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		Total No.
	Yes	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

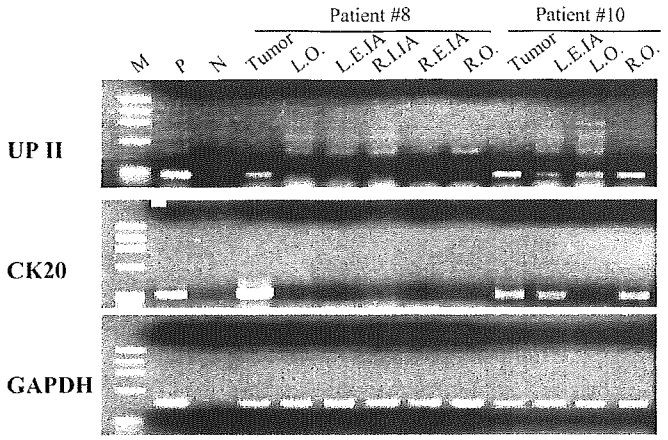

FIG. 2. UP II and CK 20 mRNA expression was detected by RT-PCR in primary bladder tumor and pelvic lymph node tissues. *Lane M*, DNA marker. *Lane P*, positive control. *Lane N*, negative control. *L*, left. *O*, obturator. *E*, external. *IA*, iliac artery. *R*, right. *I*, internal.

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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$p16^{INK4a}$ 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.

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

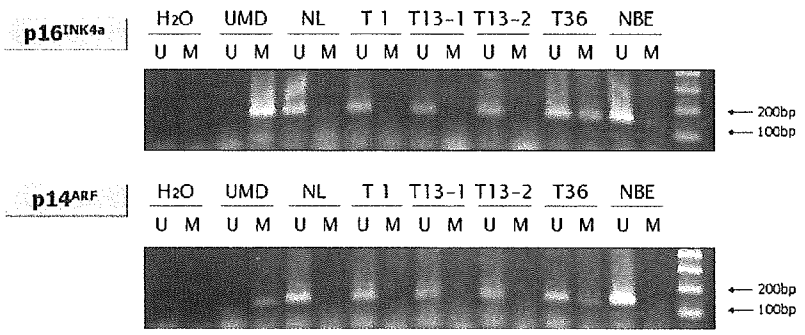

Fig. 1. Methylation-specific PCR of $p16^{INK4a}$ and $p14^{ARF}$ promoter regions. T1 is a non-recurrent tumor, T13 a sample from a patient with tumor recurrence. In both the primary (T13-1) and recurrent tumors (T13-2), $p16^{INK4a}$ was unmethylated. In one of the invasive tumors (T36), $p16^{INK4a}$ 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.

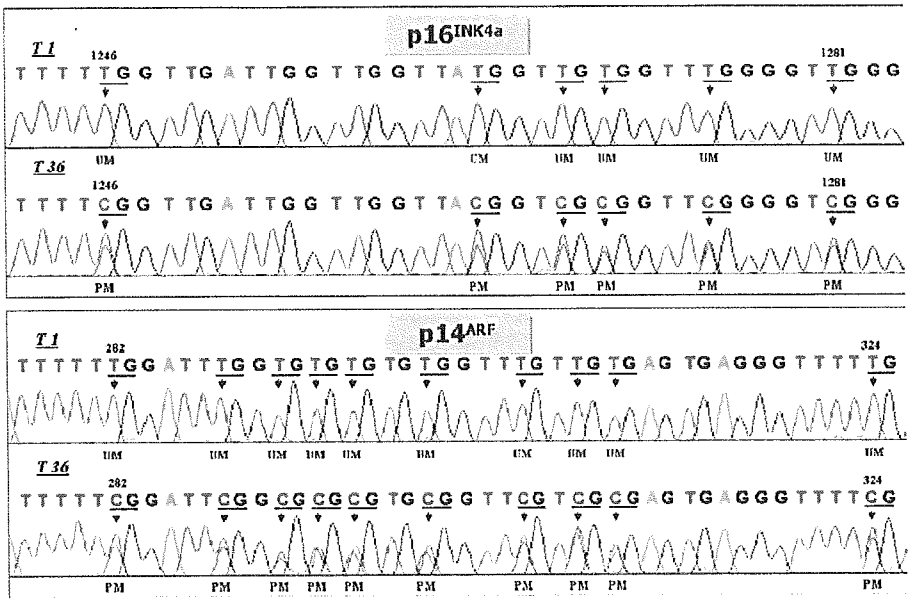

Fig. 2. Top: bisulfite DNA sequencing of the $p16^{INK4a}$ and $p14^{ARF}$ genes. Unmethylated (top lane) and partly methylated $p16^{INK4a}$ (bottom lane) samples correspond with samples labeled T1 and T36 in Fig. 1, respectively. Bottom: bisulfite DNA sequencing of $p14^{ARF}$. In unmethylated samples (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.

