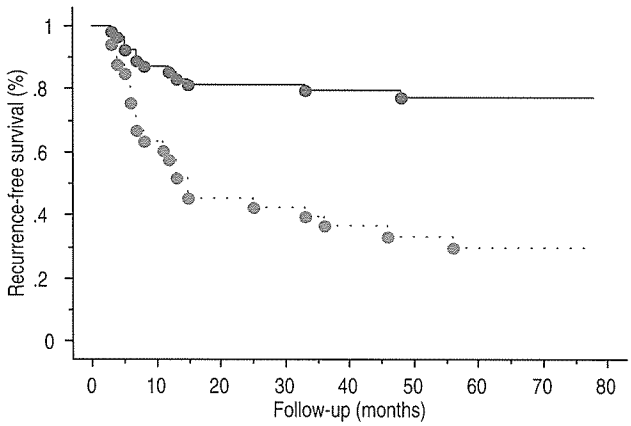

Fig. 2. Kaplan–Meier method for determining tumor recurrence-free survival rates of pTa and pT1 urothelial bladder carcinoma patients with either decreased or positive *KAI1* expression. (·····), decreased *KAI1* expression ($n = 33$); (——), positive *KAI1* expression ($n = 54$; $P < 0.0001$).

Table 2 Five-year recurrence-free survival rates according to each clinicopathologic factors in primary pTa and pT1 urothelial bladder carcinomas

Prognostic factors	Probability of recurrence (%) 1 year	3 years	5 years	*P*-value Log-rank test
Histology				
Grade 1	5 (21.7)	9 (39.1)	9 (39.1)	NS
Grade 2	15 (26.8)	20 (35.7)	22 (39.6)	
Grade 3	2 (25.0)	3 (37.5)	4 (68.8)	
Pathology				
Stage pTa	5 (15.2)	8 (24.2)	8 (24.2)	0.0158
Stage pT1	17 (31.5)	24 (44.4)	27 (51.3)	
Morphology				
Papillary	21 (28.0)	28 (37.3)	30 (40.3)	NS
Non-Papillary	1 (8.3)	4 (33.3)	5 (42.9)	
Pedunculated	14 (23.3)	16 (26.7)	18 (30.4)	0.007
Sessile	8 (29.6)	16 (59.3)	17 (63.3)	
Size (cm)				
<1	3 (14.3)	6 (28.6)	6 (28.6)	0.0001
1–3	11 (22.4)	13 (26.5)	15 (31.0)	
>3	7 (58.3)	9 (75.0)	10 (87.5)	
Unidentified	1 (20.0)	4 (80.0)	4 (80.0)	
No. tumors				
1	10 (21.7)	14 (30.4)	16 (35.2)	NS
2–4	7 (28.0)	13 (52.0)	13 (52.0)	
>5	2 (20.0)	2 (20.0)	3 (31.4)	
Unidentified	3 (50.0)	3 (50.0)	3 (50.0)	

Table 3 Independent prognostic indicators for recurrence selected by multivariate analysis with Cox's proportional hazards regression test in primary pTa and pT1 urothelial bladder carcinomas

Prognostic factors	Hazard rate	95% CI	*P*
Stage (pT1 *vs* pTa)	2.51	1.09–5.79	0.031
Size (>3 *vs* <1 cm)	3.82	1.31–11.14	0.0143
Morphology (sessile *vs* pedunculate)	3.53	1.55–8.04	0.0026
KAI1 expression (decreased *vs* positive)	3.92	1.70–9.01	0.0013

CI, confidence interval.

