Detection of Human Telomerase Reverse Transcriptase Messenger RNA in Voided Urine Samples as a Useful Diagnostic Tool for Bladder Cancer

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ABSTRACT

Activation of telomerase and stabilization of telomeres are thought to be required for cellular immortality and oncogenesis. Telomerase activity is detected in >90% of various cancers, including urothelial cancers. Of the three subunits comprising telomerase complex, human telomerase reverse transcriptase (hTERT) is a rate-limiting determinant of the enzymatic activity of telomerase. In the present study, spontaneously voided urine specimens from 33 patients with bladder cancer and 26 without bladder lesions were examined for the expression of hTERT mRNA, and the usefulness of detecting hTERT mRNA in urine samples for screening of bladder cancer was evaluated. RT-PCR analysis revealed that ~80% of urinary sediments from patients with bladder cancer expressed hTERT mRNA, regardless of clinical stage or pathological grade, whereas only 4% of sediments from patients without urothelial lesions did. Interestingly, hTERT mRNA expression was observed, even in some urine samples from bladder cancer patients with negative urinary cytology. These findings suggest that the expression of hTERT in urine sample may be a useful diagnostic marker for bladder cancer.

INTRODUCTION

Telomeres are the distal ends of eukaryotic chromosomes that protect and stabilize chromosomes (1). Because cellular DNA polymerases cannot replicate the 5′ end of linear DNA molecules, the telomeres in normal somatic cells undergo progressive shortening with cell division (2), resulting in chromosomal instability that may cause cellular senescence. Telomerase is a specialized ribonucleoprotein polymerase containing an integral RNA with a short template element that directs the *de novo* synthesis of telomeric repeats at chromosome ends (3, 4). Studies using the PCR-based TRAP[2] (5–8) have reported that telomerase is activated in a variety of malignant tumors, including urothelial cancers.

Three subunits comprising the human telomerase complex have recently been identified. Human telomerase RNA component (hTR) functions as a template for telomere elongation by telomerase (9). Telomerase-associated protein (TP1) has also been cloned (10, 11), but its function remains to be determined. Studies have demonstrated that expression of these components does not reflect the level of telomerase activity (12). Most recently, hTERT has been cloned as a catalytic component of telomerase (13, 14). Expression of hTERT is observed at high levels in telomerase-positive cell lines and malignant tumor tissues (15–17). We reported previously that the expression of hTERT but not that of hTR or that of TP1 was significantly correlated with telomerase activity in tumors including urothelial cancers (15, 18). These findings suggest that hTERT is a rate-limiting determinant of the enzymatic activity of human telomerase.

One of the major problems in the diagnosis of bladder cancer is the inaccessibility of the bladder to unaided visual examination. In addition, bladder cancers strongly tend to recur, and recurrent or metastatic lesions from upper urinary tract tumors frequently arise in the bladder. Monitoring asymptomatic patients is therefore of great importance. Cystoscopy is the most powerful method for the diagnosis of bladder cancers but is not suitable as a screening method due to its invasiveness, especially in male patients. Moreover, some lesions, such as carcinoma *in situ*, are difficult to detect with this method. Noninvasive methods with higher sensitivity are therefore urgently needed for screening for bladder cancer.

An important feature that attracts investigative attention in urothelial cancers is the frequent presence of some exfoliated cancer cells in naturally voided urine. Although urinary cytology is widely used for screening for bladder cancer, it is sometimes difficult to judge cytological specimens, particularly for low-grade cancers, because the morphological abnormality is likely to be slight in such lesions, resulting in insufficient sensitivity. Our previous study found that most bladder cancers expressed hTERT mRNA and the expression associated with telomerase activity, whereas normal bladder tissues did not (18). These findings prompted us to examine whether hTERT mRNA

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[2] The abbreviations used are: TRAP, telomeric repeat amplification assay; hTERT, human telomerase reverse transcriptase; RT-PCR, reverse transcription-PCR.

expression can be detected in urinary sediments from bladder cancer, and whether this expression is a useful diagnostic marker of bladder cancer. Our findings indicated that hTERT mRNA is frequently observed in urinary sediments from patients with bladder cancers but not in sediments from patients without bladder lesions, suggesting that detection of hTERT mRNA expression in urine sample is a useful screening method for bladder cancer.