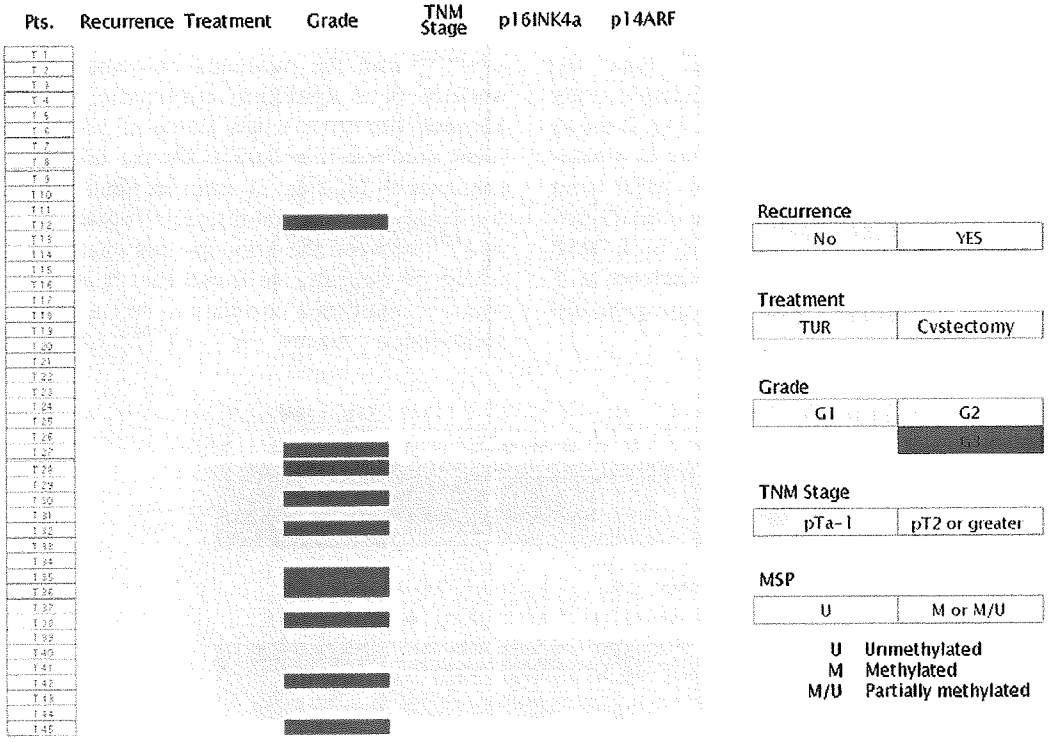

Fig. 3. Clinicopathological features and methylation status of 45 bladder cancer patients. In the $p16^{INK4a}$ and $p14^{ARF}$ columns, orange boxes correspond to complete methylation (only methylated product detected) or partial methylation (both unmethylated and methylated products detected). Green 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)	

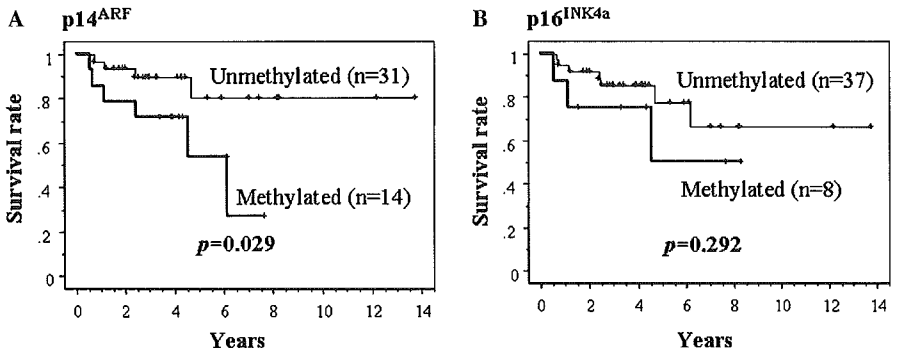

Fig. 4. Correlation of methylation status and overall survival by the Kaplan–Meier method. (A) Survival curves classified by the $p16^{INK4a}$ methylation status. (B) Survival curves classified by the $p14^{ARF}$ methylation status.

($p = 0.059$), 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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HD-MVAC 療法が著効した尿路上皮癌の2症例

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TWO CASES OF UROTHELIAL CANCER RESPONSIVE TO HIGH-DOSE-INTENSITY METHOTREXATE VINBLASTINE DOXORUBICIN AND CISPLATIN (M-VAC) THERAPY

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We report two cases of metastatic urothelial cancer treated with high-dose-intensity (HD) methotrexate vinblastine, doxorubicin and cisplatin (MVAC). After HD-MVAC, the size of primary and lymph node tumors decreased by 57–67% as compared with the standard M-VAC therapy. By shortening the dose interval, HD-MVAC may increase the anti-tumor effect without increasing side effects.

