Detection of Telomerase Activity in Exfoliated Cells in Urine From Patients With Bladder Cancer

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Background: Telomeres are specific structures located at the ends of chromosomes that help maintain chromosome stability. In most tissues, telomeres become shorter as cells divide, a phenomenon thought to be associated with limitations on normal cell proliferation. Almost all types of cancer cells, including bladder cancer cells, express the enzyme telomerase, which can maintain or extend telomere length. *Purpose:* We examined telomerase activity in tumor specimens from a cohort of patients with bladder cancer and determined whether telomerase could be detected in exfoliated cancer cells present in urine from these patients. *Methods:* Spontaneously voided urine specimens and bladder-washing fluids (obtained by propelling normal saline into the bladder through a catheter and then withdrawing the liquid contents) were taken from 45 patients before they underwent surgery. Telomerase activity was examined by means of the TRAP (telomeric repeat amplification protocol) assay on extracts of tumor samples from 42 patients and extracts of exfoliated cells in urine and bladder-washing fluid from 42 and 43 patients, respectively. Standard cytologic examination (Pap staining) of urine specimens was also used to detect exfoliated cancer cells. *Results:* Telomerase activity was found in 41 (98%; 95% confidence interval [CI] = 87%-100%) of the 42 tumor samples examined. In contrast, it was not detected in normal bladder tissue from two autopsied individuals who were free of bladder cancer and five of six individuals who had bladder cancer. Telomerase was detected in exfoliated cells in 23 (55%; 95% CI = 39%-70%) of the 42 spontaneously voided urine specimens and in 36 (84%; 95% CI = 69%-93%) of the 43 bladder-washing fluids examined. Considering voided urine specimens and bladder-washing fluids together, telomerase was detected in exfoliated cells from 40 (89%; 95% CI = 76%-96%) of the 45 patients. Telomerase activity was not detected in bladder-washing fluids from 12 cancer-free individuals. Cancer cells were detected by means of standard cytologic examination in the urine of 19 (42%; 95% CI = 28%-58%) of the 45 patients. Urine cytologic examination detected cancer cells in one (8%; 95% CI = 0%-38%) of 12 patients with grade 1 tumors and in 13 (46%; 95% CI = 28%-66%) of 28 patients with grade 2 tumors. In contrast, telomerase activity was detected in exfoliated cells (in voided urine or bladder-washing fluids) from nine (75%; 95% CI = 43%-95%) of 12 patients with grade 1 tumors and from 27 (96%; 95% CI = 82%-100%) of 28 patients with grade 2 tumors. *Conclusion and Implication:* Telomerase activity can be detected in exfoliated cells in urine from patients with bladder cancer, and measurement of this activity appears to be more sensitive in detecting the presence of cancer than standard urine cytologic examination. These findings suggest that measuring telomerase activity in exfoliated cells would be useful in the diagnosis and follow-up of patients with bladder cancer, a possibility that warrants further study. [J Natl Cancer Inst 1997;89:724-30]

Telomeres are specific structures located at the ends of chromosomes [reviewed in (*1*)]. They are believed to be important in maintaining chromosome stability and in preventing the loss of genetic information (*2*). In most somatic cells, telomeres become shorter with each cell division. This phenomenon is hypothesized to be associated with the limitations on normal cellular proliferation (*3*). Telomerase, an RNA protein complex, can enzymatically maintain or extend telomere length (*4*).

Kim et al. (*5*) have described an easy, highly sensitive method for the analysis of telomerase activity. Recent studies (*5-8*) have used this method, known as the telomeric repeat amplification protocol (TRAP), to demonstrate that telomerase activity can be detected with a frequency of more than 90% in almost all types of malignant tumors, but it is rarely detected in somatic cells, except for hematopoietic cells (*6*) and some stem cells (*9*).

Bladder cancer is a common malignant disease throughout the world, ranking as the 12th most common cancer in Japanese males and the fourth most common cancer diagnosed in the United States (*10*). Previously, we identified genetic alterations in human urothelial tumorigenesis and the role of p53 mutations in the acquisition of invasive characteristics (*11-13*). Using a mutational analysis of the p53 gene, we also demonstrated that multicentric urothelial cancers might be monoclonal in origin (*14*). This latter result suggests that exfoliated cells derived from primary tumors may contribute to the multicentric nature of many urothelial cancers. Since human bladder cancers can recur at a frequency of approximately 50% after transurethral resection (*15,16*), early detection and follow-up, using minimally invasive techniques, are important aspects of successful treatment. Cystoscopy is an essential diagnostic tool, but it involves an invasive examination, and some lesions, such as carcinomas in situ, are hard to detect. On the other hand, urine cytologic examination is noninvasive and useful in routine practice, but the sensitivity of this technique can be influenced by subjectivity and depends on the skill, knowledge, and experience of the cytopathologist. In view of these facts, a more objective and reproducible technique is required. Lin et al. (*17*) reported that 97.5% of transitional cell carcinomas are positive for telomerase activity, suggesting that telomerase may be a universal target involved in bladder carcinogenesis. In this study, we examined telomerase activity in tumor specimens from a cohort of patients with bladder

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See "Notes" following "References."

cancer, and we addressed the following question: Can bladder cancers be detected by assaying for telomerase activity in exfoliated cells in urine?

