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UROPLAKIN II AS A PROMISING MARKER FOR MOLECULAR DIAGNOSIS OF NODAL METASTASES FROM BLADDER CANCER: COMPARISON WITH CYTOKERATIN 20

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ABSTRACT

Purpose: Current methods used to determine pathological examination of the lymphatics after radical cystectomy are tedious and costly. We performed a systemic study of uroplakin II (UP II) and cytokeratin 20 (CK 20) expression in pelvic lymph nodes on multiple sides in patients with bladder cancer.

Materials and Methods: A total of 82 pelvic lymph node and 19 bladder tumor samples were obtained from 21 patients with bladder cancer by radical cystectomy with pelvic lymphadenectomy for reverse transcriptase-polymerase chain reaction assay.

Results: Of the 19 bladder tumor tissue specimens 19 (100%) and 13 (68.4%) were positive for UP II and CK 20 mRNA expression, respectively. UP II mRNA was detected in 15 of 16 pelvic lymph node samples (93.8%) with pathologically proven metastases, whereas 9 (56.6%) were positive for CK 20 mRNA. The reverse transcriptase-polymerase chain reaction assay for UP II was statistically more sensitive than that for CK 20 in detecting not only primary tumors, but also metastatic pelvic lymph nodes ($p = 0.0179$ and 0.0373, respectively). Of 66 pelvic lymph node samples without metastasis UP II was detected in 6 (10%), while CK 20 was not. In addition, UP II and CK 20 mRNA could be detected in at least 50 and 500 bladder cancer HT1197 cells, respectively.

Conclusions: These results indicate that UP II might be a more useful marker than CK 20 for detecting micrometastases of bladder cancer in the pelvic lymph nodes, although a greater number of patients and longer followup are needed to come to a definitive conclusion.

KEY WORDS: bladder, bladder neoplasms, neoplasm metastasis, uroplakin II, cytokeratin 20

The presence or absence of pelvic lymph node involvement is one of the most important prognostic factors after radical cystectomy for bladder cancer.[1,2] No less than half the patients with pathologically node negative, invasive bladder cancer could die of recurrence after radical cystectomy.[3] This indicates that there are not a few occult metastases that cannot be detected by conventional pathological examination. On the other hand, it has been shown that postoperative chemotherapy confers significant survival benefits in patients with pathologically progressive node metastases.[4] Therefore, an accurate determination of lymph node status is critically important for improving the clinical outcome after radical cystectomy. However, currently a pathologically thorough examination of excised lymphatic tissues is tedious and costly. Thus, an objective, rapid, reproducible and simple method to determine lymph node status is necessary in patients with bladder cancer after radical cystectomy.

Uroplakin II (UP II) is an urothelial differentiation related membrane protein that is expressed specifically in the urothelium and is also well preserved in transitional cell carcinoma of the urinary tract.[5,6] Recent studies using reverse-transcriptase (RT)-polymerase chain reaction (PCR) showed that UP II mRNA can be detected in bladder cancer cell lines and tumor tissues but not in prostate, skin, liver or ovary tissue specimens.[7,8] Furthermore, we and others have detected circulating cancer cells in the peripheral blood of patients with urothelial cancer using the RT-PCR assay for UP II.[7–9]

Cytokeratin 20 (CK 20) is a protein of the intermediate filament group that is selectively expressed in epithelial cells of the urinary tract, gastrointestinal tract and Merker cells.[10,11] CK 20 amplification has been extensively used to identify rare disseminated cancer cells in peripheral blood, bone marrow and lymph node samples in patients with several types of cancers, including bladder, colorectal and thyroid cancers.[12–15]

These 2 molecules may serve as biomarkers for detecting micrometastases in pelvic lymphatic nodes in bladder cancer cases. We analyzed the expression of UP II and CK 20 in pelvic lymph node and tumor tissue samples from patients with bladder cancer using RT-PCR assays.

MATERIALS AND METHODS

Patients and tissue samples. A total of 82 pelvic lymph node and 19 primary tumor tissue samples were obtained from 21 patients with bladder cancer by radical cystectomy with pelvic lymphadenectomy at Kagawa University Hospital between June 2001 and January 2005. All patients were pathologically diagnosed to have bladder transitional cell carcinoma. Table 1 lists patient clinicopathological characteristics. Primary tumors were graded and staged according to WHO criteria[16] and the TNM classification,[17] respectively. The mean number of examined lymph nodes in each patient was 4 (range 2 to 7). Of these lymph node tissue samples a total of 66 (80.5%) from 14 patients had no evidence of lymph node metastasis, whereas a total of 16 (19.5%) from 7 pa-

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TABLE 1. *Clinicopathological features in bladder cancer patients*

Pt No.—Age	Grade	TNM
1—74	3	T2
2—55	3	T2
3—49	2	T3a
4—76	2	T3b
5—76	3	T4
6—75	3	T3b
7—64	3	T2a
8—71	3	T2a
9—60	2	Tis
10—73	3	T3b
11—53	3	T2a
12—85	3	T3a
13—80	2	T3a
14—67	3	T3b
15—57	3	T3b
16—81	3	T3a
17—87	3	T3b
18—74	3	T3b
19—81	3	T2a
20—74	3	T3b
21—74	3	T3a

tients had lymphatic metastases on routine pathological examination. A hemisphere of each pelvic lymph node sample from each site was subjected to histopathological diagnosis and the other hemisphere was snap frozen in liquid nitrogen and stored at −80C until RNA extraction. Primary bladder tumor tissue samples were also prepared for RNA extraction.

Cell lines. We also analyzed the human bladder cancer cell line HT1197, the human prostate cancer cell lines LNCaP, DU145 and TSU-RP1, and the human renal cell carcinoma cell line ACHN as controls. These cells were cultured in RPMI-1640 supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum at 37C in a humidified 5% CO_2 atmosphere. Total RNA was extracted and subjected to RT-PCR assay.

RNA extraction and RT-PCR assay. Total RNA was extracted from cell lines as well as tissue samples of primary bladder tumor and pelvic lymph nodes using TRIzol® reagent. The RNA concentration was determined using a GeneQuant™ pro RNA/DNA calculator. cDNA was synthesized using a First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) at 37C for 60 minutes. Total RNA (1 μg) was added to the RT reaction.

Aliquots of the same cDNA were amplified with specific primers of UP II and CK 20. Primer sequences were 1) UP II sense 5′-TCCCCAGGGGCTGCAGACTT-3′ and UP II anti-sense 5′-GGTTTGTCACCTGGTATGCACT-3′, and 2) CK 20 step I sense 5′-CAGACACACGGTGAACTGG-3′ and CK 20 step I anti-sense 5′-GATCAGCTTCCACTGTTAGACG-3′, and CK 20 step II sense 5′-CTGTTTGTTGGCAATGAGAAAATGG-3′ and CK 20 step II anti-sense 5′-GATCTCTCTCAGTCT-CATA-3′. UP II was amplified in single step PCR,[18] whereas Ck 20 was amplified in nested PCR,[19] as described previously. The size of amplified UP II and CK 20 products was 268 and 349 bp, respectively.

The 25 μl PCR mixture consisted of 25 pmol of each primer, 200 μM deoxynucleotide triphosphates, 0.2 U Taq™ polymerase, PCR buffer containing $MgCl_2$ and 1 μl template. PCR conditions for UP II were 35 cycles of 94C for 1 minute, 55C for 1 minute and 72C for 1.5 minutes. PCR reactions for CK 20 were done for 40 cycles for 20 seconds at 94C, 20 seconds at 55C and 30 seconds at 72C in each PCR step. Amplified products were separated on 2% agarose gel and bands were analyzed with ImageMaster™.

A sample without RNA and RNA extracted from HT1197 cells served as a negative and positive control, respectively, in every RT-PCR assay. In addition, amplified products of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers served as an internal control in all analyses.[20]

Determination of detection limit. RNA was extracted from HT1197 cells and serially attenuated with distilled water to determine the detection limit. The lowest dilution of RNA was that extracted from 50,000 cells and the highest dilution was from 0.05 cells, theoretically. RT-PCR was done using each specific primer for these serially attenuated samples.

Statistics. Comparison between UP II and CK 20 RT-PCR assays in pelvic lymph nodes was assessed by Fisher's exact test with p <0.05 considered significant.

RESULTS

Sensitivity and specificity of the RT-PCR assay for UP II and CK 20. We evaluated the sensitivity of RT-PCR assay for UP II and CK 20 by testing RNA extracted from 0.05 to 50,000 HT1197 human bladder cancer cells. UP II and CK 20 mRNA expression was detected in RNA extracted from more than 50 and 500 HT1197 cells, respectively (fig. 1).

To verify the specificity of amplification products we then analyzed the prostate cancer cell lines LNCaP, DU145 and TSU-RP1, and the renal cell carcinoma cell line ACHN. Neither UP II nor CK 20 mRNA was detected in these nonbladder cancer cell lines (data not shown). We also examined 8 pelvic lymph node tissue samples from 4 patients with prostate cancer who underwent radical prostatectomy. All samples were negative for UP II and CK 20 mRNA (data not shown).

IUP II and CK 20 mRNA expression in bladder tumor and lymph node tissues. Table 2 shows the results of the UP II and CK 20 RT-PCR assay. Of the 19 bladder tumor tissue specimens analyzed 19 (100%) and 13 (68.4%) were positive for UP II and CK20 mRNA, respectively (table 3). The RT-PCR assay for UP II was more sensitive than that for CK 20 for detecting bladder tumors (p = 0.0179). Of 82 pelvic lymph node tissue samples 21 (25.6%) showed UP II mRNA and 11 of 82 (13.4%) were positive for CK 20 mRNA. In addition, we also examined UP II and CK20 mRNA expression in normal bladder tissue samples. In all 15 normal bladder tissue samples analyzed (100%) UP II and CK20 mRNA were positive. GAPDH expression was detected as the internal control in all samples studied.