(Hinyokika Kiyo **51**: 113-116, 2005)

Key words: Urotherial cancer, Chemotherapy, High dose intensity M-VAC

緒　　言

膀胱癌に対する化学療法として最も一般的な M-VAC 療法の奏功率は39～70%（CR 13～50%）と比較的高いが[1~3]，長期予後が不良など満足できる成績はえられていない[3]．

Sternberg ら[4]が2001年に報告した high-dose-intensity-MVAC（HD-MVAC）療法は，従来の MVAC 療法の15，22日目の methotrexate（MTX）と vinblastine（VBL）の投与を省略し，4～10日目の計画的な G-CSF 投与により1コースの期間を2週間へ短縮する方法である（Table 1）．今回，われわれは HD-MVAC 療法を行い，重篤な副作用なしに顕著な腫瘍縮小効果がえられた進行膀胱癌と進行尿管癌の2症例を経験したので報告する．

Table 1. HD-MVAC 療法と M-VAC 療法のメニュー比較

	HD-MVAC	M-VAC
1コースの期間	2週間	4週間
Day 1	MTX 30 mg/m^2	MTX 30 mg/m^2
Day 2	VBL 3 mg/m^2	VBL 3 mg/m^2
	ADR 30 mg/m^2	ADR 30 mg/m^2
	CDDP 70 mg/m^2	CDDP 70 mg/m^2
Day 4-10	G-CSF 240 μg/m^2	
Day 15・22	なし	MTX 30 mg/m^2
		VBL 3 mg/m^2
G-CSF	必要なら14日間投与，好中球が3.0×10^4/μL 以上で投与中止	とくに規定なし

症　　例

症例1

患者：70歳，男性

主訴：右腰背部痛

家族歴・既往歴：特記事項なし

現病歴：2001年5月上記主訴にて近医を受診．骨盤部の MRI によって，右大腿骨に転移性腫瘍と膀胱内に浸潤性腫瘍，また胸部写真にて両肺野に転移性腫瘍を認めたため，肺・右大腿骨転移を伴う膀胱癌と診断された．右大腿骨転移巣への 30 Gy の放射線照射後，7月18日当科に転科した．画像所見および生検から TCC，G2～3，T4N0M1 と診断，8月2日から HD-MVAC 療法を開始した．

G-CSF は原法と異なり顆粒球数をみながら投与した．白血球数は最低値で 2,400/μl であり，3,000/μl 未満は2日間であった．合計3コース施行したが各コース間の休薬期間は2日・9日間であり，顆粒球数が回復するのを待ってから次のコースを開始した．3コース施行後，多発肺転移は最大径 4 cm あったものを含め胸部写真上すべて消失し，CT 上も病巣の多く