Patients and Methods

Bladder Tumor Samples and Cell Lines

Tumor samples were obtained from 45 patients with bladder cancer, and the telomerase activity in 42 of these primary tumor tissues was evaluated by use of the TRAP assay. All tumors were histopathologically confirmed to be transitional cell carcinomas; tumor grade and stage were according to the World Health Organization system (*18*) and the TNM Classification system (*19*), respectively. All samples were obtained from patients who were treated in Japanese hospitals and gave written informed consent. Eight specimens were obtained as normal control tissues; six of these specimens were obtained from patients with bladder cancer (i.e., macroscopically and cystoscopically normal mucosae from one cystectomy specimen and five random biopsy specimens), and the remaining two specimens were obtained at autopsy from individuals who did not have pathologic conditions of the bladder. Each clinical specimen was immediately snap-frozen in liquid nitrogen and then stored at −80 °C. Three bladder tumor cell lines, T24 (*20*), J82 (*21*), and 1197 (*22*), were purchased from the American Type Culture Collection (Rockville, MD) and maintained according to the supplier's recommendations.

Exfoliated Cells in Urine

Telomerase activity in exfoliated cells in urine was examined for 42 spontaneously voided urine specimens and 43 specimens of bladder-washing fluid obtained from the 45 patients before they underwent surgery. We also examined telomerase activity in 12 bladder-washing specimens obtained from patients undergoing surgery for nonmalignant diseases. For bladder washing, approximately 100 mL normal saline was propelled into the bladder and drawn back a total of four times through a urethral catheter with the aid of a syringe. From each urine or bladder-washing specimen, 50 mL was used for protein extraction, and 10 mL was used for conventional cytologic examination. For protein extraction, the samples were centrifuged at 800*g* at 4 °C for 5 minutes immediately after collection. The pelleted material, including the exfoliated cells, was washed with 15 mL calcium-free, magnesium-free phosphate-buffered saline (PBS[−]) and was subjected to centrifugation again. The new pellets were resuspended in 1 mL PBS[−], transferred to 1.5-mL tubes, and then centrifuged once again. The supernatants were discarded, and these final pellets were used for protein extraction. For patients with hematuria, the samples were treated with Triton X-100. Three milliliters of 1% Triton X-100 was added to 50 mL of urine or bladder-washing fluid, and the samples were mixed gently before centrifugation; all subsequent procedures were as described above. The final concentration of Triton X-100 in these samples was negligible. In addition, we confirmed that a concentration of 0.05% Triton X-100 did not influence the outcome of the TRAP assay (data not shown).

Protein Extraction and TRAP Assay

The protein extraction procedure and the TRAP assay were performed according to methods described by Kim et al. (*5*) and Piatyszek et al. (*23*). In brief, approximately 10 mg of bladder tumor tissue was homogenized, using a Kontes micropestle (Kontes Glass Company, Vineland, NJ), in 200 µL of ice-cold 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10 m*M* Tris–HCl (pH 7.5), 1 m*M* $MgCl_2$, 1 m*M* ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.1 m*M* phenylmethylsulfonyl fluoride, 5 m*M* β-mercaptoethanol, and 10% glycerol (CHAPS lysis buffer). Extracts were incubated for 30 minutes on ice and then centrifuged at 20 000*g* for 10 minutes at 4 °C. The resulting supernatants were recovered and divided into 90-µL aliquots, which were snap-frozen in liquid nitrogen and stored at −80 °C. When exfoliated cells were used, protein was extracted, following the same procedure, in 40 µL of CHAPS lysis buffer. The protein concentration in individual lysates was determined by use of the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA); the extracts from tissues were diluted to a protein concentration of 0.3 µg/µL with additional CHAPS lysis buffer. For the TRAP assay, individual reaction tubes were prepared by placing 25 pmol of CX primer (5′-CCCTTACCCTTACCCTTACCCTTA-3′), in 2 µL 20 m*M* Tris–HCl (pH 7.5), 1.5 m*M* $MgCl_2$, 63 m*M* KCl, and 0.02% gelatin, on the bottom of the tubes and then overlaying the mixture with molten wax (Ampliwax; The Perkin-Elmer Corp., Foster City, CA). For the analysis of tissue samples, serial 10-fold dilutions of lysates were prepared such that 0.6-0.006 µg of protein was mixed with 20 µL of a buffer containing 25 pmol 5′-biotin-labeled TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 20 m*M* Tris–HCl (pH 7.5), 1.5 m*M* $MgCl_2$, 63 m*M* KCl, 0.02% gelatin, 0.005% Tween 20, 1 m*M* EGTA, 0.1 mg/mL bovine serum albumin, 50 µ*M* deoxynucleoside triphosphates, 0.5 µg bacteriophage T4 gene 32 protein (Boehringer Mannheim GmbH, Federal Republic of Germany), and 2 U of *Taq* polymerase (The Perkin-Elmer Corp.) (TRAP reaction buffer); this second mixture was placed above the wax barrier in the TRAP reaction tubes. For the analysis of exfoliated cell samples, 3-µL aliquots of cell lysates were added to the TRAP reaction buffer without measuring the protein concentration. After incubating the reaction tubes at 23 °C for 60 minutes, 32 cycles of the polymerase chain reaction were performed, using 1-minute temperature steps at 94 °C, 50 °C, and 72 °C for each cycle. The amplified products generated in TRAP assays were separated in 10% nondenaturing polyacrylamide gels and then transferred onto nylon membranes (Hybond-N+, Amersham, Bucks., U.K.) overnight by use of semidry transfer methods. Detection of the telomerase reaction products was carried out by means of chemiluminescence methods (Imaging High, TOYOBO, Osaka, Japan) (*24*); the sensitivity of this approach was almost the same as that obtained with radioisotopic detection (data not shown).