Table 4 Association between *KAI1* expression and recurrence in subgroup according to each clinicopathologic factor and intravesical treatment in primary pTa and pT1 urothelial bladder carcinomas

Prognostic factors	Expression of *KAI1* (–/+)		χ^2 test	
	Recurrence	Non-recurrence	RR (95% CI)	*P*
Histology				
Grade 1	5/4	2/12	7.5 (1.0–55.0)	0.036
Grade 2	16/6	7/27	10.3 (2.9–36.0)	0.0001
Grade 3	2/2	1/3	3.0 (0.2–59.9)	0.47
Pathology				
Stage pTa	6/2	4/21	15.8 (2.3–107.9)	0.0016
Stage pT1	17/10	6/21	6.0 (1.8–19.7)	0.0025
Morphology				
Papillary	20/10	7/38	10.9 (3.6–32.9)	<0.0001
Non-Papillary	3/2	3/4	2.0 (0.2–20.6)	0.56
Pedunculated	11/7	9/33	5.8 (1.7–19.1)	0.0028
Sessile	12/5	1/9	21.6 (2.1–218.6)	0.0023
Size (cm)				
<1	3/3	3/12	4.0 (0.5–30.8)	0.17
1–3	10/5	5/29	11.6 (2.8–48.6)	0.0003
>3	6/4	0/2	–	0.12
Unidentified	4/0	1/0	–	–
No. tumors				
1	11/5	6/24	8.8 (2.2–35.2)	0.001
2–4	8/5	3/9	4.8 (0.9–26.8)	0.07
>5	1/2	0/7	–	0.11
Unidentified	3/0	1/2	–	0.08
Intravesical				
MAC	18/10	9/40	8.0 (2.8–23.1)	<0.0001
BCG	2/1	1/1	2.0 (0.1–78.3)	0.71
Non-treatment	3/1	0/1	–	0.17

BCG, Bacillus Calmette–Guérin; MAC, mitomycin C, adriamycin and cytosine arabinoside; (–/+), decreased/positive expression of *KAI1* protein; RR, relative risk; CI, confidence interval.

higher chance of recurrence among those considered phenotypically at a low risk of recurrence.

Discussion

The *KAI1* gene encodes a protein consisting of 267 amino acids and belonging to a structurally distinct family of leukocyte surface glycoproteins, being a member of the transmembrane 4 superfamily (TM4SF). The *KAI1* gene was isolated from human chromosome 11p11.2 and was shown to suppress metastasis when introduced into rat AT6.1 prostate cancer cells.[16] Decreased expression of this gene may be involved in the malignant progression of prostate and other cancers.[16] Downregulation of the *KAI1* gene during progression of cancer does not commonly involve either mutation or allelic loss of the *KAI1* gene[17] and is not associated with methylation of the promoter or *p53* regulation.[34] Although the mechanisms of action of the

KAI1 protein have not been fully elucidated, some clues are emerging and it seems that, like cell surface glycoproteins, the *KAI1* protein may play an important role in signal transduction.[35]

In the present study, *KAI1* expression was examined using immunohistochemistry.[29,36] Because in the present study patients in both the recurrence and non-recurrence groups had the same type of urothelial carcinoma (transitional cell) and treatment was consistent with the stage of the disease, we were able to analyze the data comparatively. Because *KAI1* is a member of the TM4SF, positive staining for the protein should be observed essentially at the cell membrane. However, in formalin-fixed, paraffin-embedded samples, the modification of which appears to be substantially altered in the presence of the human T-cell leukemia virus type 1 (HTLV-1) genome, immunoreactivity for *KAI1* that could have prognostic value is observed, exhibiting specific cytoplasmic localization of the protein in the cells.[37] In the normal papilla of Vater, esophageal tissue or primary tumor,[18,38] *KAI1* immunoreactivity was also demonstrated in the cytoplasm and at the cell membranes. In the present study, we used a rabbit polyclonal antibody, with epitope mapping at the C-terminus of metastasis suppressor protein (*KAI1*) of human origin, and found that *KAI1* was expressed abundantly in normal urothelial tissue, but was downregulated in pTa and pT1 urothelial bladder carcinoma and further downregulated in muscle-invasive bladder cancers (stage pT2–4). Staining of carcinoma cells was weaker than of normal epithelial cells. We considered decreased *KAI1* protein expression to occur when a non-uniform staining pattern was observed and the proportion of stained cells was less than 50% within the tumor tissue. The non-uniform staining pattern correlates with loss of *KAI1* expression.[25,39] In advanced bladder carcinoma, an abnormal or heterogeneous immunostaining pattern was defined as negative staining and was significantly related to tumor stage and grade.[19] In the present study, these poorest staining patterns were exhibited most at the tumor surface, second at fragments or heterogeneously in fragments and at the base of the tumors. These different staining patterns were also seen at the top more than at base of large tumors, suggesting that cell desquamation maybe more pronounced in pTa and pT1 urothelial bladder carcinoma. Although the malignant activity of pTa and pT1 urothelial bladder carcinoma was lower than the muscle-invasive bladder cancers, decreased expression of *KAI1* also showed a tendency to relate to clinicopathological factors, including grade and stage, and was significantly related to tumor size. This level of *KAI1* expression has been described previously.[40] Our results suggest that this evaluation is a reasonable approach for examining the expression of the *KAI1* metastasis suppressor gene in pTa and pT1 urothelial bladder carcinoma.