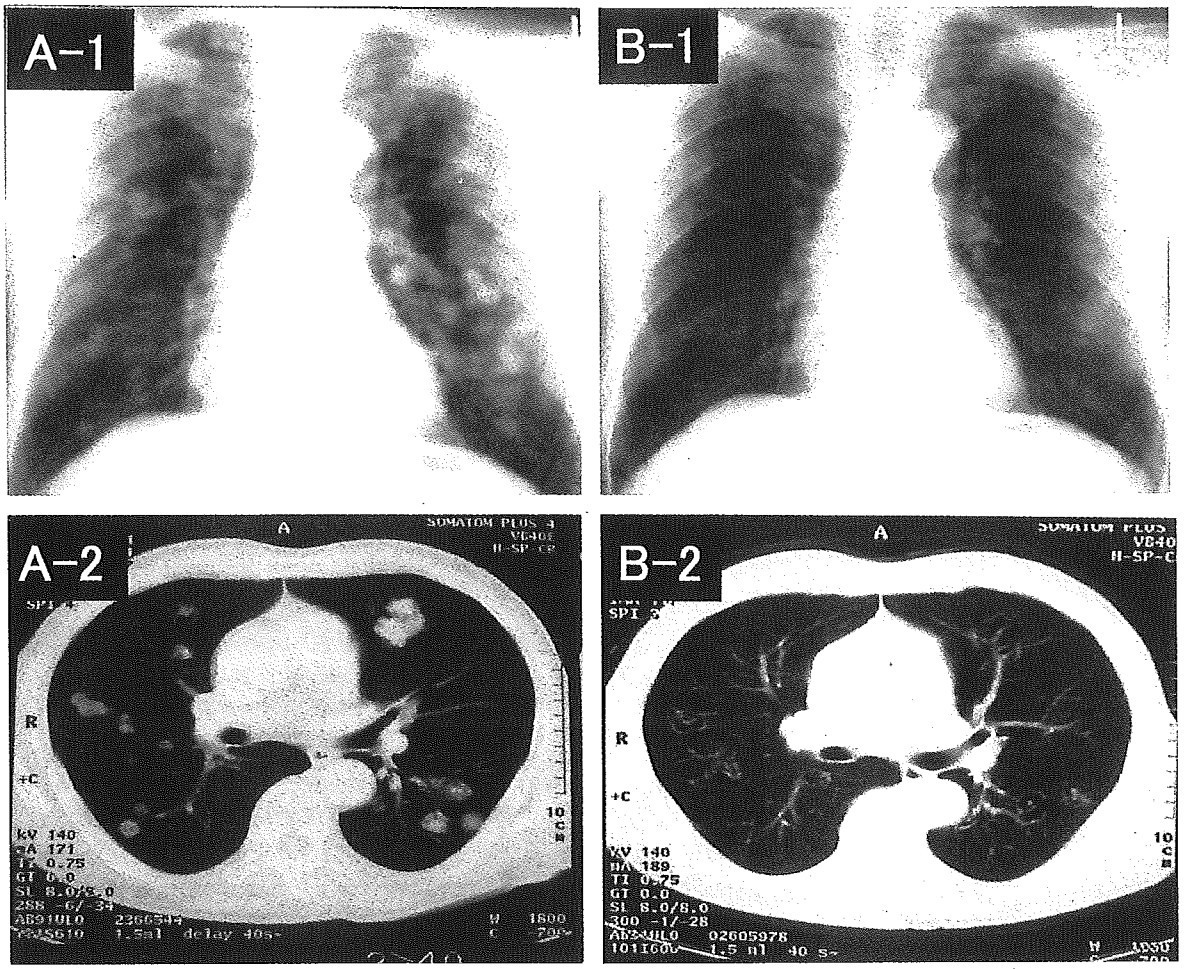

Fig. 1. ChestXP and computed tomography (CT) images before (A-1, A-2) and after (B-1, B-2) chemotherapy.

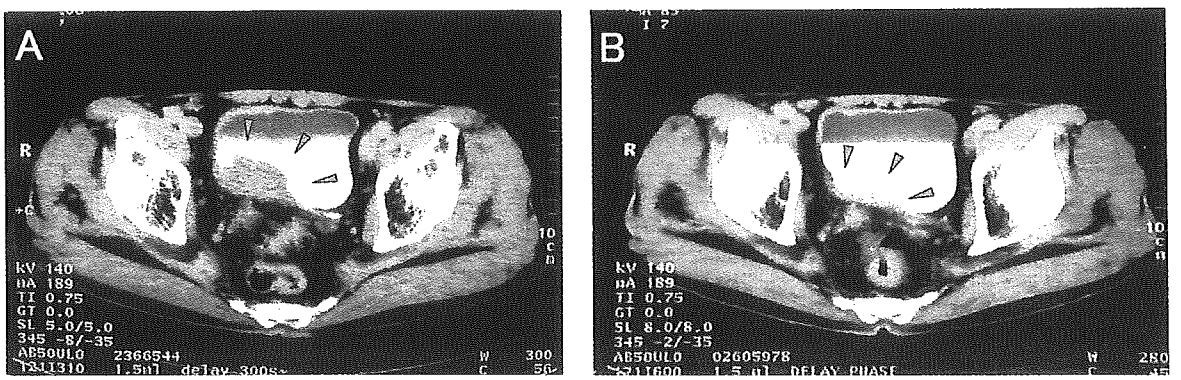

Fig. 2. Computed tomography (CT) images of the pelvis before (A) and after (B) chemotherapy in case 1. The size of bladder tumor (surrounded by arrow heads) decreased after chemotherapy.

は壊死による空洞化を呈した (Fig. 1). 骨盤部 CT では右壁に認めた最大経 5 cm の腫瘍は著明に縮小 (縮小率57%) した (Fig. 2). 患者の QOL を考慮し10月 9 日右腎尿管膀胱全摘出術および左尿管皮膚瘻造設術を施行した. 膀胱内には viable な癌細胞を認め, 術後の病理診断は TCC, G3>G2, pT3b であった.

症例 2

患者：70歳, 女性

主訴：腰背部痛, 肉眼的血尿

既往歴：高血圧

家族歴：父親が脳出血　妹が高血圧

現病歴：2001年 7 月上記主訴にて近医受診. エコーにて左水腎症と左尿管口付近に腫瘍を認め, 7 月18日に当科紹介入院となった. CT にて左下部尿管より第 4 腰椎へ直接浸潤する径約 5 cm の腫瘍と大動脈周囲リンパ節転移を認め, 尿細胞診は class IV であった.