Evaluation of Telomerase Activity

The telomerase activity assay was considered to be positive for a sample when a 6-base ladder, indicating extension of the TS primer, was detected. To compensate for the inhibitory effects of the relatively highly concentrated lysates derived from tissue specimens, TRAP assay-reaction mixtures containing serial 10-fold dilutions of protein, ranging from 0.6 to 0.006 µg protein, were prepared for all determinations as indicated above. The TRAP assay was performed at least three times on all samples, and the replicated results were verified. For confirmation that a positive TRAP assay signal was generated by telomerase activity, all of the telomerase-positive lysates were pretreated with 1 µg ribonuclease A (Wako, Osaka, Japan) to demonstrate that the signals were sensitive to ribonuclease.

Cytologic Examination

The cytologic examination of urine or bladder-washing fluid was performed by use of standard Pap staining. The cells in approximately 10-mL aliquots of voided urine or bladder-washing fluid were concentrated by centrifugation at 500*g* at room temperature for 5 minutes. The cells were then spread onto microscope slides, fixed with ethanol, and stained. The slides were screened under a microscope by cytologists, and they were reviewed by pathologists. Morphologic diagnosis was performed according to criteria used at our institute. A urine cytologic examination was performed at least twice for each patient.

Statistical Analysis

Comparison of the outcomes obtained with two procedures for individual patients was performed by use of McNemar's test (*25*), and two-sided 95% confidence intervals (CIs) were computed by use of Statistical Analysis System version 6.11 software (SAS Institute, Inc., Cary, NC) for Windows.

Results

Consistent with previously reported data (*5*), substantial telomerase activity was observed in lysates prepared from the three bladder tumor cell lines. Thus, we used a lysate obtained from the T24 cell line and CHAPS lysis buffer without added cell extract as positive and negative controls, respectively, in every TRAP assay of clinical materials. Table 1 summarizes the data for each patient. Telomerase activity was demonstrated in 41 (98%; 95% CI = 87%-100%) of the 42 bladder tumor samples tested (e.g., Fig. 1, A). The tumor with no detectable telomerase activity (No. 1) was a low-grade papillary tumor of a noninvasive nature. To examine the telomerase activity in normal bladder mucosae, we obtained macroscopically and cystoscopically normal epithelium from one cystectomy specimen and five random biopsy specimens from six of the patients with bladder tumors as well as two specimens obtained at autopsy from individuals without any pathologic condition of the bladder.

Table 1. Detection of telomerase activity and its relationship to histopathologic features and urine cytologic examination in bladder cancer*

Patient No.	Age, y/sex	Pathology		Cytologic examination of Void-U	Telomerase activity		
		Grade	TNM		Tumor	Void-U	Washing-F
1	80/F	1	pTa	–	–	–	–
2	58/M	1	pTa	–	+	+	–
3	73/M	1	pTa	Atypia	+	+	+
4	43/F	1	pTa	+	+	+	+
5	60/M	1	pTa	–	+	–	+
6	62/F	1	pTa	–	+	ND	+
7	68/M	1	pTa	–	+	+	+
8	52/M	1	pTa	–	+	–	–
9	56/M	1	pTa	–	+	–	+
10	78/M	1	pTa	–	+	–	–
11	72/F	1	pT1	–	+	+	+
12	57/M	1	pT1	–	+	–	+
13	68/M	2	pTa	+	+	ND	+
14	49/F	2	pTa	–	+	+	+
15	47/M	2	pTa	Atypia	+	–	+
16	79/M	2	pTa	–	+	–	+
17	84/M	2	pTa	Atypia	+	–	+
18	74/M	2	pTa	Atypia	+	–	+
19	69/M	2	pTa	Atypia	+	–	–
20	72/M	2	pTa	–	+	+	–
21	88/M	2	pTa	+	+	+	+
22	59/F	2	pTa	+	+	+	+
23	79/M	2	pTa	Atypia	+	+	+
24	55/M	2	pTa	–	+	–	+
25	82/F	2	pTa	Atypia	+	–	+
26	79/F	2	pTa	+	+	–	+
27	64/M	2	pTa	–	+	+	+
28	66/F	2	pTa	+	+	+	ND
29	85/M	2	pTa	–	+	–	+
30	70/M	2	pT1	+	+	+	+
31	68/M	2	pT1	+	+	+	+
32	72/M	2	pT1	Atypia	+	–	+
33	63/M	2	pT1	+	+	+	+
34	58/F	2	pT1	Atypia	+	–	+
35	84/F	2	Insufficient	Atypia	+	+	+
36	69/M	2	pTis	+	+	+	+
37	65/F	2	pTis	+	ND	+	+
38	69/F	2	pTis	+	ND	ND	+
39	73/M	2	pT1	+	+	+	+
40	66/M	2	pT2	+	+	+	+
41	67/M	3	pTa	+	+	–	+
42	75/M	3	pT1	+	+	–	–
43	49/F	3	pT2N1	+	+	+	+
44	62/M	3	pT2	+	ND	+	ND
45	60/M	3	pT1	+	+	+	+