Identification of gene expression patterns in superficial and invasive bladder cancer gives us a better understanding of those genes related to encoding proteins involved in cell proliferation, oncogenes and growth factors, cell adhesion, immunology, transcription, proteinases and ribosomes.[41] Recurrence of pTa and pT1 urothelial bladder carcinoma is a complex process in which many genes and steps participate and is related to invasiveness, spread, transplant, adhesion and the proliferation ability of neoplastic cells. The function of the *KAI1* gene is closely correlated to the invasive and reimplantation characteristics of tumor cells in the bladder wall and/or the spreading of tumor cells via expansion within the urothelium, which was thought to be the main cause of the recurrence of pTa and pT1 urothelial bladder carcinoma.[12–15] Univariate and multivariate analysis of results of the present study demonstrated that decreased *KAI1* expression is significantly related to the recurrence of tumors and that it is also an independent prognostic factor of recurrence in pTa and pT1 urothelial bladder carcinoma.

Recurrent tumors have a similar biological potential as the original tumor and behave in a similar manner.[14] To prevent tumor recurrence, it is important to recognize various biological factors and clinicopathological characteristics in each individual case. Previously, we examined the correlation between the tumor repressor gene *p53*, *Ki-67* antigen, *c-erbB-2* oncoprotein and tumor recurrence in pTa and pT1 urothelial bladder carcinoma. When all these factors became negative, the tumor recurrence rate was significantly low, combinational analysis of two factors resulting in a larger significance than analysis of a single factor, but there was no significant correlation between decreased expression of *KAI1* protein and each of these factors. In the present study, subgroup analysis according to each clinicopathological factor showed that patients with decreased *KAI1* expression have a high relative risk of recurrence, even for patients with a low grade, stage pTa, papillae, tumor diameter 1–3 cm and single tumor, which are supposed to indicate a lower recurrence rate. Intravesical treatment, especially BCG instillation, is able to prevent the recurrence of some tumors, but when decreased expression of *KAI1* protein is detected, the follow-up regimen should be evaluated. Although the clinical material available for subgroup analysis is small, tumors were phenotypically similar and the treatment consistent with the characteristics of the tumors, which makes the results more relevant for testing new markers. Thus, analysis of the *KAI1* protein can be useful for medical

practice, because decreased *KAI1* expression occurs during the progression of cancer, when the biological behavior of the tumor changes from low risk to high risk, permitting the identification of those patients in whom the cancer is likely to recur.

In conclusion, decreased expression of *KAI1* protein was related to stage and morphology of the tumor stem and was significantly correlated with tumor size. Decreased *KAI1* expression was associated with the degree of invasiveness and progression of cancer and was an independent prognostic factor for tumor recurrence in primary pTa and pT1 urothelial bladder carcinoma. Additional larger studies are needed to corroborate the validity of these observations and the role of this metastasis suppress gene in the evaluation of primary pTa and pT1 urothelial bladder carcinoma.