Comparison of RT-PCR results with pathological diagnosis of lymph nodes. Tables 2 and 4 list the results of the RT-PCR

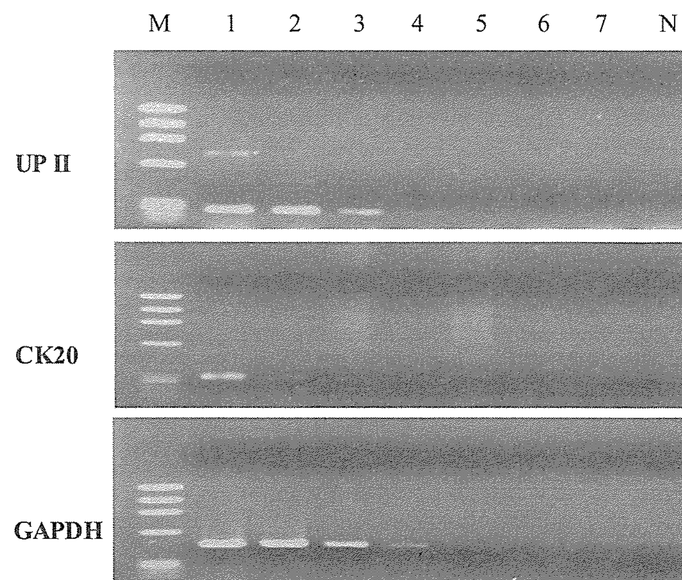

FIG. 1. UP II and CK 20 mRNA expression was detected in more than 50 and 500 cancer cells per sample, respectively, in HT1197 bladder cancer cell line under RT-PCR conditions. *Lane M*, DNA marker. *Lanes 1* to *7*, 50,000 to 0.05 HT1197 cells. *Lane N*, negative control.

TABLE 2. *Molecular features in patients with bladder cancer*

Pelvic Lymph Node				Primary Bladder Tumor	
Location	Metastasis	UP II	CK 20	UP II	CK 20
Pt 1:				Pos	Pos
Lt obturator	No	Neg	Neg		
Rt obturator	Yes	Pos	Neg		
Pt 2:				Pos	Neg
Lt obturator	No	Neg	Neg		
Lt external iliac artery	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Pt 3:				Pos	Pos
Lt external iliac artery	Yes	Pos	Pos		
Rt obturator	Yes	Pos	Pos		
Rt common iliac artery	Yes	Pos	Pos		
Presacral	Yes	Pos	Pos		
Pt 4:				Pos	Pos
Lt obturator	No	Neg	Neg		
Lt external iliac artery	No	Pos	Neg		
Rt obturator	No	Neg	Neg		
Pt 5:				Pos	Pos
Lt obturator	No	Neg	Neg		
Lt external iliac artery	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Rt external iliac artery	No	Neg	Neg		
Rt internal iliac artery	No	Neg	Neg		
Pt 6:				Pos	Neg
Lt obturator	No	Neg	Neg		
Lt external iliac artery	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Rt common iliac artery	No	Neg	Neg		
Pt 7:				Pos	Pos
Lt obturator	No	Neg	Neg		
Lt internal iliac artery	No	Neg	Neg		
Rt obturator (1)	No	Neg	Neg		
Rt obturator (2)	No	Neg	Neg		
Rt external iliac artery	No	Pos	Neg		
Rt common iliac artery	No	Neg	Neg		
Pt 8:				Pos	Pos
Lt obturator	No	Neg	Neg		
Lt external iliac artery	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Rt internal iliac artery	No	Neg	Neg		
Rt external iliac artery	No	Neg	Neg		
Pt 9:				Pos	Pos
Lt obturator	No	Neg	Neg		
Lt external iliac artery	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Rt external iliac artery	No	Neg	Neg		
Pt 10:				Pos	Pos
Lt obturator	Yes	Pos	Neg		
Lt external iliac artery	Yes	Pos	Pos		
Rt obturator	Yes	Pos	Pos		
Pt 11:				Pos	Neg
Lt obturator	No	Neg	Neg		
Lt external iliac artery	No	Neg	Neg		
Rt internal iliac artery	No	Neg	Neg		
Rt external iliac artery	No	Neg	Neg		
Pt 12:				Pos	Pos
Lt obturator	Yes	Pos	Pos		
Rt obturator	No	Neg	Neg		
Rt internal iliac artery	No	Neg	Neg		
Pt 13:				Pos	Pos
Lt obturator	No	Neg	Neg		
Lt external iliac artery	No	Neg	Neg		
Lt internal iliac artery	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Rt external iliac artery	No	Neg	Neg		
Pt 14:				Not determined	Not determined
Lt internal iliac artery	No	Neg	Neg		
Rt external iliac artery	No	Neg	Neg		
Lt external iliac artery	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Lt obturator	No	Neg	Neg		
Pt 15:				Pos	Neg
Rt external iliac artery	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Lt obturator	No	Neg	Neg		
Pt 16:				Pos	Neg
Lt internal iliac artery	No	Neg	Neg		
Lt obturator	No	Neg	Neg		
Rt internal iliac artery	No	Neg	Neg		
Pt 17:				Pos	Pos
Lt obturator	No	Neg	Pos		
Rt obturator	No	Neg	Neg		

TABLE 2. *Continued*

Location	Pelvic Lymph Node Metastasis	UP II	CK 20	Primary Bladder Tumor UP II	Primary Bladder Tumor CK 20
Pt 18:				Pos	Neg
Lt internal iliac artery	No	Neg	Neg		
Lt obturator	No	Neg	Neg		
Rt obturator	No	Neg	Neg		
Pt 19:				Pos	Pos
Lt external iliac artery	Yes	Neg	Neg		
Rt obturator	Yes	Pos	Neg		
Rt external iliac artery	No	Neg	Neg		
Presacral	No	Neg	Neg		
Pt 20:				Pos	Pos
Lt obturator	No	Pos	Neg		
Lt external iliac artery	No	Neg	Neg		
Rt obturator	Yes	Pos	Neg		
Rt external iliac artery	No	Pos	Neg		
Rt common	No	Pos	Neg		
Pt 21:				Not determined	Not determined
Lt obturator	No	Pos	Pos		
Lt external iliac artery	Yes	Pos	Pos		
Lt common	Yes	Pos	Neg		
Rt internal iliac artery	Yes	Pos	Neg		
Rt common	Yes	Pos	Pos		

TABLE 3. *RT-PCR sensitivity for UP II and CK 20 in primary tumors and pelvic lymph nodes with metastases*

	No./Total No. Sensitivity (%)	p Value
Primary tumors:		
UP II	19/19 (100)	0.0179
CK 20	13/19 (68.4)	
Lymph nodes:		
UP II	15/16 (93.8)	0.0372
CK 20	9/16 (56.3)	

TABLE 4. *Histopathological diagnosis vs RT-PCR assay for UP II and CK 20 in pelvic lymph nodes*

	No. Pelvic Lymph Node Metastasis Yes	No. Pelvic Lymph Node Metastasis No	Total No.
UP II:			
Pos	15*	6	21
Neg	1	60	61
CK 20:			
Pos	9	2	11
Neg	7	64	71
Totals	16	66	82

* UP II greater than CK 20 for detecting pelvic lymph node metastasis (Fisher's exact test p = 0.373).

assay for UP II and CK 20 in comparison to the pathological diagnosis in pelvic lymph node tissue samples from patients with bladder cancer. UP II mRNA was detected in a total of 15 of 16 pelvic lymph node tissue samples (93.8%) from 7 patients with pathologically proven metastases, whereas 9 of 16 (56.3%) were positive for CK 20 mRNA (fig. 2). The RT-PCR assay for UP II was greater than that for CK 20 for detecting metastatic pelvic lymph nodes (p = 0.0373). Interestingly UP II mRNA was also positive in a total of 6 pelvic lymph node tissue samples from 4 patients without pathological metastasis. In contrast, CK 20 mRNA was not detected in the same samples.

DISCUSSION

The presence or absence of pelvic lymph node involvement is one of the most important prognostic factors after radical cystectomy for bladder cancer.[1,2] Moreover, no less than half the patients with pathologically node negative, invasive bladder cancer could die of recurrence after radical cystectomy.[3] Recurrence might be relevant to pelvic lymph node micrometastases that were undetected by routine histological examination. On the other hand, postoperative chemotherapy in node positive patients, particularly those with minimal node involvement, has proved to provide survival benefits.[4] Therefore, assays with high sensitivity and specificity for detecting node involvement that supplement routine pathological diagnosis are urgently needed in patients with bladder cancer after radical cystectomy.

UP II mRNA expression was reported in bladder tumor tissue samples and in bladder cancer cell lines but it was not detected in prostate, skin, liver or ovary tissue specimens.[7,8] It was suggested that the detection of UP II mRNA positive cells in peripheral blood might be used as a tumor marker for molecular staging of urothelial cancers.[8,9] Thus, it is worthwhile to test the clinical usefulness of UP II mRNA for molecular surveillance of micrometastases in bladder cancer lymph node tissues. In contrast to the fact that only 1 site of pelvic lymph node per case was examined for UP II mRNA in an early study,[18] we performed a systemic study of UP II mRNA in regional lymph nodes on multiple sides in patients with bladder cancer treated with radical cystectomy with pelvic lymphadenectomy. Simultaneously we also compared RT-PCR assay for CK 20 to detect micrometastasis in bladder cancer pelvic lymph nodes since it was reported that CK 20 was used to identify rare disseminated cancer cells in the lymph node samples of patients with colorectal and thyroid cancers.[13,14]

In this study UP II mRNA expression was detected in all 19 bladder tumor specimens (100%) and in 15 of 16 pelvic lymph node tissue samples (93.8%) from patients with pathologically proven pelvic lymphatic metastases. The sensitivity of the RT-PCR assay for UP II was significantly higher than that for CK 20 for detecting primary tumors and metastatic pelvic lymph nodes (p = 0.0179 and 0.0373, respectively). These results suggest that detecting UP II mRNA expression in metastatic lymphatic nodes is a more useful diagnostic method for bladder cancer than detecting CK 20 mRNA.