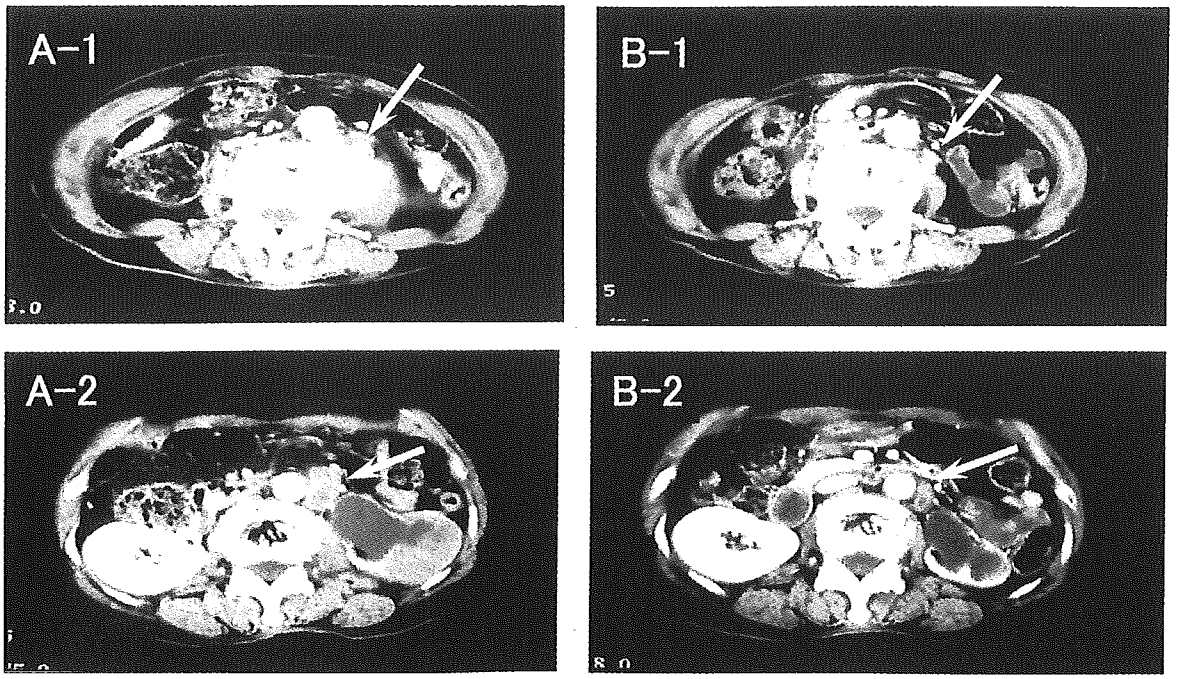

Fig. 3. Computed tomography (CT) images before (A-1, A-2) and after (B-1, B-2) chemotherapy in case 2. The maximal diameters of ureteral tumor and para-aortic lymph node metastasis (A-2 and B-2, arrow) decreased from 5.0 to 3.2 cm and 3.0 to 2.1 cm, respectively.

膀胱鏡では左尿管口内部より膀胱内に突出する腫瘍を認めた．腫瘍生検を2箇所で施行したが，検体が壊死組織であったため病理診断が不能であった．画像診断および細胞診より尿管癌 T4N1M1 と診断し，8月1日より HD-MVAC 療法を開始した．2コース目開始後7日目に白血球数 900/μl，血小板数9.9万/μl と骨髄抑制をみたが，G-CSF 投与にて改善した．消化器症状は1コース目から生じたが，対処療法で軽快した．3コース目の実施は十分可能と判断したが，食指不振などに対する患者の不安が強く，2コースでの終了となった．尚，各コース間の休薬期間は0日間であった．2コース後の CT で原発巣は64%，腎門部リンパは67%の縮小率を示し，主訴の腰痛は消退した (Fig. 3)．

考　　察

M-VAC 療法はその奏効率の高さから，尿路上皮癌化学療法の中心となっている．しかし当初発表された71%（CR 50%）という高い奏効率は[1]，大規模追試では39%（CR 13%）と当初の成績より低く，さらに2年生存率が20%と近接効果が予後に反映されないことが問題になっている[3]．Scher ら[5]が述べるように，一定以上の治療効果をえるには3コース以上の施行が必要である．しかし，本邦では副作用のため多くの症例で投与量が70%以下に減量され，原法通りに3コース以上施行できることが少ないのが実情である[6]．そのため，副作用を増大せず治療効果をあげる新しい試みが模索されている．

抗癌剤の一回投与量を増やす dose escalation は抗腫瘍効果と同時に副作用も増大する[7]ことから，M-VAC 療法原法を越える標準療法とはなっていない．また，新規抗癌剤として taxol 系や gemcitabine などが尿路上皮癌に有効と報告されているが，現時点では M-VAC 療法を明らかに上回る成績は報告されておらず[8]，保険適用の面からも日常診療で広く用いられていない．これに対し HD-MVAC 療法は，使用薬剤の用法は原法とほぼ同じだが投与間隔を半分にすることで dose intennsity をあげ効果増強を図ったものである．今回の経験のみで M-VAC 療法と比較することはできないが，肺転移の消失（CR）や57～67%の腫瘍縮小率など十分に効果増強を期待できる結果と言える．しかし，HD-MVAC 療法を行っても評価病巣が PD などである症例について，当院では現在 taxol 系や gemcitabine を用いた治療を行っており，HD-MVAC 後に肝転移の出現した症例に gemcitabine，carboplatin にて CR となった症例を経験している[9]．

HD-MVAC 療法には期間当たりの抗癌剤投与量増加による副作用増悪が懸念された．我々が経験した HD-MVAC 療法と M-VAC 療法の最近の5例（2コース施行が3例，3コースが2例）について，骨髄抑制と消化器症状の程度を共通毒性基準にて評価比較した (Table 2)．HD-MVAC 治療症例は70歳という高齢にもかかわらず，M-VAC 療法症例と骨髄抑制に明らかな差を認めず，むしろ消化器症状については軽度であった．消化器症状が軽度に抑えられた理由と