*Pathology according to World Health Organization classification (*18*) and TNM classification (*19*); T = tumor, N = lymph node, and M = metastasis. M = male; F = female; Void-U = spontaneously voided urine; Washing-F = bladder-washing fluid; ND = not done; + = cancer cells detected by Pap staining (cytologic examination) or telomerase activity detected; – = no cancer cells or telomerase activity detected.

Telomerase activity was not detected in five of the six normal urothelial specimens from the patients with bladder tumors or in either of the two autopsy specimens. Telomerase activity was present in one cystoscopically normal specimen of bladder epithelium that was isolated from a patient with a papillary grade 2 tumor.

To test whether telomerase activity was detectable in exfoliated urinary cells, we collected 42 spontaneously voided urine samples and 43 samples of bladder-washing fluid from the 45 patients with bladder tumors. Table 2, A, shows the sensitivities and specificities of conventional cytologic examination and the telomerase analysis of samples collected by different procedures. Telomerase activity was observed in 23 (55%; 95% CI = 39%-70%) of the 42 voided urine samples and in 36 (84%; 95% CI = 69%-93%) of the 43 samples of washing fluid from the patients with bladder tumors. When the results from spontaneously voided urine samples and the samples of bladder-washing fluid were combined, telomerase activity was detected in specimens from 40 (89%; 95% CI = 76%-96%) of the 45 patients. In contrast, the sensitivity of conventional cytologic examination was 42% (19 of 45 samples; 95% CI = 28%-58%). Representative autoradiograms of TRAP assay results with exfoliated cells are shown in Fig. 1, B. Telomerase activity was not detected in any of the specimens of bladder-washing fluid from 12 patients who underwent surgery for urologic diseases other than cancer (specificity = 100%; 95% CI = 74%-100% [Table 2, A]).

Eighteen (95%; 95% CI = 74%-100%) of the 19 patients with positive urine cytologic findings were positive for telomerase activity in the exfoliated cells in urine or bladder-washing fluid (Table 2, B). We also detected telomerase activity in nine (90%; 95% CI = 55%-100%) of the 10 patients with atypical cells in their urine. In addition, 13 (81%; 95% CI = 54%-96%) of 16 patients showed positive telomerase activity in either spontaneously voided urine or bladder-washing fluid in spite of negative cytologic findings. Conventional urine cytologic examination or urine telomerase activity measurement was not positive in four patients with bladder cancer, including the patient (No. 1; Table 1) whose tumor showed no telomerase activity.

We examined the relationship between the pathologic grade of the bladder tumors, positivity in the cytologic examination, and the detection of telomerase activity in the exfoliated cells in voided urine or bladder-washing fluid (Table 2, C). The sensitivity of measuring telomerase activity in exfoliated cells was high (75%-96%), regardless of the histopathologic grade of the bladder tumors, whereas the sensitivity of urine cytologic examination declined with decreasing tumor grade. For grades 1 and 2 tumors, the sensitivity of telomerase analysis of exfoliated cells was significantly higher than that of cytologic examination: 75% versus 8% for grade 1 tumors and 96% versus 46% for grade 2 tumors (both $P<.01$, McNemar's test).