In 1 of 16 lymph nodes (6.2%) the UP II RT-PCR assay failed to detect tumor cells despite their positive identification by pathological study. A possible explanation for the false-negative results might be sampling error, which means that only half of each lymph node was tumor infiltrated, while the other part was tumor-free.

It is interesting that UP II mRNA was also detected in a total of 6 pelvic lymph node tissue samples from 4 patients

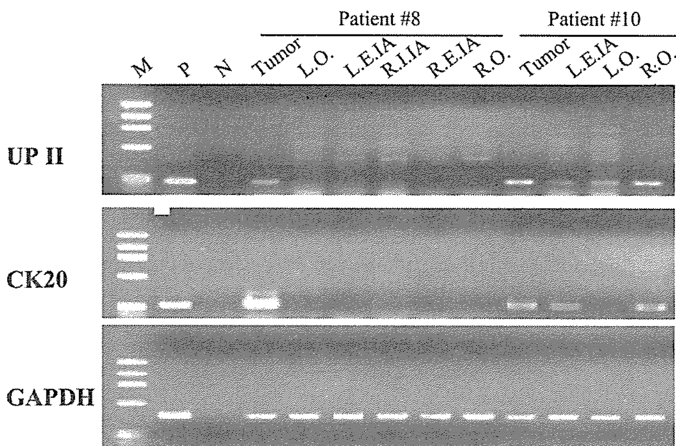

FIG. 2. UP II and CK 20 mRNA expression was detected by RT-PCR in primary bladder tumor and pelvic lymph node tissues. *Lane M*, DNA marker. *Lane P*, positive control. *Lane N*, negative control. *L*, left. *O*, obturator. *E*, external. *IA*, iliac artery. *R*, right. *I*, internal.

without pathologically metastasis, although CK 20 mRNA was not detected. This positive UP II mRNA expression may be attributable to pelvic lymph node micrometastases that were undetectable by routine histology. Long-term followup in these patients is needed to reveal whether they will have clinical recurrence. In addition, neither UP II nor CK20 mRNA was detected in the prostate cancer cell lines, in a renal cell cancer cell line or in pelvic lymph node samples from patients without urothelial cancers, while they were detected in bladder cancer cells. These results indicate that the RT-PCR assay for UP II or CK 20 might be specific for the detection of bladder cancer cells, although sensitivity for CK 20 is relative low.

In the current study we also compared the detectability of RT-PCR assays for UP II and CK 20 using a serial dilution of RNA extracted from the bladder cancer cell line HT1197. UP II and CK 20 mRNA could be detected in RNA extracted from more than 50 and 500 HT1197 cells, respectively. These data further confirm that the sensitivity of RT-PCR for UP II was greater than that for CK 20.

CONCLUSIONS

Comprehensive assessment of the current study from the viewpoints of sensitivity, specificity and detectability suggest that UP II be a more promising molecular marker for detecting pelvic node micrometastasis at radical cystectomy than CK 20, although more patients and longer followup are needed to come to a definitive conclusion.

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EDITORIAL COMMENT

Pelvic lymph node metastasis is an independent prognostic indicator of clinical failure in patients with bladder cancer. Adjuvant chemotherapy immediately following cystectomy may benefit patients with lymph node involvement. However, histological examination may not reveal occult lymph node metastases. Accurate prognostic indicators that correlate with lymph node metastases could help identify patients who would benefit from early systemic therapy. These authors used RT-PCR to analyze the expression of UP II and CK 20 mRNA in primary bladder tumor and pelvic lymph node tissues. UP II expression accurately detected lymph node

metastases with 93% sensitivity and 90% specificity, while CK 20 expression was less accurate. Since long-term followup data are not presented, it is unknown whether the 10% false-positive UP II cases developed metastasis. It is too early to conclude that UP II expression can detect occult metastases. However, based on study results a prospective trial involving more specimens and longer followup is warranted.

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REPLY BY AUTHORS

We agree with that it remains unclear whether UP II RT-PCR can detect occult lymph nodal involvement due to the small number of cases and short followup period. However, to date 1 of 4 false-positive cases has already shown recurrence and progression of disease 6 months after surgery. A prospective study of a larger number of cases and longer followup is needed to verify the usefulness of molecular diagnosis of UP II mRNA for detecting occult metastasis.

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Long-term Functional Outcome and Late Complications of Studer's Ileal Neobladder

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Objective: The purpose of this study was to evaluate the long-term functional outcome and late complications of Studer's ileal neobladder.
Methods: The study included 57 patients who underwent radical cystectomy and bladder reconstruction with Studer's ileal neobladder, and were followed-up for at least 3 months after surgery. The voiding and storage function, and late complications were evaluated. The times of evaluation after surgery were categorized into periods I (3–23 months), II (24–59 months), III (60–95 months) and IV (⩾96 months).
Results: Daytime and night-time continence rates were 95.6 and 88.6%, respectively. The averages of functional capacity (439 ml), maximum flow rate (15.7 ml/s) and residual urine (35 ml) evaluated in period I were maintained in period IV. Of the 57 patients, intermittent self-catheterization was needed in five (8.8%) due to incomplete emptying or urinary retention. Urethroileal anastomotic stricture was found in two patients (3.5%), who were successfully treated by transurethral intervention. Inguinal hernia was found in seven patients (12.8%), five of whom developed it within 2 years after surgery.
Conclusions: Our results indicate that Studer's ileal neobladder had a favorable long-term functional outcome. Although late complication rates were low, the incidence of inguinal hernia was relatively high, and this was considered as a definite late complication in our study.

Key words: bladder substitutes – urinary diversion – cystectomy – bladder neoplasms – complications

INTRODUCTION

Orthotopic bladder substitutions have become standard for urinary reconstruction after radical cystectomy in patients who do not have neoplastic lesions of the urethra. Several types of orthotopic bladder substitutions have been developed, of which Studer's ileal neobladder is one of the most common procedures (1).

Studer's ileal neobladder is easily constructed and provides unchanged voiding habits with good continence and upper urinary tract preservation, with relatively low rates of complication (2,3), even compared with the intermediate-term results of an ileal conduit (4). However, only a few reports are available on the long-term results of this operation. In this study, we reviewed the clinical outcomes of patients who underwent Studer's ileal neobladder operation and were followed-up for a long time to elucidate whether the voiding function was maintained and to clarify what complications developed in the late period.

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PATIENTS AND METHODS

Between February 1991 and September 2003, 62 patients underwent bladder reconstruction with a Studer's ileal neobladder after radical cystectomy for high risk T1 or Tis and invasive bladder cancer. Indications for this procedure consisted of no evidence of neoplastic lesions of the prostatic urethra of male patients and bladder neck of female patients, which was histopathologically confirmed by biopsy before cystectomy.

We used the original operative procedures for construction of the ileal neobladder reported by Studer et al. (2). However, ureters were implanted in the afferent limb of the ileum with the Le Duc–Camey technique, as previously reported, in all but five patients (3).

Of the 62 patients who received Studer's ileal neobladder, 57 patients who were followed-up for at least 3 months after the operation were analyzed retrospectively. All complications in the periods were reviewed. Continence rates were estimated by the Kaplan–Meier method. The follow-up period was categorized into four groups, depending on the period after surgery: period I consisting of 57 patients who were followed-up from 3 months to 2 years; period II, 40 with follow-up for 2–5 years; period III, 23 patients, for 5–8 years; and period IV,

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comprising 13 patients for ⩾8 years. In each period, we evaluated the functional capacity of the neobladder with a frequency/volume chart, the maximum flow rate (Qmax) with conventional uroflowmetry and the post-void residual urine volume (PVR) with catheterization. Changes in these parameters over the periods were statistically examined with the Kruskal–Wallis test. *P*-values <0.05 were considered to be statistically significant.

RESULTS

Patient Characteristics

A total of 53 males (93.0%) and four females (7.0%) were included in this study (Table 1). The mean follow-up period was 57.0 months with a range of 5–136. Patients who were followed-up for ⩾5 years accounted for 40% of the total. More than 90% of patients had invasive disease.

Storage and Voiding Function

Patients achieved 95.6% daytime continence and 88.6% for the night-time, when continence was defined as that with no use of a pad (Fig. 1). Most patients achieved daytime continence within 6 months after the operation. Night-time continence recovered more slowly than that in the daytime. Functional capacity was maintained at 400–500 ml for each period, with no significant change (Table 2). However, two patients developed a neobladder capacity >1000 ml 5 years after the operation. There were no significant changes of Qmax and PVR during the follow-up periods, with the mean rates being maintained at 10–20 ml/s and the mean PVR at <60 ml.