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IN SITU GELATINOLYTIC ACTIVITY CORRELATES WITH TUMOR PROGRESSION AND PROGNOSIS IN PATIENTS WITH BLADDER CANCER

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ABSTRACT

Purpose: Degradation of the extracellular matrix by malignant tumor cells has an essential role in the process of tumor invasion and metastasis. The 2 gelatinolytic matrix metalloproteinases (MMPs) MMP-2 and MMP-9 are believed to be key enzymes in this process. We investigated the possible relationship between *in situ* gelatinolytic activity of MMPs and clinicopathological factors in patients with bladder cancer to clarify whether these proteins would be critical for tumor advancement in this disease.

Materials and Methods: We evaluated the intensity of gelatinolytic activity in 25 bladder cancer tissues by film *in situ* zymography (FIZ). To clarify the MMP(s) responsible for gelatinolytic activity in bladder cancer tissues we examined MMP-2 and MMP-9 expression in bladder tissues by gelatin zymography. MMP expression was also confirmed by reverse transcriptase-polymerase chain reaction and Western blotting. We then investigated the association between MMP expression detected by gelatin zymography and the intensity of gelatinolytic activity determined by FIZ.

Results: FIZ demonstrated that all tumor tissues had *in situ* gelatinolytic activities. There was a statistically significant correlation between the intensity of gelatinolytic activity, and tumor grade, stage, vessel invasion and cause specific survival ($p < 0.05$). Stronger *in situ* gelatinolytic patterns were documented in cases with higher pro and active MMP-2 expression.

Conclusions: FIZ enables the direct assessment of *in situ* gelatinolytic activity in bladder cancer tissues. The intensity of activity appears to affect the biology of carcinoma tissues. Our results indicate a major role for MMP-2 in *in situ* gelatinolysis in bladder cancer.

KEY WORDS: bladder, bladder neoplasms, matrix metalloproteinases, gelatinases

Bladder cancer is the second common malignant disease in the urogenital organs and its incidence is still increasing. There are several therapeutic options for bladder cancer, such as transurethral resection, radical cystectomy, instillation of some chemoagents or bacillus Calmette-Guerin, chemotherapy and combinations of these therapeutic modalities. Tumor stage is a strong prognostic factor and it provides information when selecting therapeutic modalities. However, there seems to be some discrepancy between tumor stage and malignant potential in this disease. Therefore, the establishment of adequate biological parameter is mandatory to add useful information on stage.

Tumor cell invasion and metastasis biologically depend on the proteolytic destruction of surrounding matrix components. Matrix metalloproteinases (MMPs) are a family of enzymes that are capable of degrading the basement membrane and extracellular matrix. To date about 20 MMPs have been recognized. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are believed to have an important role in destruction of the basement membrane. These enzymes are considered to contribute to tumor invasion and metastasis. There have been several reports regarding the prognostic significance of increased MMP expression in cancers in several human organs.[1–12]

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Film *in situ* zymography (FIZ)[13–17] can directly show *in situ* gelatinolytic activity in tissue sections. We have already reported that gelatinolytic activity on FIZ correlates with the size, grade and vessel invasion of renal cell carcinomas, and MMP-2 activity correlates with gelatinolytic activity on FIZ.[14] In this study we investigated the possible relationship between FIZ findings and various clinicopathological factors in patients with bladder carcinoma. We also investigated the correlation between MMP expression detected by gelatin zymography (GZG) and the intensity of *in situ* gelatinolytic activity.

MATERIALS AND METHODS

Patients and tissue specimens. A total of 25 patients with bladder carcinoma treated at the department of urology at our institution between 1995 and 2002 were included in the current study. Mean age was 66.7 years (range 41 to 88). The study group comprised 24 men and 1 woman. All samples were obtained with informed consent. All patients had undergone total cystectomy or transurethral bladder tumor resection. Tumor samples were snap frozen in liquid nitrogen, embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and stored at −80C. All hematoxylin and eosin stained slides from each case were reviewed and all tumors were histologically classified as transitional cell carcinoma (TCC). All Victoria blue stained

slides were also reviewed to confirm vessel invasion (positive or negative).