Discussion

In this study, 98% (41 of 42) of the tested bladder tumors were positive for telomerase activity. This frequency is entirely consistent with previous reports of telomerase activity in cancers (*5-8,26*) and supports the findings of Lin et al. (*17*), who demonstrated that 39 of 40

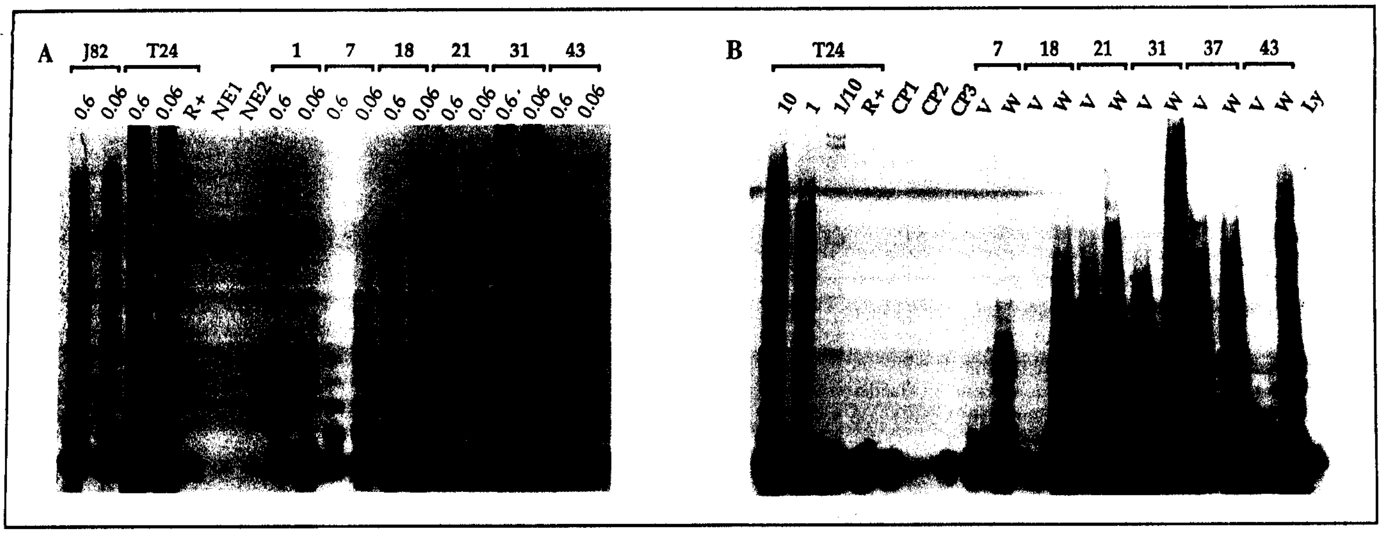

Fig. 1. Telomerase activity in bladder cancer tissues and exfoliated cells in urine. A) The telomeric repeat amplification protocol (TRAP) assay was performed with serial 10-fold dilutions of extracts of tumor tissue or cultured bladder tumor cells (protein range tested = 0.6-0.006 μg). The assay products were separated in polyacrylamide gels, transferred to nylon membranes, and detected by use of chemiluminescence methods (*see* "Patients and Methods" section for details). Telomerase activity was detected in cells of the bladder cancer lines J82 and T24 as well as in the tumor tissue from patient Nos. 7, 18, 21, 31, and 43. Activity was not detected in normal bladder epithelium (NE) or the tumor tissue from patient No. 1. The sensitivity of the activity to pretreatment with ribonuclease A (R+) was examined for all telomerase-positive specimens. **B)** Telomerase activity was detected in exfoliated cells in spontaneously voided urine (V) and bladder-washing fluid (W) from the patients with bladder cancer, whereas it was not detected in the bladder-washing fluid from control patients (CP). The voided urine from patient No. 18 was negative for telomerase. Although the signal intensity was weak for the voided urine specimens from patient Nos. 7 and 43, the presence of telomerase was confirmed by longer x-ray film exposures. The numbers above the T24 lanes indicate the cell equivalents of protein assayed in the corresponding reactions. In every series of assays, we used an extract from T24 cells and extraction (i.e., cell-lysis [Ly]) buffer without added cell extract as a positive and a negative control, respectively.

bladder cancers and one of two dysplasias showed positive telomerase activity, whereas none of seven normal tissues displayed this activity. The high frequency of telomerase positivity in bladder cancer, regardless of the clinical stage, may indicate that telomerase is reactivated at an early stage during tumorigenesis.

One of the eight normal epithelial tissues examined showed positive telomerase activity. However, it is possible that this specimen contained dysplastic or malignant cells with telomerase. Because the specimen was obtained from the cystoscopically normal mucosa of a patient with a bladder tumor, malignant or dysplastic cells may have been present or contaminated the samples during biopsy.

In the development of advanced diagnostic and treatment technologies, the early detection of human cancers, using minimally invasive techniques, has become an important issue. We have demonstrated that bladder cancers can be detected at high frequency by evaluating the telomerase activity in exfoliated cells in urine. Telomerase activity was demonstrated in 55% of the spontaneously voided urine samples and in 89% of the spontaneously voided urine samples and bladder-washing fluids combined, whereas none of the 12 bladder-washing fluids obtained from control patients were positive for telomerase (Table 2, A). It is important to note that the frequency of detecting telomerase activity in urine was significantly higher than the frequency of detecting carcinoma by the method of conventional urine cytologic examination, especially for patients with low-grade bladder cancers (Table 2, C).