Late Complications

The most frequent complication was transient or long-lasting metabolic acidosis, which had to be continuously treated with potassium/sodium citrate in nine patients (Table 3). Neobladder stones, which were found in seven patients, were successfully treated with endoscopic lithotripsy in all but one patient who had spontaneous passage of a stone. All stones seemed to be formed with a nucleus consisting of mucus and debris from the intestine. A unique late complication was inguinal hernia, which seven patients developed in our study. Most of these patients developed the condition within 2 years after the operation. Because incomplete emptying and urinary retention that resulted in a large amount of PVR (>150 ml) developed during follow-up, five patients needed to undergo clean intermittent catheterization (CIC). No patients had urethroileal anastomotic stricture when starting CIC. Of these patients, two had a poor voiding condition in the early period after the operation. Two other patients gradually developed a poor voiding condition without apparent cause. Urinary retention occurred in one female patient a year after operation, though she had achieved better voiding and continence before this episode. Urethroilael anastomotic stricture was seen in two patients in period I. Balloon dilation or internal urethrotomy under direct vision was effective for management of the stricture. Febrile urinary tract infection occurred in one patient (1.8%) who received Le Duc–Camey ureterointestinal anastomosis. No ureteroileal anastomotic stricture and impaired renal function was observed during follow-up in our series.

Table 1. Patients characteristics (n = 57)

Characteristics	
Sex	
Male (%)	53 (93.0)
Female (%)	4 (7.0)
Mean age: years (range)	60.1 (34–75)
Mean follow-up period: months (range)	57.0 (5–136)
Clinical stage: no. of patients (%)	
T0	1 (1.8)
T1	1 (1.8)
Tis	2 (3.5)
T2	35 (61.4)
T3	18 (31.5)

Table 2. Changes in functional capacity, maximum flow rate and post-void residual urine volume after surgery

Periods (months)	I (3–23)	II (24–59)	III (60–95)	IV (96+)	*P*-value
No. of patients	57	40	23	13	
Functional capacity (ml)	439 (109)	447 (115)	509 (270)	405 (153)	0.741
Qmax (ml/s)	15.7 (8.2)	13.7 (7.7)	16.8 (8.0)	16.7 (7.9)	0.636
PVR (ml)	35 (60)	36 (83)	60 (115)	34 (71)	0.386

Values in parentheses are the SD.
Qmax, maximum flow rate; PVR, post-void residual urine volume.
The *P*-value was determined with the Kruskal–Wallis test.

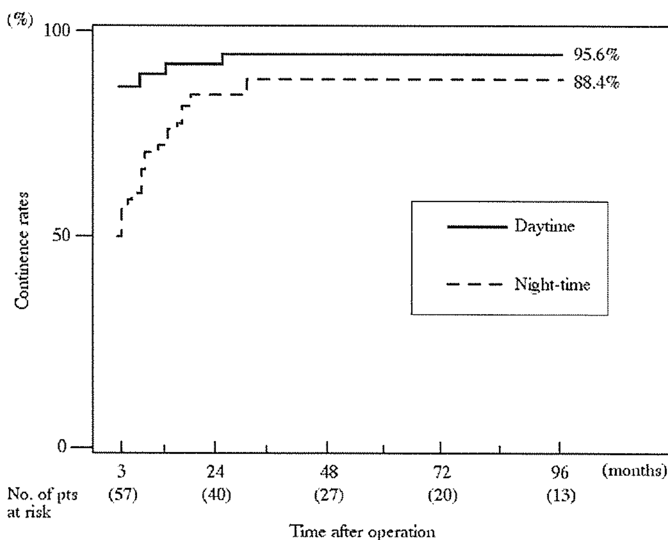

Figure 1. Daytime and night-time continence rates.

Table 3. Late complications

	Overall no. of patients (%)	No. of onsets in each period (months)			
		I (3–23)	II (24–59)	III (60–95)	IV (96+)
Metabolic acidosis	9 (15.8)	4	4	1	0
Inguinal hernia	7 (12.3)	5	1	1	0
Need for intermittent catheterization	5 (8.8)	4	0	1	0
Neobladder calculi	4 (7.0)	1	3	0	0
Upper urinary tract calculi	3 (5.3)	0	1	2	0
Urethroileal anastomotic stricture	2 (3.5)	2	0	0	0
Febrile urinary tract infection	1 (1.8)	1	0	0	0

DISCUSSION

The results of this study indicated that Studer's ileal neobladder maintained favorable voiding and storage functions for many years after the operation. Although the neobladder capacity is insufficient for the first 3 months after operation, it increases to 400–500 ml at 6 months (3,5,6). In this study, the appropriate capacity was maintained even >8 years after the operation. This tendency is comparable with that observed in Studer's series (6,7). However, in our study, two patients developed a capacity of >1000 ml over 5 years after the operation. Periodic assessment with a frequency/volume chart and reinstruction of neobladder management are required to avoid its overextension and too large a storage volume.

Although we did not identify the specific factors, our recommendations for patients to wake up and void at least once in the middle of the night, and to refrain from drinking an excessive amount of water before going to sleep may have contributed to the reduction of incontinence frequency.

Qmax was 10–20 ml/s immediately after the operation, as has been reported by others (8,9), and it was stable in the long term. Urinary retention occurred in one female patient. It was associated with neither anastomotic stricture nor urethral recurrence of carcinoma. Although urinary retention is rare, it occurs more frequently in female patients (10). One of the speculated causes of urinary retention in females is kinking of the urethra (6,7,10), which is probably caused by denervation of the proximal urethra and is considered to be the main cause (10,11). However, neither voiding cystourethrography nor cystourethroscopy revealed such an apparent cause for retention in our patient. Thus the episode was due to other, as yet unknown functional or anatomical causes.

The percentage of our patients who needed intermittent catheterization was 8.8%, which was comparable with that in other reports (9,12). Of those patients, one had a PVR that increased to >150 ml 5 years after the operation. Although a large PVR was reported to be a result of inguinal or incisional hernias (7,10), our patient had neither inguinal nor incisional hernia, and had a functional capacity >800 ml. These findings suggest that overextension is a cause of increased PVR. Mikuma et al. pointed out that in patients with a low Qmax and a high PVR, the anastomosis between the neobladder and membranous urethra was not located at the bottom of the pouch and a cystocele-like change was observed (13). Although that was not confirmed by radiographic examination, in our patient, a cystocele-like change resulting from overextension of the neobladder that occurred several years after the operation might have been involved in the increase of PVR.

The incidence of inguinal hernias was unexpectedly high in this study. Studer et al. reported that the incidence of inguinal or abdominal wall hernias was 7% in their series with a median follow-up period of 30.2 months (5). Our longer follow-up, 57 months, might be related to the difference in the rate from that of others, although we did not find any specific explanations for the incidence. Ichioka et al. reported that 21.3% of patients who underwent radical retropubic prostatectomy developed inguinal hernia. On the other hand, in patients with cystectomy and mainly incontinent urinary diversion in their series, the incidence of inguinal hernias was 5.4% (14). When compared with the rate in radical prostatectomy, the lower rate in their cystectomy series was explained by the increased volume of the abdominal cavity after operation and lesser abdominal pressure provided by the operative procedure so that the peritoneum was left open. However, patients who receive an ileal neobladder need to strain to void. We speculate that this situation has inherent potential to increase to some extent the incidence of inguinal hernias in patients with an ileal neobladder.

CONCLUSIONS

Studer's ileal neobladder had a favorable long-term functional outcome in our study. Although the late complication rate was generally low and all complications were already known to occur, there were several patients who had to undergo CIC for their poor voiding condition resulting in a larger PVR. A unique complication was inguinal hernia, the rate of which was relatively high in our series. This is considered to be one of the definite late complications in patients with an ileal neobladder.

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$p16^{INK4a}$ and $p14^{ARF}$ methylation as a potential biomarker for human bladder cancer

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Abstract

Promoter hypermethylation is one of the putative mechanisms underlying the inactivation of negative cell-cycle regulators. We examined whether the methylation status of $p16^{INK4a}$ and $p14^{ARF}$, genes located upstream of the RB and p53 pathway, is a useful biomarker for the staging, clinical outcome, and prognosis of human bladder cancer. Using methylation-specific PCR (MSP), we examined the methylation status of $p16^{INK4a}$ and $p14^{ARF}$ in 64 samples from 45 bladder cancer patients (34 males, 11 females). In 19 patients with recurrent bladder cancer, we examined paired tissue samples from their primary and recurrent tumors. The methylation status of representative samples was confirmed by bisulfite DNA sequencing analysis. The median follow-up duration was 34.3 months (range 27.0–100.1 months). The methylation rate for $p16^{INK4a}$ and $p14^{ARF}$ was 17.8% and 31.1%, respectively, in the 45 patients. The incidence of $p16^{INKa}$ and $p14^{ARF}$ methylation was significantly higher in patients with invasive ($\geqslant$pT2) than superficial bladder cancer ($\leqslant$pT1) ($p = 0.006$ and $p = 0.001$, respectively). No MSP bands for $p16^{INK4a}$ and $p14^{ARF}$ were detected in the 8 patients with superficial, non-recurrent tumors. In 19 patients with tumor recurrence, the $p16^{INK4a}$ and $p14^{ARF}$ methylation status of the primary and recurrent tumors was similar. Of the 22 patients who had undergone cystectomy, 8 (36.4%) manifested $p16^{INKa}$ methylation; $p16^{INK4a}$ was not methylated in 23 patients without cystectomy ($p = 0.002$). Kaplan–Meier analysis revealed that patients with $p14^{ARF}$ methylation had a significantly poorer prognosis than those without ($p = 0.029$). This is the first study indicating that MSP analysis of $p16^{INK4a}$ and $p14^{ARF}$ genes is a useful biomarker for the pathological stage, clinical outcome, and prognosis of patients with bladder cancer.
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Keywords: Bladder cancer; Methylation; $p16^{INK4a}$; $p14^{ARF}$

Urothelial cancers of the urinary bladder are characterized by synchronous or metachronous multiplicity. At initial presentation, 70–80% of bladder cancer patients have superficial Ta or T1 tumors; the remainder presents with muscle-invading T2–T4 tumors [1]. About 70% of patients with superficial tumors suffer disease recurrence within 2 years; approximately one-third show grade- or stage progression [2–4]. Morphologically similar tumors can behave differently and it is currently not possible to identify patients who will experience tumor recurrence or disease progression. Synchronous or metachronous bladder cancers derive from monoclonal- rather than polyclonal origins [5,6], suggesting that genetic alterations may occur during early bladder carcinogenesis and that they are retained in the recurrent tumors. As cancer is characterized by alterations in cell-cycle regulation, changes in cell-cycle regulator genes at initial diagnosis may be useful for predicting tumor recurrence or progression.