Table 2. 当科症例における HD-MVAC 症例と M-VAC 症例における主要な副作用についての比較

	M-VAC (n=5)	HD-MVAC (n=2)
骨髄抑制		
白血球 Grade 3	1-6 (2.8) 日	0-2 (1) 日
Grade 4	0-6 (1.2) 日	0-1 (0.5) 日
血小板 Grade 3	0-8 (1.8) 日	0-2 (1) 日
Grade 4	0 (0) 日	0 (0) 日
G-CSF 投与回数	2-39 (20) 回	10-31 (20.5) 回
G-CSF 投与割合	0.67-13 (8.63)	5-10.3 (7.65)
消化器症状		
食思不振 Grade 3	3-21 (10.5) 日	1-2 (1.5) 日
Grade 4	8-19 (12.8) 日	1-10 (5.5) 日
嘔吐回数	0-4回	0-1回
嘔吐日数	0-7 (3) 日	0-3 (1.5) 日

( ) 内は平均値を示す. G-CSF 投与割合は1コース当たりに投与した G-CSF の投与回数を示す.

してはシスプラチンの投与量が同じでメソトレキセートとビンブラスチンの投与量が少ないことが考えられる. しかし Sternberg ら[4]の報告では HD-MVAC は MVAC と比較して消化器系副作用はわずかに重い傾向があり, われわれの結果は症例数が少ないためである可能性は否定できない.

また, 顆粒球減少については, 両療法ともにほぼ同量の G-CSF にて十分コントロール可能であった点が注目される. HD-MVAC 療法の原法通りに G-CSF の計画的投与をしたならば, 顆粒球数はより高く維持できたと考えられる.

HD-MVAC 療法は抗癌剤の投与が各コースの第1日目・2日目のみであり, 次コースまでの連続した G-CSF 投与が行える点でも骨髄抑制のコントロールが容易と思われる.

以上より HD-MVAC 療法は Sternberg[4] の報告のように, 副作用の増悪なく効果増強の可能性が有り, M-VAC 療法に代わる化学療法として症例を重ねて長期成績を検討したい.

結　　語

High-Dose-Intensity-M-VAC (HD-MVAC) 療法の著効した尿路上皮癌の2例を報告した. 通常の M-VAC 療法に比して副作用の明らかな差がなく効果増強が期待できる化学療法と思われた.

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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]

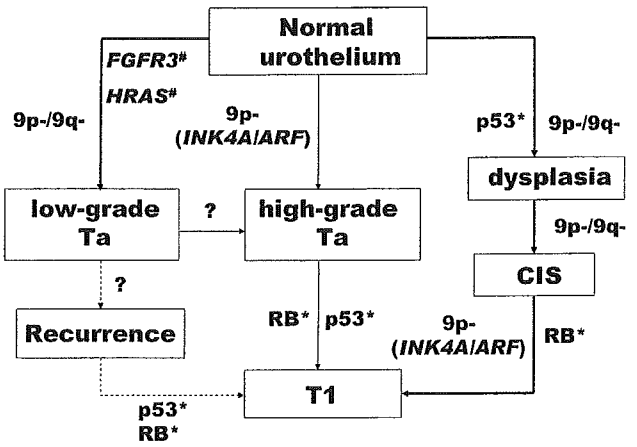

Fig. 1. Molecular pathogenesis and progression of superficial bladder cancer. *CIS*, carcinoma in situ; #, mutations; *, structural and functional defects; *thick arrows*, frequent pathway; *fine arrows*, infrequent pathway; *dotted arrows*, rare pathway

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

with long-term follow-up (see Table 1). Although TaG1 and TaG2 disease has lower progression and cancer death rates, T1G3 has much higher rates, which is one of the reasons why early radical cystectomy is recommended for the disease.[22] Even when the disease is treated by TUR-Bt, postoperative intravesical treatment with BCG and careful observation of the clinical course are mandatory.

CIS is another type of superficial bladder cancer. Most of the disease is found in association with papillary or solid high-grade disease. Primary CIS defined clinically as bladder cancer without any visible tumor by cytoscopy. The final diagnosis is only made with histopathological examination. Intravesical BCG treatment can eradicate cancer cells in the bladder in more than 70% of patients with the disease. The 5-year recurrence-free rate is 60%. However, the rate of progression to muscle-invasive or metastatic disease is significant.

How do we detect early disease?

Usual presentation and detection

Urinalysis, urine cytology, and cystoscopy are well accepted as the standard methods for detecting bladder cancer (Fig. 2). Even if urinalysis reveals microscopic hematuria, however, it is unclear whether the hematuria is derived from bladder cancer. Moreover, hematuria in bladder cancer may be intermittent. Cytology has high specificity but the diagnostic value is far from satisfactory, particularly for low grade or Ta superficial bladder cancer. Cytoscopy can identify most superficial disease, but it sometimes fails to detect a small or flat lesion. Its greatest disadvantage is that it is an invasive diagnostic procedure. Thus, flexible fiberscopy is the current procedure of choice as it provides a clear intravesical view with less invasiveness. In this context, novel specific markers for bladder cancer are expected.