Urine cytologic examination involves the assessment of morphologic changes in tumor-derived cells among the exfoliated cells in urine. This morphology-based examination inevitably demonstrates low sensitivity in patients with low-grade tumors because the cytologic abnormality is slight. The reported sensitivity of urine cytologic examination varies with tumor grade, although the sensitivity for tumors of low grade has been controversial (*27,28*): less than 10%-70% for grade 1 transitional cell carcinomas, 40%-80% for grade 2 tumors, and more than 80% for grade 3 tumors (*15,29-31*). These sensitivities are similar to those obtained in the present study. The specificity of cytologic examination at our institute is 99%, based on data from 126 patients with hematuria who were free of bladder cancer (Table 2, A). In this study, urine cytologic examination was also performed on the bladder-washing fluids of 27 patients who were selected at random. The sensitivity of this examination was almost the same as that of a similar examination of voided urine samples (data not shown). Bladder washing increases the cellular content of urine samples, but it is thought to contribute little to the diagnosis of low-grade tumors (*27*).

In combination with conventional urine cytologic examination, the determination of telomerase activity in both voided urine and bladder-washing fluids substantially improved the sensitivity of detecting grades 1 and 2 tumors: from 8% to 75% and from 46% to 96% for grades 1 and 2 tumors, respectively (Fig. 2). However, catheterization to collect bladder-washing fluid is an invasive procedure, and it is not an adequate method alone for the screening of bladder tumors. When telomerase analysis was performed on spontaneously voided urine specimens only, the sensitivity was improved by 20%-40% compared with that of cytologic examination alone (Fig. 2). On the basis of these observations, we believe that the analysis of voided urine may be useful, especially in the follow-up of patients with low-grade bladder cancers.

Table 2, A. Sensitivity and specificity of various telomerase analyses and cytologic examination

Procedure	Sensitivity, %*	95% CI, %†	Specificity, %‡	95% CI, %
Telomerase activity				
Tumor tissues	98 [41/42]	87-100	88 [7/8]	47-100
Exfoliated cells§				
Void	55 [23/42]‖	39-70	ND	ND
Washing	84 [36/43]‖,¶	69-93	100 [12/12]	74-100
Void or washing	89 [40/45]#	76-96	100 [12/12]	74-100
Cytologic examination	42 [19/45]¶,#	28-58	99 [125/126]**	96-100

*Numbers in brackets = positive telomerase findings/number of specimens tested from patients with cancer.
†95% CI = 95% confidence interval.
‡Numbers in brackets = negative telomerase findings/number of normal specimens tested from patients with cancer or patients without cancer.
§Void = spontaneously voided urine; washing = bladder-washing fluid; ND = not done.
‖Significantly different from one another (McNemar's test; two-sided P = .003).
¶Significantly different from one another (McNemar's test; two-sided P = .001).
#Significantly different from one another (McNemar's test; two-sided P = .001).
**This specificity value was based on data from 126 patients with hematuria but not bladder cancer visiting our institute.

Table 2, B. Relationship between cytologic findings and sensitivity of measuring telomerase activity in urine*

Cytologic findings	No. of patients	No. with positive telomerase activity/No. tested (%; 95% CI)		
		Void	Washing	Void or washing
Positive	19	14/17 (82; 57-96)	16/17 (94; 71-100)	18/19 (95; 74-100)
Atypia	10	3/10 (30; 7-65)†	9/10 (90; 55-100)†	9/10 (90; 55-100)
Negative	16	6/15 (40; 16-68)	11/16 (69; 41-89)	13/16 (81; 54-96)

*95% CI = 95% confidence interval; Void = spontaneously voided urine; washing = bladder-washing fluid.
†Two groups showing a statistically significant difference; two-sided P = .014 (McNemar's test).

Table 2, C. Sensitivity of telomerase analysis of exfoliated cells in urine according to histologic grade of bladder cancer*

Histologic grade†	No. of patients	No. with positive telomerase activity/No. tested (%; 95% CI)			No. with positive cytology/ No. tested (%; 95% CI)
		Void	Washing	Void or washing	
1	12	5/11 (45; 17-77)‡	8/12 (67; 35-90)§	9/12 (75; 43-95)‖	1/12 (8; 0-38)‡,§,‖
2	28	15/26 (58; 37-77)¶	25/27 (93; 76-99)¶,#	27/28 (96; 82-100)**	13/28 (46; 28-66)#,**
3	5	3/5 (60; 15-95)	3/4 (75; 19-99)	4/5 (80; 28-99)	5/5 (100; 48-100)

*95% CI = 95% confidence interval; Void = spontaneously voided urine; washing = bladder-washing fluid.
†Grading according to World Health Organization classification (*18*).
‡Significantly different from one another (McNemar's test; two-sided P = .046).
§Significantly different from one another (McNemar's test; two-sided P = .008).
‖Significantly different from one another (McNemar's test; two-sided P = .005).
¶Significantly different from one another (McNemar's test; two-sided P = .007).
#Significantly different from one another (McNemar's test; two-sided P = .001).
**Significantly different from one another (McNemar's test; two-sided P = .001).