The cell-cycle markers altered in bladder cancer include $p16^{INK4a}$, $p14^{ARF}$, MDM2, p53, p21, pRB, cdk4, and cyclin D1 [7–12]; all members of the RB and p53 pathway. However, the prognostic value of these factors remains to

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be determined. Among them, p16^{INK4a} and p14^{ARF} function as tumor suppressors by interrupting responses to hyperproliferative signals and modulating the activities of the Rb and p53 pathways. p16^{INKa} protein binds both *cdk4* and *cdk6*, and inhibits the catalytic activity of the cdk4-cdk6/cyclin D enzyme complex required for pRb phosphorylation which, during G1, disrupts the association with histone deacetylase and E2F transcription factors, thereby allowing the transcription of genes involved in cell-cycle progression. The p14^{ARF} protein induces G1 and G2 phase arrest in a p53-dependent manner, interacts with the Mdm2 oncoprotein, and inhibits the nuclear export of Mdm2 by tethering it in the nucleolus. This prevents Mdm2-p53 association and blocks Mdm2-induced p53 degradation in the proteosome, thereby stabilizing p53 [13].

CpG hypermethylation of the DNA promoter is important in the genetic regulation of mammalian cells [14]. Aberrant methylation of promoter regions associated with gene silencing is one of the major mechanisms underlying the inactivation of tumor suppressor genes; it has been observed in various cancers [15–20] including bladder cancer [7,21,22].

In the present study, we determined the methylation status in the promoter region of *p16^{INK4a}* and *p14^{ARF}*, analyzed the relationship between the methylation status and various clinicopathological parameters, and investigated whether promoter methylation of *p16^{INK4a}* and *p14^{ARF}* is a predictor of disease progression and of the prognosis of patients with bladder cancers.

Materials and methods

Tissue samples. We obtained 64 pathologically proven transitional cell carcinoma (TCC) samples from 45 bladder cancer patients who underwent transurethral resection (TUR) or radical cystectomy between March 1997 and December 2002 at Kagoshima University Hospital (Kagoshima, Japan). Of the 64 samples, 38 were from 19 patients with recurrence; we had samples from their primary and recurrent tumors, 8 specimens were from patients with non-recurrent tumors, and 18 from patients with invasive bladder cancers. The median follow-up was 34.3 months (range 27.0–100.1 months). The patients' background and clinicopathological characteristics are summarized in Table 1. Each tumor was staged and graded according to the TNM staging system [23] and the Japanese Urological Association and the Japanese Society of Pathology [24]. Normal human lymphocytes and normal bladder epithelium from the patients with non-cancerous disease served as the control. The study was approved by the Institutional Review Board of our institution; written prior informed consent was obtained from all patients for use of their samples and clinical and pathological data.

DNA extraction. DNA was extracted from formalin-fixed, paraffin-embedded, microdissected sections as described previously [25,26]. Briefly, 10-μm sections were mounted on microscope slides and one section from each sample was stained with hematoxylin and eosin. Cancerous regions of the stained tissues were identified and marked under a light microscope using 100-fold magnification. Microdissection of tissue on the remaining slides was performed by carefully scraping the cancerous areas into Eppendorf tubes with a surgical scalpel, using the stained slide as a template. In each case, we used fifteen 10-μm thick sections for DNA extraction. Genomic DNA from all samples was extracted with a QIAamp DNA Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The concentration of DNA was determined with a spectrophotometer; integrity was checked by gel electrophoresis.

Table 1
Patient characteristics

Parameters	No.
Patients	45
Age (years)	
Median	67.8
Range	45–88
Gender	
Female	11 (24.4)
Male	34 (75.6)
Tumor grade (%)	
G1	5 (11.1)
G2	30 (66.7)
G3	10 (22.2)
Pathological stage (%)	
pTa	9 (20.0)
pT1	17 (37.8)
pT2	6 (13.3)
pT3	10 (22.2)
pT4	3 (6.7)
Procedures	
TUR single[a]	8
TUR repeated	15
Cystectomy following TUR	4
Immediate cystectomy	18

[a] TUR, transurethral resection of bladder tumor.

Methylation analysis. The DNA methylation patterns in the CpG islands of *p16^{INK4a}* and *p14^{ARF}* genes were determined by methylation-specific PCR (MSP) as described previously [27]. Briefly, DNA (1 μg) from tumor samples was denatured with NaOH and then treated (50 °C, 16 h) with hydroquinone and sodium bisulfite. Modified DNA samples were purified with Wizard DNA purification resin according to the manufacturer's instructions (Promega, Madison, WI) and eluted into 50 μl water. NaOH was added to complete the modification and this was followed by ethanol precipitation. Resuspended DNA was used for PCR assay. The primers used for MSP and unmethylation-specific PCR (USP) were: p16^{INK4a} MSP primers, 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (forward), 5′-GACCCCG AACCGCGACCGTAA-3′ (reverse); p16^{INK4a} USP primers, 5′-TTATTAGAGGGTG GGGTGGATTGT-3′ (forward), 5′-CAACCCCAAACCACAACCATAA-3′ (reverse); p14^{ARF} MSP primers, 5′-GTGTTAAAGGGCGGCGTAGC-3′ (forward), 5′-AAAA CCCTCACTCGCGACGA-3′ (reverse); p14^{ARF} USP primers, 5′-TTT TTGGTGTTAAAGGGTGGTGTAGT-3′ (forward), 5′-CACAAAAA CCCTCACTCACAACAA-3′ (reverse). The 5′ end of the forward p16^{INK4a} MSP and p16^{INK4a} USP primers corresponds to base number 1132 in GenBank Accession No. X94154. The 5′ end of the forward p14^{ARF} MSP and p14^{ARF} USP primers corresponds to base numbers 195 and 201 in GenBank Accession No. L41934. The annealing temperature used for p16^{INK4a} MSP, p16^{INK4a} USP, p14^{ARF} MSP, and p14^{ARF} USP primers was 65, 60, 60, and 60 °C, respectively; 35 PCR cycles were applied [15,28]. CpGenome Universal Methylated DNA (UMD) (Intergen, Purchase, NY) was used as a positive control for methylated alleles. DNA from normal lymphocytes was the negative control for methylated genes [13]. DNA from normal ureteral epithelium of patients who had undergone nephrectomy for renal cell carcinoma was used as normal urothelial DNA. Amplified products were electrophoresed on 2% agarose gels, stained with SYBR Green I Nucleic Acid Gel Stain (TAKARA BIO, Tokyo, Japan), and visualized under UV illumination.

Bisulfite DNA sequencing analysis. Bisulfite-modified DNA (1 μl) was amplified in a total volume of 20 μl using a pair of universal primers as

follows: $p16^{INK4a}$ universal primers, 5′-GAGGAAGAAAGAGG AGGGGTT-3′ (forward), 5′-CTACAAACCCTCTACCCAC CTAAA-3′ (reverse); $p14^{ARF}$ universal primers, 5′-GTTGTTTATTTTTGGT GTTAAAGGG-3′ (forward), 5′-AACCTTTCCTACCTAATCTTCT AAAAAAC-3′ (reverse). These primers were designed without CpG sites in either the forward or reverse direction. The annealing temperature was 58 °C, 40 PCR cycles were used. Direct bisulfite DNA sequencing of the PCR products using forward universal primer was performed according to the manufacturer's instructions (Macrogen, Seoul, Korea).

Statistical analysis. We carried out a descriptive statistical study in which the analyzed variables were contrasted by Fisher's exact test. Kaplan–Meier survival curves of patients with bladder cancer classified according to the methylation status were prepared and compared using the log-rank test. p values of less than 0.05 were regarded statistically significant. All statistical analyses were performed using the StatView version 5.0 for Windows.

Results

Methylation status of the $p16^{INK4a}$ and $p14^{ARF}$ promoter in clinical samples

We analyzed the methylation status of $p16^{INK4a}$ and $p14^{ARF}$ promoter regions in normal urothelial- and bladder cancer specimens. Representative results of MSP and USP assays for $p16^{INK4a}$ and $p14^{ARF}$ are shown in Fig. 1. One of the superficial tumors (T1) had only one USP band. Both the primary (T13-1) and recurrent (T13-2) tumors from patient 13 also had only one USP band for $p16^{INK4a}$ and $p14^{ARF}$. One of the invasive tumors (T36) showed a band for $p16^{INK4a}$ and $p14^{ARF}$ on both MSP and USP. Normal human lymphocytes (NL) and normal ureteral epithelium (NUE) demonstrated only a USP band for $p16^{INK4a}$ and $p14^{ARF}$. The results of the methylation study were confirmed by bisulfite DNA sequencing. Fig. 2 shows the results typical for bisulfite DNA sequencing in a bladder cancer sample. In sample T1 (Fig. 1), no MSP band was observed; the CpG sites were completely unmethylated. Sample T36 (Fig. 1), which demonstrated both MSP and USP bands for $p16^{INK4a}$ and $p14^{ARF}$, disclosed a T peak and a C peak at the CpG sites, indicating partial methylation.