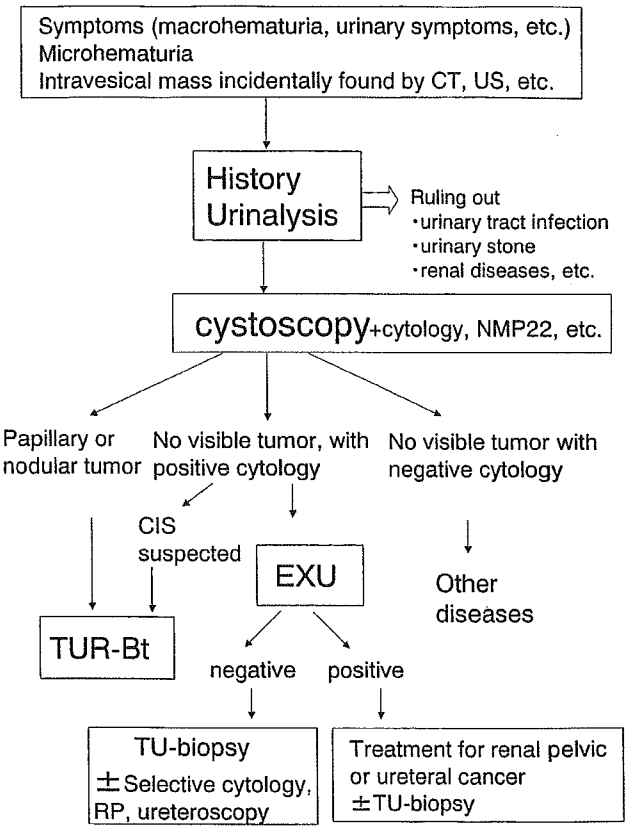

Fig. 2. Algorithm for diagnosis of bladder cancer. *EXU*, excretory urography; *TUR-Bt*, transurethral resection of bladder tumor; *TU-biopsy*, transurethral biopsy; *RP*, retrograde pyelography

Innovative methods for detection

Commercially available molecular markers

Several urinary markers for bladder cancer have been investigated in recent years. Unfortunately, no serum marker is as yet available for clinical use.

Bladder tumor antigen (BTA)stat, BTA TRAK, nuclear matrix protein (NMP)22, fibrinogen degenerative product (FDP), ImmunoCyt, and fluorescence in situ hybridization (FISH) (UroVysion) tests for bladder cancer have been approved by the U.S. Food and Drug Administration.[23,24] Bladder tumor antigen (BTA) is a human complement factor H-related protein that is similar in structure and function to human complement factor H.[25,26] The BTAst at test (Mentor, Santa Barbara, CA, USA) is an immunoassay using two monoclonal antibodies recognizing two epitopes on human complement factor H-related protein.[27] It is a one-step, qualitative, immunochromatographic assay whereas BTA TRAK (Polymedco, Cortlandt Manor, NY, USA) is a quantitative two-step enzyme-linked immunosorbent assay (ELISA).[24] When the role of BTAstat was compared with that of BTA TRAK in detection of bladder cancer, BTAstat was reported to have higher specificity and lower sensitivity.[28] Which test is better remains to be determined, although with BTA TRAK there is slightly higher sensitivity in high-grade tumors.[29,30]

NMP22 (Matritech, Newton, MA, USA) is a nuclear mitotic apparatus protein involved in the distribution of chromatin to daughter cells. It is located in the nuclear matrix in all cell types and is released from the nuclei of tumor cells during apoptosis.[29] The NMP22 test is an immunoassay that detects complexed and fragmented forms of the nuclear mitotic apparatus protein in urine.[31] This protein has an up to 25-fold-greater intracellular concentration in bladder cancer cells than in normal urothelium and is released in soluble form during apoptosis.[32]

FDP is released into the circulation and is detectable in the urine of patients with bladder cancer.[33] Bladder cancer cells produce vascular endothelial growth factor (VEGF), an angiogenic factor involved in the maintenance and induction of vascular endothelial cells, which increases the vessel wall permeability of blood and plasma proteins such as plasminogen, fibrinogen, and other clotting factors.

Recent assay systems that use monoclonal antibody immunoassays to detect FDP consistently achieve overall sensitivities ranging from 68% to 83%.[33-35] The urinary FDP level tends to become higher in patients with cancer as grade and stage increase, although its utility in the detection of CIS remains unproved.[35] Although inflammatory conditions of the urinary tract generate detectable amounts of FDP in urine, the FDP levels are far lower than those in urine of patients with bladder cancer. However, FDP does not seem to have additional value over cytology for recurrent disease.[24]

ImmunoCyt (DiagnoCure, Sainte-Foy, QC, Canada) is a test combining cytology with an immunofluorescence assay using three antibodies: 19A211 against a high molecular weight form of carcinoembryonic antigen, and M344 and LDQ10 against mucins, which are expressed in bladder cancer but not in normal epithelium.[36] Pfister et al.[37] performed a multicenter study and reported that the ImmunoCyt test improved the diagnostic accuracy of cytology in daily practice. The sensitivity of ImmunoCyt for low-grade and low-stage cancer is controversial.[38,39] However, Toma et al.[39] performed comprehensive analysis of several urine markers for noninvasive bladder cancer and concluded that ImmunoCyt in combination with conventional cytology offered better sensitivity than other tests.