FIZ. Frozen sections were cut 4 μm thick using a cryotome and mounted on polyethylenetelephthalate film coated with 7% gelatin (FIZ-GN) (Fuji Photo Film Co., Ltd., Tokyo, Japan). As a control, these sections were mounted on polyethylenetelephthalate film coated with 7% gelatin including MMP inhibitor (FIZ-GI, 1,10-phenanthroline, Sigma Chemical Co., St. Louis, Missouri). At the same time hematoxylin and eosin staining was performed. They were incubated in a moist chamber at 37C for 12 hours and the films were stained with 0.5% Amido Black 10B (Wako Co., Ltd., Osaka, Japan) for 15 minutes. The films were then destained for 15 minutes with a solution containing 70% methanol and 10% acetic acid. Gelatinolytic activity was determined as a clearly visible area after unstaining by Amido Black 10B. The intensity of *in situ* gelatinolysis was classified into 4 patterns according to visualized degradation on FIZ-GN films, namely pattern A—no gelatinolytic activity, pattern B—weak activity with gelatin films scarcely dissolved, pattern C—focal complete degradation and pattern D—diffuse complete degradation (fig. 1).

GZG. The GZG method was described in the previous report. Enzymatic activities of MMP-2, proMMP-2, MMP-9 and proMMP-9 were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gelatin zymography. Normal and malignant frozen bladder tissues were homogenized. Supernatants were extracted and adjusted to a protein concentration of 1 μg/ml. We used supernatants of HT1080 (fibrosarcoma) cells as a positive control and an MMP sample (Yagai Laboratories, Yamagata, Japan) as a marker. All samples were subjected to nonreduced SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels containing 7.5% gelatin. Subsequently SDS was removed by soaking gels in 2.5% Triton X-100 solution for 1 hour at room temperature. The gels were re-incubated at 37C for 24 hours in reaction buffer containing 50 mmol/ml tris-HCl (pH 7.6), 100 mmol/l NaCl, 10 mmol/ml $CaCl_2$, 0.05% Brij-35 and 0.02% NaN_3, stained with Coomassie brilliant blue and destained. Gels were then dried for use with Gel Dry Solution (Tefco, Tokyo, Japan).

The gelatinolytic activities of proMMP-2, MMP-2, proMMP-9 and MMP-9, as detected by GZG were quantified using a densitometer (Model GS-700 Imaging Densitometer, BioRad Laboratories, Hercules, California). The ratio of band density of each sample to that of the standard marker was determined.

RNA extraction and reverse transcriptase (RT)-polymerase chain reaction (PCR). Total RNA was isolated from 2 tissue samples of each FIZ pattern group using Trizol reagent (Invitrogen, Carlsbad, California). RNA (1 μg) was reverse transcribed to cDNA in a final volume of 20 μl containing oligo deoxythymidine primers, 25 mmol/l $MgCl_2$, tris-HCl, 10 mmol/l deoxynucleoside triphosphate mix and SuperScript II RT (Invitrogen). PCR primers for human MMP-2 were forward 5′-AAG GCC AAG TGG TCC GTG TGA A-3′ and reverse 5′-AAC AGT GGA CAT GGC GGT CTC AG-3′, amplifying a 371 bp fragment, and for MMP-9 they were forward 5′-GGC GCT CAT GTA CCC TAT GT-3′ and reverse 5′-TCA AAG ACC GAG TCC AGC TT-3′, amplifying a 468 bp fragment.