Methylation status of $p16^{INK4a}$ and $p14^{ARF}$ in bladder cancer

We performed MSP and USP to investigate the methylation status of $p16^{INK4a}$ and $p14^{ARF}$ promoter regions in normal urothelial- and bladder cancer specimens. The latter included 19 pairs of primary and recurrent tumor samples (38 samples); each pair obtained from the same patient (Fig. 3). In Table 2, we present the correlation between the methylation status of $p16^{INK4a}$ or $p14^{ARF}$ and several clinicopathological parameters. Overall, the methylation rate was 17.8% for $p16^{INK4a}$ and 31.1% for $p14^{ARF}$ in the 45 patients. Our series included 26 patients with superficial ($\leqslant$pT1) and 19 patients with invasive bladder cancer ($\geqslant$pT2). The incidence of $p16^{INK4a}$ methylation was significantly higher in patients with invasive (7/19, 36.8%) than superficial bladder cancer (1/26, 3.8%) ($p = 0.006$). The rate of $p14^{ARF}$ methylation was also significantly higher in patients with invasive (11/19, 58.0%) than superficial bladder cancer (3/26: 11.5%) ($p = 0.001$). Interestingly, during a median follow-up of 34.3 months, no methylation of $p16^{INK4a}$ or $p14^{ARF}$ was detected in patients 1–8 (Fig. 3) with superficial bladder cancer without tumor recurrence. Among 22 patients (patients 24–45, Fig. 3) who had received cystectomy, 8 (36.4%) demonstrated $p16^{INK4a}$ methylation; no $p16^{INK4a}$ methylation was found in 23 patients without cystectomy (Fig. 3). On the other hand, $p14^{ARF}$ methylation was found in 10 of 22 (45.5%) patients with cystectomy and in 4 of 23 (17.4%) who did not undergo cystectomy (Fig. 3). As shown in Table 2, there was a significant correlation between the methylation status of $p16^{INK4a}$ and the treatment outcome ($p = 0.002$). In the absence of a significant correlation, there was a trend between the methylation status of $p14^{ARF}$ and the treatment outcome ($p = 0.057$). During follow-up, 19 patients (patients 9–27, Fig. 3) suffered tumor recurrence. When we compared the methylation status of $p16^{INK4a}$ and $p14^{ARF}$ between the primary- and recurrent tumor in each of the 19 patients, we found that it was the same. There was no significant correlation, but a trend, between the methylation status of $p16^{INK4a}$ and tumor grade

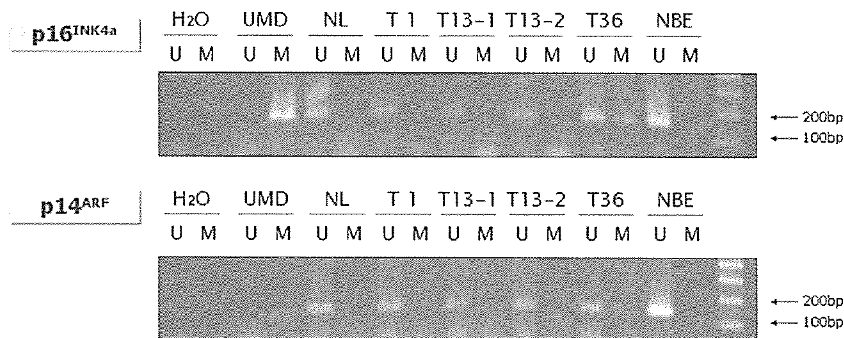

Fig. 1. Methylation-specific PCR of $p16^{INK4a}$ and $p14^{ARF}$ promoter regions. T1 is a non-recurrent tumor, T13 a sample from a patient with tumor recurrence. In both the primary (T13-1) and recurrent tumors (T13-2), $p16^{INK4a}$ was unmethylated. In one of the invasive tumors (T36), $p16^{INK4a}$ was both methylated and unmethylated. A similar result was obtained by MSP analysis of $p14^{ARF}$. Universal methylated DNA (UMD) with $p16^{INK4a}$ and $p14^{ARF}$ methylation served as the positive- and normal human lymphnode (NL) and normal bladder epithelium (NBE) as the negative control. M and U indicate methylation and unmethylation, respectively.

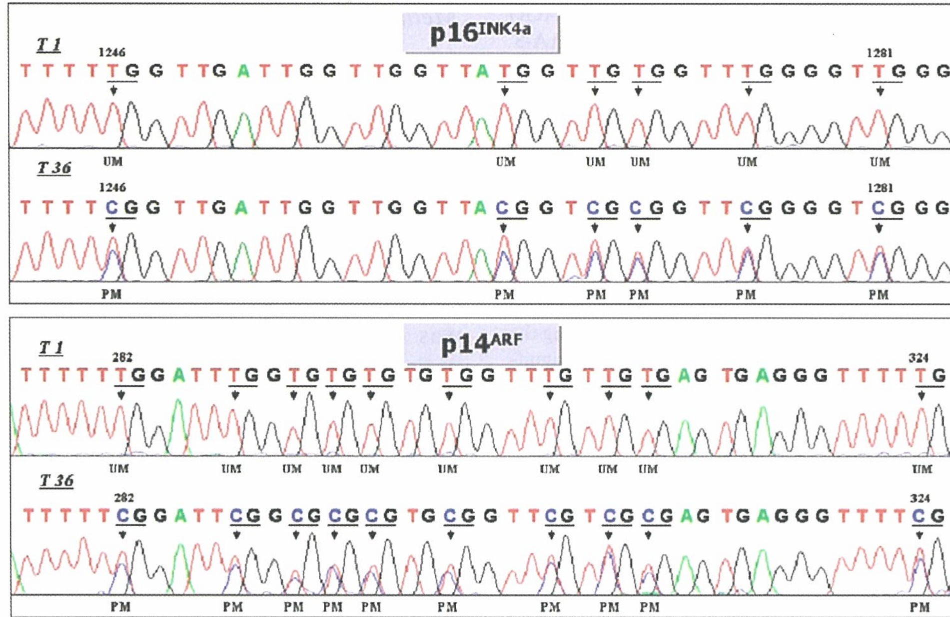

Fig. 2. Top: bisulfite DNA sequencing of the $p16^{INK4a}$ and $p14^{ARF}$ genes. Unmethylated (top lane) and partly methylated $p16^{INK4a}$ (bottom lane) samples correspond with samples labeled T1 and T36 in Fig. 1, respectively. Bottom: bisulfite DNA sequencing of $p14^{ARF}$. In unmethylated samples (T1, top lane), all CpG sites were unmethylated. In partly methylated samples (T36, bottom lane), there were a T- and a C peak at the CpG sites.

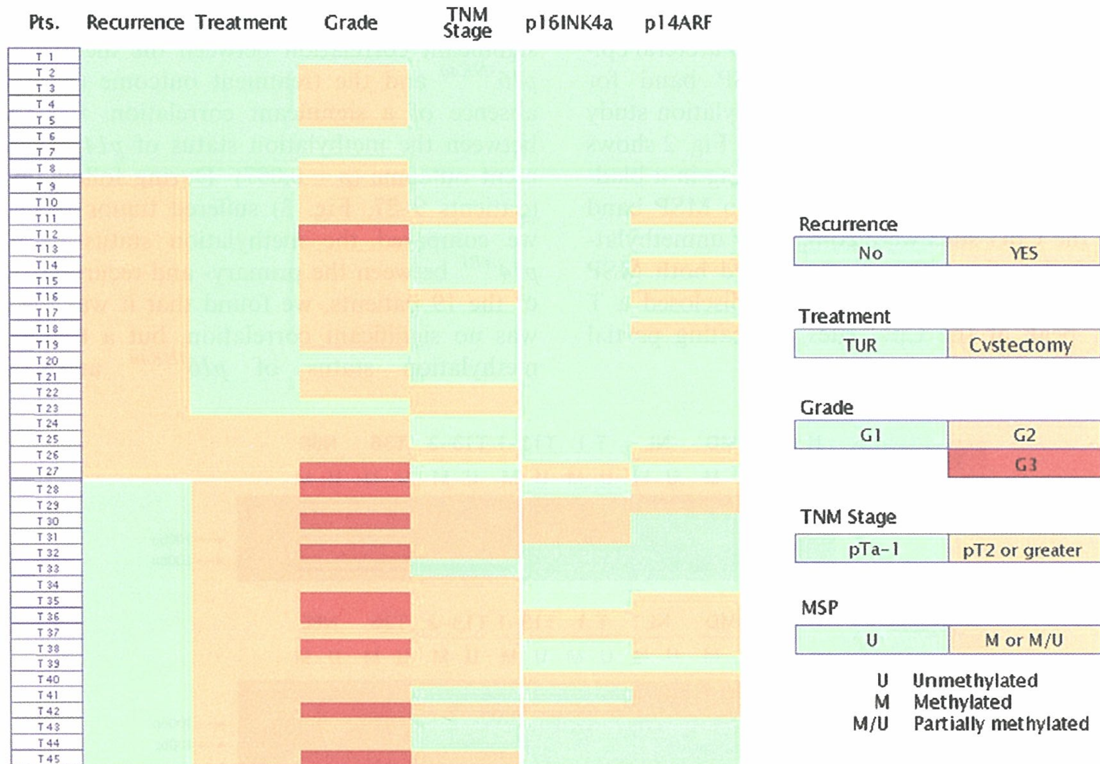

Fig. 3. Clinicopathological features and methylation status of 45 bladder cancer patients. In the $p16^{INK4a}$ and $p14^{ARF}$ columns, orange boxes correspond to complete methylation (only methylated product detected) or partial methylation (both unmethylated and methylated products detected). Green boxes indicate that only unmethylated product was detected. Of the 45 patients, 8 (T1–T8) had superficial bladder tumors without recurrence, 19 (T9–T27) suffered tumor recurrence during follow-up, and 18 (T28–T45) had invasive bladder cancers at initial diagnosis and had undergone immediate cystectomy.