Telomerase analysis of voided urine samples is so simple and so low in cost that it may be applicable to the screening of bladder cancer as well.

A possible disadvantage in using telomerase activity measurements for the detection of bladder tumors is that a large number of exfoliated cancer cells is required to obtain reproducible results. Telomerase activity can be detected in an amount equivalent to the protein content of a single cell when telomerase-positive tumor cell lines are studied (*32*). However, in clinical samples containing both intact and degraded cells, much more protein is thought to be required in each reaction mixture to obtain stable results. We examined the number of exfoliated cells in randomly selected samples and found that approximately 500-4000 cells were present in 50 mL of voided urine, and 1000-10 000 cells were present in the same volume of fluid obtained by the bladder-washing technique. These numbers varied considerably from patient to patient (data not shown). The differences in the numbers of exfoliated cells among patients may have influenced the detectability of telomerase activity. Supporting the idea that the number of exfoliated tumor cells is important in our assay, the sensitivity of measuring telomerase activity in bladder-washing fluid (84%) was significantly higher than that obtained with spontaneously voided urine (55%) (P = .003, McNemar's test).

Another possible disadvantage is that of obtaining a false-positive result because of the presence of contaminating inflammatory cells in the urine samples. Peripheral leukocytes have been reported to exhibit weak telomerase activity (*6,23*). In this study, we found moderate cystitis in only three of 45 patients with bladder tumors. Furthermore, urine samples from

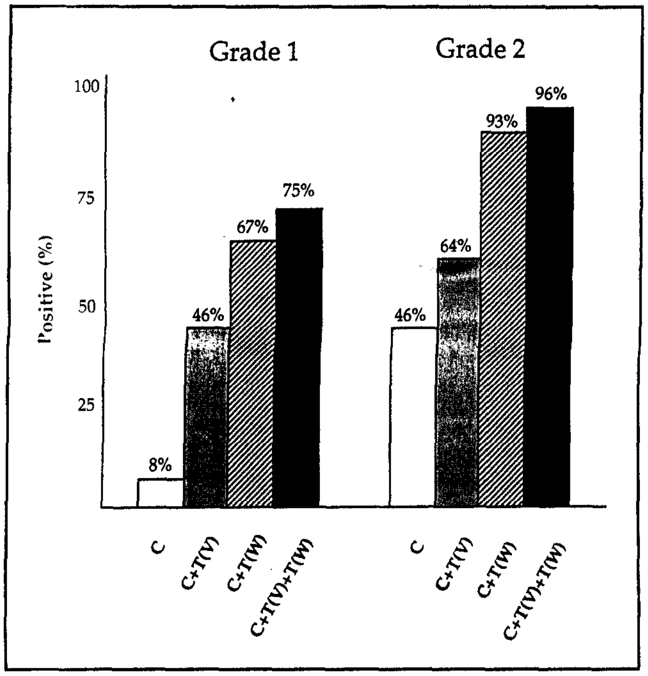

Fig. 2. Percent of bladder cancers detected (positive) by means of standard urine cytologic examination (C) (i.e., with Pap staining) with or without a determination of telomerase activity in exfoliated cells in spontaneously voided urine [T(V)] or bladder-washing fluid [T(W)] from patients with grades 1 or 2 bladder tumors [World Health Organization classification (*18*)].

two control patients with moderate-to-severe cystitis, caused by urolithiasis and a urethral stricture, showed no telomerase activity. Consequently, we conclude that leukocyte contamination did not have much influence on our data. However, it is still possible that severe urinary tract inflammation might cause false-positive results, and this possibility should be considered if the TRAP assay is applied to the detection of bladder cancer. To avoid this possibility, telomerase analysis should be performed only after antibiotic treatment if the leukocyte contamination is significant.

In conclusion, our findings suggest that the determination of telomerase activity in exfoliated cells in voided urine is an objective and noninvasive method of detecting bladder cancer. In addition, this method is more sensitive than conventional urine cytologic examination, and it might be particularly useful in the follow-up of patients with low-grade bladder cancers. To confirm the usefulness of telomerase analysis as a diagnostic tool for bladder tumors, we are currently using the assay in a study involving a large number of patients.

References

(*1*) Blackburn EH. Structure and function of telomeres. Nature 1991;350:569-73.

(2) Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 1992;11:1921-9.

(*3*) Harley CB. Telomere loss: mitotic clock or genetic time bomb? Mutat Res 1991;256:271-82.

(*4*) Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 1989;59:521-9.

(*5*) Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011-5.

(*6*) Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S. Telomerase activity in normal leukocytes and in hematologic malignancies. Blood 1995;85:2315-20.

(*7*) Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA, Shay JW. Correlating telomerase activity levels with human neuroblastoma outcomes. Nat Med 1995;1:249-55.

