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Telomerase Activity: A Potential Marker of Bladder Transitional Cell Carcinoma in Bladder Washes

Seung-Choul Yang[1], Dong-Hyeon Lee[1], Sung-Joon Hong[1], Byung-Ha Chung[1], and Isaac Yi Kim[2]

The enzyme telomerase maintains a constant telomere length in immortalized cells, allowing unlimited cell proliferation. Almost all cancer cells express telomerase activity. However, little data is available regarding the role of telomerase activity in the detection of bladder cancer with a bladder wash specimen. We detected telomerase activity in a bladder wash specimen of bladder cancer and normal tissues, and compared them with final pathologic diagnosis. Tewnty-three patients with transitional cell carcinoma (TCC) of the bladder were enrolled in our study. A bladder wash specimen was obtained before transurethral resection of the bladder (TURB) and normal and cancer tissues from the same patients during TURB. Telomerase activity was analyzed in each specimen a using telomeric repeat amplification protocol (TRAP) assay based on polymerase chain reaction (PCR) technique. Cytologic diagnosis was performed using Papanicolaou's stain with cytocentrifuged cytology preparation. We observed telomerase activity in 95.7% (22/23) of both cancer tissues and bladder wash specimens; only one case did not express telomerase activity. Telomerase activity was undetected in all normal tissues except one, which was obtained from a patient with carcinoma in situ. A total of 69.6% (16/23) of wash specimens were positive in cytopathologic diagnosis. The accuracy of cytopathologic diagnosis in pathologic grade 2 or 3 was relatively high (83.3%, 15/18). However, in five cases of grade 1 TCC only 20% (1/5) of cytologic diagnosis was positive whereas the telomerase activity of wash specimens was detected in 80% (4/5). Our data demonstrates that not only the majority of human bladder cancer tissues, but also the bladder wash specimens obtained from patients with TCC, expressed telomerase activity. It indicates that telomerase activity may be a reliable marker in detecting bladder cancer especially in cases with a low grade that bladder wash cytology can miss.

Key Words: Telomerase activity, bladder wash cytology, bladder transitional cell carcinoma

Bladder transitional cell carcinoma (TCC) is a common urologic malignancy that affected 50,000 people in the US in 1996 (Stadler and Vogelzang, 1996). At initial presentation, approximately 20% is invasive while 80% is superficial. However, about 20% of the latter group will eventually progress to muscle invasive cancer. To diagnose and to monitor patients with bladder cancer, bladder wash cytology is usually used because it is a minimally invasive diagnostic tool. However, only 40% of superficial TCC is diagnosed by this method, as repeated bladder manipulations, urinary tract infections, and stone disease complicate the diagnosis of bladder cancer in wash cytology by causing morphological changes

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Department of Urology[1], Yonsei University College of Medicine, Seoul, Korea, Department of Urology[2], Northwestern University Medical School, Chicago, Illinois 60611, USA
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Address reprint request to Dr. S.C. Yang, Department of Urology, Yonsei University College of Medicine, C. P.O. Box 8044, Seoul 120-752, Korea

in the exfoliated cells of urine (Badalament *et al.* 1987). In addition, the accuracy of cytopathologic analysis of bladder washes depends on the experience of the cytopathologists (Wiener *et al.* 1993). Therefore, to enhance the sensitivity of bladder wash cytology, additional markers of bladder TCC must be identified and investigated. In this regard, it has recently been demonstrated that the enzyme telomerase is readily detected in human bladder cancer tissues (Lin *et al.* 1996). Telomerase allows unlimited cell proliferation by maintaining a constant length of telomeres which are specialized structures at the end of all eukaryotic chromosomes that protect genomic DNA from degradation and deleterious recombination events. Telomeric DNA consists of hundreds-to-thousands of tandem repeats of the sequence (TTAGGG)n. Normal human somatic cells have 5~15 kbp of telomere in a chromosomal end and lose 50~100 bp during each cycle of DNA replication (Harley *et al.* 1990; Hastie *et al.* 1990). Recently, it has been demonstrated that many types of malignant cells, including bladder TCC cells, express telomerase (Kim *et al.* 1994). These results suggest that telomerase may be a reliable marker of malignant transitional cells in bladder washes. Therefore, in the present study, we have investigated the presence of telomerase activity in bladder washes of 23 patients with pathologically diagnosed bladder TCC.

MATERIALS AND METHODS

Tissue samples

Malignant and normal bladder tissues were obtained from 23 patients with primary bladder cancer by transurethral resection at Yonsei University Medical Center from June to September of 1996. Patients were classified according to the TNM classification system after obtaining detailed clinical and pathological data. Prior to transurethral resection of a bladder tumor (TURBT), 100 ml bladder wash was obtained from each patient by irrigation with an Ellik evacuator. Fifty ml of each bladder wash was immediately centrifuged and the cell pellet was used for telomerase activity assay. The remaining portion of each bladder wash was sent to the pathology department for cytopathologic diagnosis using Papanicolau's stain. The cytopathology was reviewed by one pathologist. Samples of normal bladder mucosa were obtained by cold-cup biopsy in a normal-appearing area before TURBT.