Table 2
Correlation between methylation status and clinicopathological parameters in 45 bladder cancer patients

Clinicopathologocal outcome	Variable	Number of cases	$p16^{INK4a}$ [number (%)]			$p14^{ARF}$ [number (%)]		
			Methylated ($n = 8$)	Unmethylated ($n = 37$)	p value	Methylated ($n = 14$)	Unmethylated ($n = 31$)	p value
Histological type	G1/G2	35	4 (11)	31 (89)	0.059	9 (26)	26 (74)	0.244
	G3	10	4 (40)	6 (60)		5 (50)	5 (50)	
pT Stage	⩽pT1	26	1 (4)	25 (96)	0.006	3 (12)	23 (88)	0.001
	⩾pT2	19	7 (37)	12 (63)		11 (58)	8 (42)	
Recurrence	Recurrence(+)	19	1 (5)	18 (95)	>0.999	5 (26)	14 (74)	0.280
	Recurrence(−)	8	0 (0)	8 (100)		0 (0)	8 (100)	
Treatment outcome	Cystectomy(+)	22	8 (37)	14 (63)	0.002	10 (46)	12 (54)	0.057
	Cystectomy(−)	23	0 (0)	23 (100)		4 (17)	19 (83)	

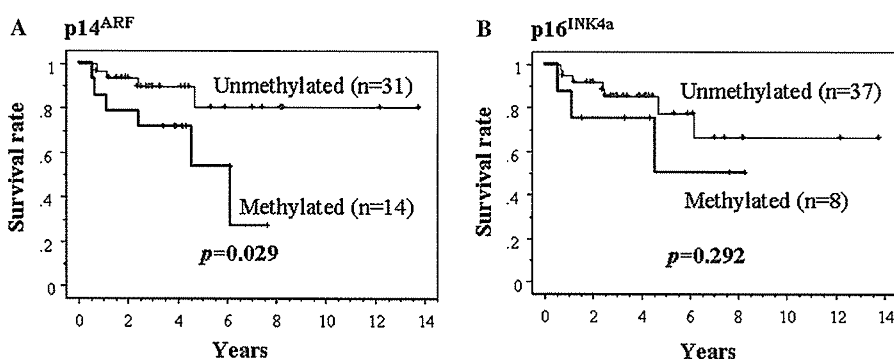

Fig. 4. Correlation of methylation status and overall survival by the Kaplan–Meier method. (A) Survival curves classified by the *p16*^{INK4a} methylation status. (B) Survival curves classified by the *p14*^{ARF} methylation status.

($p = 0.059$), and no correlation between the tumor grade and the methylation status of *p14*^{ARF} (Table 2).

Correlation between the methylation status of p16^{INK4a} *and p14*^{ARF}*, and prognosis*

We analyzed the connection between the methylation status of *p16*^{INK4a} and *p14*^{ARF}, and patient survival by Kaplan–Meier analysis. We found that *p14*^{ARF} methylation had a significant adverse effect on the prognosis ($p = 0.029$) (Fig. 4A). Similarly, patients with *p16*^{INK4a} methylation tended to have a poorer prognosis than those without although the difference was not statistically significant ($p = 0.292$) (Fig. 4B).

Discussion

Chromosome region 9p21 harbors genes *p16*^{INK4a} and *p14*^{ARF}, which have growth-suppressive activities [29,30]. p16^{INK4a} is a cyclin-dependent kinase inhibitor that functions upstream of Rb [31]. p14^{ARF} plays a major role in the p53 pathway by binding specifically to MDM2; it stabilizes both MDM2 and p53 [32,33], and thereby facilitates p53-mediated cell-cycle arrest and apoptosis [8]. The unique genomic structure and compact organization of these genes, which have common reading frames, may be essential for maintaining a balanced Rb and p53 pathway function [34]. CpG hypermethylation of the *p16*^{INK4a} or *p14*^{ARF} promoter can lead to cancer growth through the Rb or p53 pathway. Dominguez et al. [13] found a promoter hypermethylation rate of 18% for *p16*^{INK4a} and 56% for *p14*^{ARF} in bladder cancer patients; these rates were 15% [7] and 35% [35], respectively, in other reports, and 18% and 31%, respectively, in our study. These findings consistently show a higher incidence of *p14*^{ARF}- than *p16*^{INK4a} hypermethylation.

Bladder cancer recurrence has been attributed to an accumulation of genetic and epigenetic changes in tumor suppressor- and drug-resistance-related genes [36,37]. Our comparison of the primary and recurrent bladder cancers in 19 patients showed that their methylation status was the same. This observation suggests that the methylation of *p16*^{INK4a} and *p14*^{ARF} genes may be an early event in the tumorigenesis of human bladder cancer and supports the hypothesis that metachronous bladder cancers are derived from single progenitor cells rather than having polychronal origins [5,6].

We also found that the frequency of *p16*^{INK4a} and *p14*^{ARF} methylation was significantly higher in patients with invasive- than superficial bladder cancer ($p = 0.006$ and $p = 0.001$, respectively). This indicates that tumors with *p16*^{INK4a} and *p14*^{ARF} methylation are of a more aggressive phenotype. It is interesting that *p16*^{INK4a} and *p14*^{ARF} were unmethylated in 8 patients with superficial,

non-recurrent bladder cancers. In 22 patients who had undergone cystectomy, the rate of $p16^{INK4a}$ methylation was significantly higher than in 23 patients without cystectomy ($p = 0.002$). This suggests MSP analysis of $p16^{INK4a}$ and $p14^{ARF}$ as a useful biomarker not only for bladder cancer diagnosis and staging, but also for monitoring these patients for tumor recurrence. We found that $p14^{ARF}$ methylation was significantly associated with a poorer survival rate ($p = 0.029$). Dulaimi et al. [35] reported that MSP assay of urine detected bladder cancer more sensitively than conventional urine cytology. A method that can detect methylated genes in voided urine represents a marked advance over the currently used uncomfortable, invasive, and expensive procedures to diagnose bladder cancer. We are in the process of performing MSP analysis of $p16^{INK4a}$ and $p14^{ARF}$ in urine samples from patients with bladder cancer.

In conclusion, ours is the first study indicating a significant correlation between the results of MSP analysis of $p16^{INK4a}$ or $p14^{ARF}$ and the pathological stage, clinical outcome, and prognosis of patients with bladder cancer. Studies are underway in our laboratory to examine the practical implications of MSP analysis of $p16^{INK4a}$ and $p14^{ARF}$ in tissue and urine samples from patients with bladder cancer.

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REVIEW ARTICLE

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Early bladder cancer: concept, diagnosis, and management

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Abstract Recent evidence on molecular pathways helps us to understand the pathogenesis of bladder cancer. However, the molecular mechanisms of recurrence of the disease and progression into muscle-invasive disease are not fully understood. The diagnostic accuracy and specificity of innovative markers for detection of the disease currently available in the clinical setting are still far from the level where cystoscopy would not be needed. Although risk factors for progression to muscle-invasive disease have been identified, we still cannot predict accurately the clinical behavior of superficial bladder cancer. In this review article, we summarize recent evidence on molecular pathogenesis, risk factors for recurrence and progression, urine markers for detection, and treatments in superficial bladder cancer.

Key words Bladder · Cancer · Recurrence · Progression · Urine markers · Treatment

Introduction

Bladder cancer is the sixth most frequent malignant disease in the world. The age-adjusted incidence rates per 100000 persons in Japan were 12.5 for men and 2.9 for women in 1998, and almost 5000 patients died of the disease in 2001 (National Cancer Center. Cancer Statistics, http://www.ncc.go.jp/jp/statistics/index.html). More than 75% of patients with bladder cancer are diagnosed as having superficial disease that is confined to the mucosal and submucosal layers of the bladder at the time of the initial evaluation. The remaining bladder cancers are muscle-invasive disease or disease with cancer extending outside or beyond the bladder. Although the 5-year cause-specific survival rate for the superficial disease is expected to be higher than 95%,[1] the rate of intravesical recurrence is more than 50%, and up to 20% of the disease will become muscle invasive or metastatic over time, depending on clinical features, if the disease is untreated. In such cases, the 5-year survival of patients is less than 50% for muscle-invasive disease and 10% for metastatic disease.[2] In this contex, early diagnosis of the disease and prevention of intravesical recurrence and muscle-invasive progression after the initial treatment are crucial for providing a better prognosis.

Herein we summarize recent advances in the diagnosis and treatment of superficial bladder cancer, which is usually considered to be the early stage of the disease.

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What is early bladder cancer and how does it behave clinically?

Definition of early bladder cancer

As already stated, superficial bladder cancer is defined as disease that is confined to the mucosal and submucosal layers of the bladder (Ta and T1, respectively, in the TNM classification). It encompasses a wide range of disease from noninvasive low-grade papillary cancer (Ta) with almost no progression to muscle-invasive disease to superficially invasive (T1) high-grade papillary or solid cancer with significant frequency of progression into the muscle of the bladder and subsequent cancer death (Table 1).[3–5] Carcinoma in situ (CIS) is also included in superficial bladder cancer.