Fluorescence in situ hybridization (FISH) is a technique that uses fluorescently labeled DNA probes to assess the centromeres of chromosomes 3, 7, 17, and 9p21 (p16/CDKN2A). FISH can detect genetic alterations in the urine sediments of patients with urothelial carcinoma.[40,41] UroVysion (Abbott Laboratories, Downers Grove, IL, USA), a multicolored FISH probe set developed to detect bladder cancer, has significantly higher sensitivity than cytology, while maintaining the high specificity of cytology and low sensitivity for Ta tumors.[41,42] FISH also appears to be useful for monitoring the response to intravesical treatment in patients with superficial bladder cancer.[42] However, the meticulous technique necessary hampers its wide application in the clinical setting.

Several studies show that a combination of these tests offers superior sensitivity. To date, however, none of these assays has been shown to replace cystoscopy or provide substantial, additional information for making the diagnosis of recurrent bladder carcinoma.

Other markers under investigation

The urine markers that are commercially available now are somewhat limited in clinical use by low sensitivity and specificity. Various newer markers have been investigated because more-sensitive, more-specific, and less-invasive methods for detecting cancer are desired.

The hyaluronic acid (HA)-hyaluronidase (HAase) test is based on an enzyme-linked immunosorbent like-assay.[43] HA is a nonsulfated glycosaminoglycan made of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine.[44] Lokeshwar et al.[45] reported that HA levels are three- to fivefold elevated in tissue extracts of bladder cancer when compared with the levels in normal tissues. It is suggested that HA is synthesized mostly by stromal fibroblasts of cancer, and that the cancer cells activate these fibroblasts to synthesize high levels of HA.[44] HAase, an endoglycosidase, degrades HA into small fragments that promote angiogenesis. Hautmann et al.[46] reported the expression of HA and HAase in bladder cancer tissue and indicated that patients with bladder cancer were positive for the HA-HAase test because of secretion of HA and HAase in urine. The overall sensitivity and specificity are 91.2% and 84%, respectively.[26]

Survivin is a member of the inhibitor of apoptosis protein (IAP) family. This gene is overexpressed in various human malignancies, including cancers of the lung, colon, pancreas, prostate, and breast, but not in normal adult tissues.[47,48] It is a protein with a single baculoviral IAP repeat (BIR) domain and a COOH-terminal ring domain and can inhibit apoptosis by binding to caspases 3 and 7.[49] Survivin expression has been associated with features of biologically aggressive disease, resistance to therapy, and poor clinical outcome in patients with various malignancies.[48] Some investigators have reported that survivin expression in urine specimens has high sensitivity (64%–100%) and efficiency (93%–100%) for the diagnosis of bladder cancer.[50-52]

The telomeric repeat amplification protocol (TRAP) assay can detect and measure the activity of telomerase, an enzyme helping to synthesize telomeres and maintain chromosomal ends.[53] The overall sensitivity of the TRAP assay is between 70% and 86%, although the false-positive rate of 21%–76% is not negligible.[54,55] Recent studies suggested that by using the reverse transcriptase-polymerase chain reaction (RT-PCR) for the mRNA of human telomerase reverse transcriptase (hTERT), the assay has a sensitivity between 74% and 92% and specificity between 70% and 93%.[56-58]

Microsatellites are inherited short tandem repeat DNA sequences that are widely interspersed through the genome with low mutation rates unique to individuals.[58] Mutations of the gene can lead to loss of heterozygosity and/or microsatellite instability[59] and can be useful as markers of neoplasia. The microsatellite assay is performed by PCR using DNA primers for a panel of various microsatellite markers.[60,61] Although this assay has high sensitivity and high specificity, it is a very complex and expensive technique. Blunt-end single-strand DNA conformation polymorphism (blunt-end SSCP) analysis is also useful for diagnosis of bladder cancer by detecting loss of heterozygosity (LOH) on chromosome 9 in urine samples.[62]

Cytokeratins (CKs) are intermediate filament proteins specific for epithelial cells. CK18, CK19, and CK20 were found to be overexpressed in urothelial cancer cells. CYFRA21-1, an ELISA assay for detecting fragments of CK19, is a promising test with sensitivity of 76.2% and specificity of 84.2%.[63]

Lewis X antigen belongs to the Lewis system, occurring in secretions and serum lipoproteins.[64] Papilloma and urothelial cancer cells, but not normal adult urothelial cells, express this antigen, regardless of the blood group, secretory status, and grade and stage of the tumor.[48] The test for