Prepartion of cell extract

Cells from bladder washes were washed with phosphate buffered saline (PBS) and centrifuged at 4°C. After removing PBS, samples were rinsed with wash buffer (10 mM Tris-HCl, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM DTT) and centrifuged again at 4°C. Subsequently, wash buffer was removed and 80~160 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% 3-[(3-chloramidopropyl) dimethyl ammonio]-1-propanesulfonate, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mecaptoethanol, and 10% glycerol) was added based on the sample volume and placed on ice for 30 min. Finally, lysates were centrifuged and supernatants were collected for telomerase activity assay.

Bladder cancer and normal tissues were washed sequentially with PBS and wash buffer. Then, 80~160 μl of ice-cold lysis buffer was added to each sample and ground with a homogenizer. Subsequently, lysates were prepared as described above. All samples were diluted to 3 mg/ml after measuring the protein concentration using Bradford assay (Bradford, 1976).

Telomerase activity assay

Telomerse activity assay was performed as previously described using telomeric repeat amplification protocol (TRAP) (Kim *et al.* 1994). Briefly, each extract was added to a cocktail mixture containing 50 μl of TRAP mixture (50 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween, 1 mM EGTA, 50 μM deoxynucleotide triphosphates, 0.5 μM T4 gene 32 protein, and 0.1 mg/ml BSA), 2.5 unit of Taq polymerase (Bioneer, Seoul), 0.1 μM of TS oligonucleotide (5'-AATCCGTCGAGCAGAGTT-3'), and 0.3 μl of [α-^{32}P]dCTP (3000 Ci/mmole, Amersham, Buckinghamshire, UK). The mixture was incubated for 30 min at room temperature for telomerase-mediated extension of the TS

primer. Next, CX primer (5'-CCCTTACCCTTACC-CTTACCCTAA-3') was added to each tube and PCR was performed as follows: 30 sec at 94°C, 30 sec at 50°C, and 90 sec at 72°C for 31 cycles followed by a 2 min incubation at 72°C. T-24 bladder cancer cell line extract was used as a positive control because telomerase activity was previously demonstrated in these cells while lysis buffer was used as a negative control. PCR products were electrophoresed in 15% nondenaturing acrylamide gel and visualized by autoradiography.

Statistics

Statistical analysis was performed using a Fisher's exact test to evalutate the significance of the differences. $P < 0.05$ was considered statistically significant.

RESULTS

The summary of patients characteristics is shown in Table 1. The mean age was 62.4 years with a range of 54 to 89; 20 (87%) were men and 3 (13%) were women. Fifteen (62.5%) were at stage Ta or T1, 7 (30.4%) were stage T2 or higher, and 1 (4.4%) was carcinoma *in situ* (CIS).

Table 1. Patients characteristics

Pathologic stage	Tumor grade			
	Grade 1	Grade 2	Grade 3	Total
Ta	4		0	5
T1		6	3	10
T2 or higher	0	4	3	7
CIS	0	0	1	1
Total	5	11	7	23

Table 2. Telomerase activity in patients with bladder TCC

Samples	Telomerase activity		
	Positive	Negative	Total
Normal mucosa		22	23
Bladder TCC tissues	22	1	23
Bladder washes	22	1	23

The representative result of the TRAP assay is shown in Fig. 1. As demonstrated, telomerase activity was consistently detected in malignant bladder tissues and bladder washes. Table 2 shows the summary of the results. Telomerase activity was positive in 22 of 23 bladder tumor tissues and bladder washes. On the other hand, telomerase was detected only in 1 of 23 normal bladder mucosa samples. Thus,

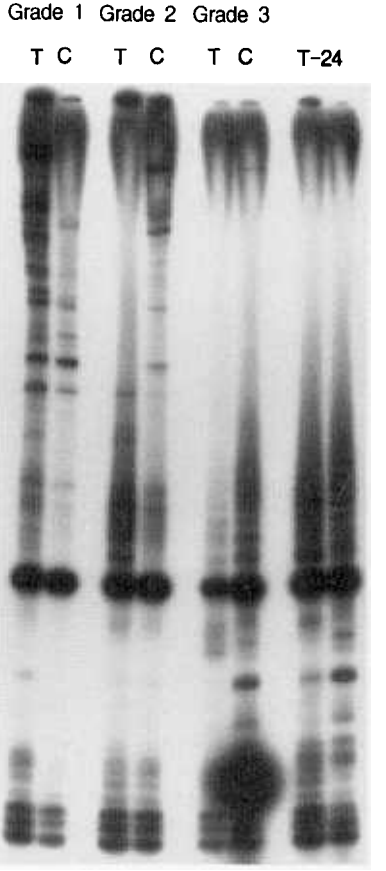

Fig. 1. The representative results of a TRAP assay. Telomerase activity is detected in bladder cancer tissues (T) and wash cytologic specimens (C) of all tumor grades. Extracts of a T-24 human bladder cancer cell line having telomerase activity were used as the positive standard.