According to the World Health Organization/International Society of Urological Pathology Consensus Classification of Superficial Bladder Cancer,[3] noninvasive diseases consist of papilloma, papillary urothelial neoplasm with low malignant potential, and papillary cancer of Ta grade 1 (G1) or high grade (Ta grade 2 or 3). Grade 3 (G3) cancer is well accepted as high-grade disease, but grade 2 (G2) encompasses a wide range of biological characteristics, some resembling G1 and others G3. However, because G2 disease can potentially mimic G3 disease, clinical under-

Table 1. Classification of superficial bladder cancers and their potential for disease progression

TNM classification[a]	World Health Organization (WHO) grade[a]	Progression (%)[b]	Cancer death (%)
Ta (confined to the mucosa)	Grade 1	<10%	<5%
	Grade 2	20%	10%
	Grade 3	20%–40%	10%–25%
T1 (invasion to the submucosa)	Grade 1	20%	20%
	Grade 2	25%	25%
	Grade 3	40%–50%	30%–40%
Carcinoma in situ			
Primary		>50%	–
Secondary			

Each figure was estimated from the results reported in references 3, 4, and 5
[a]General Rules for Clinical and Pathological Studies on Bladder Cancer (3rd edition, 2001)
[b]Progression to muscle-invasive or metastatic disease

standing is that the disease is characterized as high-grade cancer. Ta disease accounts for 70%–80% of superficial bladder cancer. Because T1G1 disease is rare, a case of it consists of high grade (G2 or G3) cancer, which comprises the remaining 20%–30% of superficial disease.[4] Primary CIS is not so common, but the secondary type is often associated with high-grade T1 superficial cancer or muscle-invasive disease. Thus, in CIS, the primary type accounts only for 10% and the secondary type for 90%.

Molecular pathogenesis of superficial bladder cancer

Receptor tyrosine kinase-Ras pathway, deletion of chromosome 9, and mutation of fibroblast growth factor receptor 3 and HRAS genes

Superficial bladder cancer has at least two discrete pathways for development and progression (Fig. 1), which may explain the differences in the invasive and metastatic potential of the disease. Constitutive activation of the receptor tyrosine kinase (RTK)-Ras pathway and deletion of chromosome 9 are mainly responsible for early development of papillary Ta superficial bladder cancer.[6–8] The former category includes mutation of fibroblast growth factor receptor 3 (*FGFR3*), found in 60%–70%, and *HRAS*, in 30%–40%, of the disease.[9,10] These changes are mutually exclusively detected in the disease, indicating that they are pivotal events for its development.[7,8,11] Partial or total deletion of chromosome 9 occurs in urothelial hyperplasia and low-grade papillary Ta cancer. Both 9q and 9p losses are involved in development of these neoplastic lesions. Indeed, a tumor suppressor gene may be located in the region at 9q32–33. In addition, the 9p21 region in the *INK4A/ARF* locus is also speculated to have a tumor suppressor gene because the region encodes both p16 and p14 and homozygous deletion of the region downregulates both the RB and p53 pathways. Furthermore, homozygous deletion at the *INK4A/ARF* locus is associated with superficial cancer having higher grade, larger size, and more predominant recurrence than that without these features.[12]

Although chromosome 9 deletions were initially indicated to be early events in the development of low-grade Ta bladder cancer, they were subsequently revealed to participate in development of high-grade cancer and, sometimes, of dysplasia and CIS.[13]

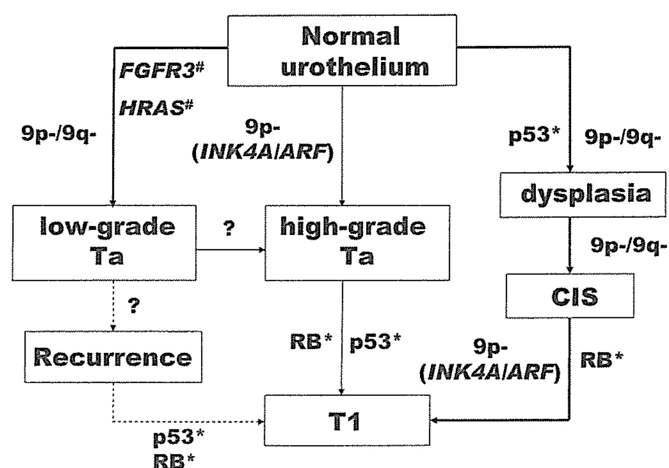

Fig. 1. Molecular pathogenesis and progression of superficial bladder cancer. *CIS*, carcinoma in situ; #, mutations; *, structural and functional defects; *thick arrows*, frequent pathway; *fine arrows*, infrequent pathway; *dotted arrows*, rare pathway

Inactivation of p53 and RB functions

Although chromosome 9 deletions may be partly involved in development of high-grade Ta or T1 bladder cancer and CIS, the pivotal pathway to the diseases is the inactivation of both p53 and RB functions. The inactivation of the p53 function is demonstrated by mutation of *TP53* or homozygous deletion of p14. A lack of RB expression or hyperphosphorylated RB protein may result in dysfunctional RB. Thus, both the p53 and RB dysfunctions are simultaneously found in more than 50% of high-grade T1 bladder cancers. Figure 1 illustrates simply molecular pathways in the development of superficial bladder cancer consisting of low-grade or high-grade disease with Ta or T1 and CIS.[6–8]

Above all, activation of the RTK-Ras pathway and deletion of chromosome 9 may be responsible for the development of low-grade Ta disease. The deletion of 9p may be involved in development of high-grade Ta disease. For the

progression of Ta disease or CIS to T1, functional defects of the p53 and/or RB pathways may be required.

Natural history of early bladder cancer and risk factors for recurrence and progression

Natural history

Superficial (Ta and T1 disease) bladder cancer is initially treated by transurethral resection of the bladder tumor (TUR-Bt). Once histopathological examination of the specimen reveals the disease to be muscle invasive, radical cystectomy is basically indicated. If examination determines that the disease is confined to the mucosal (Ta) or submucosal (T1) layer of the bladder, intravesical instillation of an anticancer agent (intravesical chemotherapy) or bacillus Calmette–Guerin (BCG) may be indicated, depending on the clinical and pathological features. When primary carcinoma in situ (CIS) is found, intravesical treatment with BCG is the standard therapy. The intravesical treatment is employed for patients after TUR-Bt, with the hope that the treatment can reduce intravesical recurrence and prevent developing muscle-invasive disease. Indeed, the major clinical issue in treatment of superficial bladder cancer is how to prevent these unfavorable events and to identify patients who need intensive treatment after TUR-Bt. In some situations, even patients with superficial disease can potentially be candidates for radical surgery. Such examples are the disease with frequent recurrence with a short interval and high-grade T1 disease refractory to intravesical chemotherapy or BCG therapy after TUR-Bt.

Mechanism of intravesical recurrence

The cellular and molecular biological mechanisms of intravesical recurrence have not been fully elucidated. Two hypotheses have been proposed to explain the mechanism of intravesical recurrence.[14–17] The first hypothesis, the "field defect," stems from the speculation that endogenous and exogenous carcinogenic agents are the main causes of bladder cancer development. According to this speculation, carcinogen-elicited genetic changes of individual cells in the bladder are responsible for synchronous and metachronous development of cancer, which translates into intravesical recurrence in the clinical setting. The intravesical seeding or intraepithelial spread of cancer cells is the second explanation for the development of recurrence. This hypothesis depends on the concept that an individual cancer arises from a single transformed cell. In the situation of intravesical seeding, floating cancer cells that detach from the primary site are implanted in the mucosa of the bladder and proliferate into full-blown cancer. The rationale for this is the clinical finding that a single course of immediate intravesical chemotherapy after TUR-Bt significantly reduces the intravesical recurrence of cancer.[18] Cancer cells can also spread in the intraepithelial layer, which results in the development of overt cancer at a remote site. Based on the results of molecular studies currently available, most bladder cancers are of monoclonal (or oligoclonal) origin, but the "field defect" may be found in a minority of cancers.[16] In addition, epigenetic modification and changes of cancer cells in the cellular microenvironment are also suggested to be crucial for cancer evolution.[19]

As for the type of recurrence, Hinotsu et al.[20] proposed the hypothesis that the recurrence had two patterns, consisting of those in early and late phases after TUR-Bt. They designated the early type as "true recurrence" and the late type as a "new second recurrence, i.e., a second primary cancer." Most recurrences in the early phase occurred within 500 days post-TUR-Bt, whereas the late ones occurred more than 500 days postsurgery. Thus, early recurrence may response to intravesical treatment, resulting in the prevention or delay of intravesical recurrence. However, the standard intravesical treatment, usually lasting less than a year, does contribute to the prevention and delay of late recurrence. Unfortunately, it is not fully elucidated how this type of recurrence is regulated by the cellular and molecular biological characteristics of cancer cells. Thus, many molecular events in the development of bladder cancer have been clarified during the past 15 years. However, we still do not know what molecular events are actually involved in the establishment of a recurrence, irrespective of its mechanism.

Risk factors for recurrence and progression

Landmark work done by the National Bladder Cancer Collaborative Group in the 1980s revealed the clinical course of patients who were treated only with TUR-Bt. The study indicated that more than 50% of superficial disease developed intravesical recurrence, and that more than 20% of progression was characterized by muscle invasion, depending mainly on the grade and pathological stage of the disease.[21] Indeed, the 3-year recurrence-free survival rate is higher in Ta disease than in T1 disease (50% vs. 30%). The rates were also affected by tumor grade, with 50% in G1 disease, 40% in G2, and 20% in G3. The pathological stage affected the progression rate of disease to the muscle layer. In Ta disease, the 3-year progression-free survival rate was 96%, whereas survival decreased to 70% in T1 disease. The rate of G3 disease (55%) was distinctly different from that of G1 (100%) and G2 (89%) disease. Thus, the pathological stage and grade are factors predictive of recurrence and progression in short- or intermediate-term follow-up of superficial bladder cancer. Multicentricity and tumor size and dysplasia found at a site in the bladder other than that of overt cancer are also listed as clinical and pathological factors affecting recurrence and progression of the disease.

As indicated earlier, the stage and grade of cancer greatly influence the clinical behavior of superficial bladder cancer. In this context, T1G3 disease has a definitely different behavior from TaG1 or TaG2. TaG3 disease has intermediate character between these diseases. The different clinical behavior is clearly demonstrated when we consider the rate of progression to muscle-invasive disease in studies