(*8*) Hiyama K, Hiyama E, Ishioka S, Yamakido M, Inai K, Gazdar AF, et al. Telomerase activity in small-cell and non-small-cell lung cancers. J Natl Cancer Inst 1995;87:895-902.

(*9*) Harle-Bachor C, Boukamp P. Telomerase activity in the regenerative basal layer of the epidermis in human skin and in immortal and carcinoma-derived skin keratinocytes. Proc Natl Acad Sci U S A 1996;93:6476-81.

(*10*) Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics, 1996. CA Cancer J Clin 1996;46:5-27.

(*11*) Habuchi T, Kinoshita H, Yamada H, Kakehi Y, Ogawa O, Wu WJ, et al. Oncogene amplification in urothelial cancers with p53 gene mutation or MDM2 amplification. J Natl Cancer Inst 1994;86:1331-5.

(*12*) Habuchi T, Ogawa O, Kakehi Y, Ogura K, Koshiba M, Hamazaki S, et al. Accumulated allelic losses in the development of invasive urothelial cancer. Int J Cancer 1993;53:579-84.

(*13*) Habuchi T, Takahashi R, Yamada H, Ogawa O, Kakehi Y, Ogura K, et al. Influence of cigarette smoking and schistosomiasis on p53 gene mutation in urothelial cancer. Cancer Res 1993;53:3795-9.

(*14*) Habuchi T, Takahashi R, Yamada H, Kakehi Y, Sugiyama T, Yoshida O. Metachronous multifocal development of urothelial cancers by intraluminal seeding. Lancet 1993;342:1087-8.

(*15*) Heney NM, Ahmed S, Flanagan MJ, Frable W, Corder MP, Hafermann MD, et al. Superficial bladder cancer: progression and recurrence. J Urol 1983;130:1083-6.

(*16*) Fitzpatrick JM, West AB, Butler MR, Lane V, O'Flynn JD. Superficial bladder tumors (stage pTa, grades 1 and 2): the importance of recurrence pattern following initial resection. J Urol 1986;135:920-2.

(*17*) Lin Y, Miyamoto H, Fujinami K, Uemura H, Hosaka M, et al. Telomerase activity in human bladder cancer. Clin Cancer Res 1996;2:929-32.

(*18*) Mostofi FK, Sobin LH, Torloni H. Histological typing of urinary bladder tumours. International classification of tumours, No. 10. Geneva: World Health Organization, 1973.

(*19*) Hermanek P, Sobin LH. TNM classification of malignant tumours. 2nd rev. 4th ed. Springer-Verlag, International Union Against Cancer (UICC), 1992.

(*20*) Bubenik J, Perlmann P, Helmstein K, Moberger G. Cellular and humoral immune responses to human bladder carcinomas. Int J Cancer 1970;5:310-9.

(*21*) O'Toole C, Price ZH, Ohnuki Y, Unsgaard B. Ultrastructure, karyology and immunology of a cell line originated from a human transitional-cell carcinoma. Br J Cancer 1978;38:64-76.

(*22*) Rasheed S, Gardner MB, Rongey RW, Nelson-Rees WA, Arnstein P. Human bladder carcinoma: characterization of two new tumor cell lines and search for tumor viruses. J Natl Cancer Inst 1977;58:881-90.

(*23*) Piatyszek MA, Kim NW, Weinrich SL, Hiyama K, Hiyama E, et al. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). Methods Cell Sci 1995;17:1-15.

(*24*) Beck S, O'Keeffe T, Coull JM, Koster H. Chemiluminescent detection of DNA: application for DNA sequencing and hybridization. Nucleic Acids Res 1989;17:5115-23.

(*25*) McNemar Q. Note on the sampling error of the differences between correlated proportions or percentages. Psychometrika 1947;12:453-7.

(*26*) Counter CM, Hirte HW, Bacchetti S, Harley CB. Telomerase activity in human ovarian carcinoma. Proc Natl Acad Sci U S A 1994;91:2900-4.

(*27*) Koss LG. Diagnostic cytology and its histopathologic bases. 4th ed. Philadelphia: Lippincott, 1992:951-63, 983-92.

(*28*) Murphy WM. Current status of urinary cytology in the evaluation of bladder neoplasms. Hum Pathol 1990;21:886-96.

(*29*) Esposti PL, Zajicek J. Grading of transitional cell neoplasms of the urinary bladder from smears of bladder washings. A critical re-view of 326 tumors. Acta Cytol 1972;16:529-37.

(*30*) Murphy WM, Soloway MS, Jukkola AF, Crabtree WN, Ford KS. Urinary cytology and bladder cancer. The cellular features of transitional cell neoplasms. Cancer 1984;53:1555-65.

(31) Koss LG, Deitch D, Ramanthan R, Sherman AB. Diagnostic value of cytology of voided urine. Acta Cytol 1985;29:810-6.

(32) Wright WE, Shay JW, Piatyszek MA. Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. Nucleic Acids Res 1995; 23:3794-5.

Notes

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