PCR was done in a 20 μl volume containing first strand cDNA, forward primer, reverse primer, buffer, $MgCl_2$ and Taq polymerase (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). For amplification 35 cycles of a 3-step temperature profile was performed, including denaturation at 94C for 60 seconds, annealing at 54C for 30 seconds and extension for 60 seconds with a final extension for 5 minutes at 72C. As a control, the internal standard β-actin was used according to manufacturer instructions (Invitrogen). A 6 μl volume of PCR mixture was electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

Western blot analysis. Two tissue samples from each FIZ pattern group were used to prepare protein extracts. After extraction tumorous and normal samples were adjusted to include 100 μg protein, electrophoresed in 10% SDS-polyacrylamide gel and transferred to polyvinylidine difluoride (PVDF) membranes by electroblotting. PVDF membranes were incubated with primary antibodies at 1:1,000 dilution overnight at 4C. Two primary antibodies against human MMP-2 and MMP-9 (Sigma Chemical Co.) were used. After extensive washing of PVDF membranes antibodies were incubated with peroxidase conjugated horseradish antirabbit IgG (Amersham Pharmacia Biotech) at 1:1,000 dilution for 1 hour at 4C. MMP bands were detected using echoluminescence Western blotting detection systems (Amersham Pharmacia Biotech).

Statistical analysis. Statistical significance was calculated using the Mann-Whitney U-test, Kruskal-Wallis test, 1-way ANOVA or simple regression. Survival curves were computed using the Kaplan-Meier method with the log rank test with $p < 0.05$ considered significant.

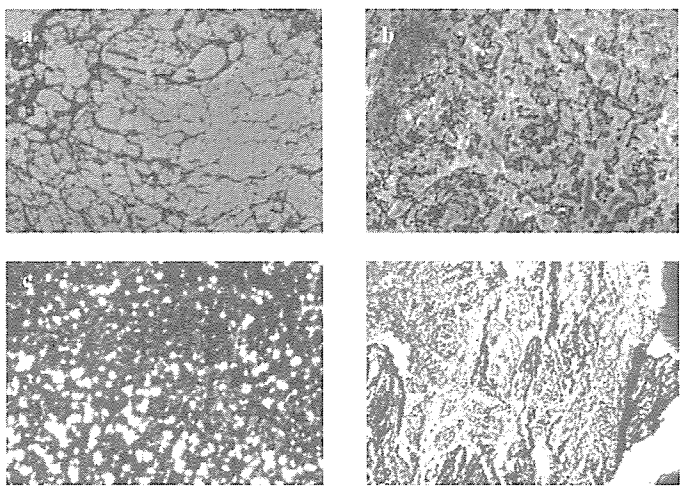

FIG. 1. Intensity patterns of *in situ* gelatinolytic activity exerted by bladder cancer tissues. *a*, pattern A—no activity. *b*, pattern B—weak activity. *c*, pattern C—focal complete gelatinolysis. *d*, pattern D—diffuse complete gelatinolysis. Reduced from ×100.

RESULTS

Gelatinase activity was observed in all cancerous tissues by FIZ. Gelatinolytic activity was decreased on FIZ-GI films in all tissues compared to FIZ-GN films (fig. 2), indicating that MMPs have a major role in the *in situ* gelatinolytic activity of tumorous tissue in bladder cancer. Table 1 shows the correlation between gelatinolytic activity patterns and clinicopathological factors. There was a statistically significant correlation between *in situ* gelatinolytic activity patterns and prognostic factors such as tumor grade, stage and vessel invasion ($p < 0.05$, table 1). However, no relationship was observed between the patterns and intraluminal recurrence ($p \geqq 0.05$).

We divided *in situ* gelatinolytic activity patterns into 2 groups, that is nondiffuse—patterns A to C and diffuse—pattern D. When the patterns were grouped in this manner, cause specific survival was significant in the diffuse activity group ($p < 0.05$, fig. 3).

The pro and active forms of MMP-2, and pro-MMP-9 were detected in all tissues by GZG. However, the active form of MMP-9 was not detected in all bladder samples examined (fig. 4). The intensity of MMP-2 and pro-MMP-2 expression on GZG clearly corresponded to that of the *in situ* gelatinolytic activity pattern. On the contrary, proMMP-9 or the MMP-2-to-proMMP-2 ratio did not correlate to *in situ* gelatinolytic activity patterns (table 2).

RT-PCR and Western blot analysis confirmed the expression of MMP-2 and MMP-9 mRNA and protein in all tumorous and normal bladder tissues examined (figs. 5 and 6).