Table 3. Cytopathological diagnosis and telomerase activity in bladder washes according to tumor grade

Tumor grade	No. of Pts.	Cytologic diagnosis			Telomerase activity	
		Normal	Equivocal	Malignant	Negative	Positive
Grade 1	5	2	2	1*	1	4*
Grade 2	11	0	3	8	0	11
Grade 3	7	0	0	7	0	7
Total	23	2	5	16	1	22

*: $P<0.05$

Table 4. Cytopathological diagnosis and telomerase activity in bladder washes according to pathologic stage

Tumor grade	No. of Pts.	Cytologic diagnosis			Telomerase activity	
		Normal	Equivocal	Malignant	Negative	Positive
Ta	5	2	1	2*	1	4*
T1	10	0	3	7	0	10
T2 or higher	7	0	1	6	0	7
CIS	1	0	0	1	0	1
Total	23	2	5	16	1	22

*: $P<0.05$

telomerase may be a reliable marker of bladder TCC in bladder biopsies and washes.

Table 3 shows the relationship between tumor grade and the presence of telomerase activity in bladder washes. As a comparison, the results of bladder wash cytology is also shown. Telomerase activity was detected in 22 of 23 (95.7%) patients with TCC; the bladder wash sample that had undetectable telomerase activity was obtained from a patient with stage Ta and grade 1. In contrast, cytologic diagnosis was positive for malignancy in only 69.6% of patients with known bladder cancer. Moreover, in patients with grade 1 TCC, the positive cytologic rate was only 20% (1 of 5). With respect to stages, telomerase activity was detected in 93.3% (14/15) of washes obtained from patients in stage Ta and T1 cancer whereas positive wash cytology was present in 60% (9/15) of the same patients (Table 4). Interestingly, the normal bladder mucosa sample that had telomerase activity was obtained from the patient who was diagnosed with CIS. These results demonstrate that the presence of telomerase activity may be a more sensitive marker of bladder TCC than an abnormal cytology in bladder washes.

DISCUSSION

Results of the present study demonstrated that telomerase activity is consistently detected in bladder washes obtained from patients with known bladder malignancy. In addition, it was shown that telomerase activity is a more sensitive marker of bladder TCC than bladder wash cytology. These results, taken together, suggest that telomerase is a marker of bladder TCC that may potentially augment the cytopathologic diagnosis.

Telomerase is a ribonucleoprotein DNA polymerase involved in telomere synthesis in vertebrates (Zakian, 1995). Telomerase activity has been detected in many different types of malignant tumors and cell

lines; however, telomerase has not been detected in normal somatic cell lines and tissues (Kim *et al.* 1994). Thus, it has been suggested that telomerase may play an important role during carcinogenesis. Such a hypothesis is consistent with the results of the present study in which telomerase activity was detected in more than 95% of bladder tumor tissues. Previously, Lin *et al.* have also demonstrated that telomerase is consistently detected in human bladder cancer tissues (Lin *et al.* 1996). Therefore, these results suggest that telomerase activity is a potential marker of bladder TCC.

In the present study, it was also shown that telomerase activity is frequently detected in bladder washes of bladder cancer patients. Moreover, telomerase activity assay was more sensitive than bladder wash cytology as telomerase activity was detected in the majority of bladder washes that were obtained from patients with negative cytopathologic diagnosis. Specifically, in low grade TCC, the sensitivity of cytopathologic diagnosis was only 20% while that of telomerase activity was 80%. The bladder wash sample that did not show telomerase activity was obtained from a patient with stage Ta, grade 1 TCC. Interestingly, in this sample, the cytopathologic study was also negative for malignant cells. Thus, it is likely that this particular sample contained either normal or early stage tumor cells. Likewise, in bladder washes obtained from patients with low stage TCC, cytology was abnormal in only 40% while telomerase activity was positive in 80%. Thus, particularly in low grade and low stage cancer, the presence of telomerase activity may be a more sensitive marker of bladder TCC than cytology in bladder washes.

Finally, in this study, it was demonstrated that telomerase activity is also a specific marker of bladder TCC as a TRAP assay was negative in 22 of 23 samples of normal bladder mucosa. It should be pointed out, though, that the one normal bladder tissue with positive telomerase activity was obtained from the patient with CIS. Thus, it is possible that this biopsy contained a small portion of cancer or was contaminated either during the intraoperative procedure or while carrying out the telomerase activity assay.

In summary, results of the present study demonstrated that telomerase activity is consistently detected in bladder TCC tissues and in bladder washes from patients with bladder TCC. It was also demonstrated that telomerase activity may be a more reliable marker of bladder TCC than bladder wash cytology, especially in low grade, low stage disease where cytology showed difficulty in differentiating benign from malignant cells due to a similarity in the shape and color of the nucleus and cytoplasm. In the future, telomerase activity will be assayed in a larger number of patients with bladder TCC to further study the sensitivity and specificity of this method.

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