MATERIALS AND METHODS

Tissue Samples. Urinary sediments were obtained from 33 patients with bladder cancer and 26 patients without urothelial cancer from 50-ml samples of naturally voided urine at the Department of Urology, Kanazawa University Hospital, Tonami General Hospital, and Kouseiren Takaoka Hospital. Approximately 5 ml of the sample were submitted for cytology. The sediments from the remaining sample were soaked in PBS, and cell pellets were recovered. These samples were refrigerated at 4°C and obtained within 12 h after voiding and were then frozen and stored at −80°C until use.

Cell Lines. T24, RT4, and KK47 cells (derived from bladder cancers), which express hTERT mRNA, were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum in the presence of 5% CO_2 at 37°C and used as a positive control.

RT-PCR Analysis. Total RNA was isolated from the tissues using Isogen (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's protocol. cDNA was synthesized from 4 μg of RNA using an RNA PCR kit Version 2 (TaKaRa, Otsu, Japan) with random primers. Analysis of the expression of hTERT was performed by RT-PCR amplification as described previously (16) with slight modification. To amplify the cDNA, 1-μl aliquots of reverse-transcribed cDNA were subjected to PCR in 10 μl of 1× buffer containing 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and 0.6 unit of ExTaq DNA polymerase (TaKaRa). A 0.45-μl portion of DMSO (Sigma Chemical Co.) was added to the buffer. hTERT mRNA was amplified using the primer pair 5′-CGGAAGAGTGTCTGGAGCAA-3′ (LT5) and 5′-GGATGAAGCGGAGTC TGGA-3′ (LT6). The reaction conditions were 31 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 90 s, with a final extension time of 5 min at 72°C. PCR products for hTERT were electrophoresed in 7% polyacrylamide gel and stained with SYBR Green (FMC BioProducts, Rockland, ME). The efficiency of cDNA synthesis from each sample was estimated by PCR with β-actin-specific primers. Existence of epithelial cells was confirmed by RT-PCR for cytokeratin-19, as Burchill *et al.* (19) and Klein *et al.* (20) reported.

Cytological Examination. Cytological examination of urine was performed by standard Papanicolaou's staining. The slides were screened under a microscope by cytologists and were reviewed and diagnosed according to the Papanicolaou's classification by pathologists. Urine cytological examinations were performed at least twice for each patient.

Table 1 Clinicopathological backgrounds of bladder cancer patients and the results of cytology and the expression of hTERT

Patient no.	Sex	Age	Histology	Stage	Grade	Urinary cytology	Urinary hTERT mRNA
1	F	78	TCC[a]	T_4	G_3	V	+
2	M	80	TCC	T_3	G_3	V	+
3	F	83	TCC	T_1	G_3	V	+
4	F	73	TCC	T_1	G_3	V	+
5	M	79	TCC	T_1	G_3	V	−
6	F	68	TCC	T_a	G_3	V	−
7	M	52	TCC	T_3	G_2	V	+
8	F	72	TCC	T_3	G_2	V	−
9	M	87	TCC	T_2	G_2	V	+
10	F	79	TCC	T_1	G_2	I	+
11	F	70	TCC	T_1	G_2	V	+
12	M	66	TCC	T_1	G_2	I	+
13	M	76	TCC	T_1	G_2	I	−
14	M	74	TCC	T_1	G_2	V	+
15	M	76	TCC	T_1	G_2	V	+
16	M	78	TCC	T_1	G_2	V	+
17	F	52	TCC	T_1	G_2	I	+
18	M	50	TCC	T_1	G_2	V	+
19	M	75	TCC	T_a	G_2	V	+
20	M	77	TCC	T_a	G_2	II	+
21	M	83	TCC	T_a	G_2	V	+
22	M	84	TCC	T_a	G_2	III	+
23	M	53	TCC	T_a	G_2	I	−
24	M	57	TCC	Tis	G_2	V	+
25	M	68	TCC	T_3	G_1	V	+
26	F	90	TCC	T_1	G_1	III	+
27	M	81	TCC	T_1	G_1	I	+
28	M	73	TCC	T_1	G_1	I	+
29	M	72	TCC	T_1	G_1	I	−
30	M	75	TCC	T_1	G_1	V	+
31	M	80	TCC	T_1	G_1	V	−
32	M	82	TCC	T_1	G_1	II	−
33	M	67	TCC	Tis	G_1	I	+

[a] TCC, transitional cell carcinoma.

RESULTS

A total of 33 urinary sediments from patients with bladder cancer as well as 26 from patients without urothelial cancers (11 with urinary tract infection, 10 with cancers originating outside the urinary tracts, 4 with benign prostatic hypertrophy, and one with amyloidosis of the bladder) were examined for the expression of hTERT mRNA (Table 1). Three cancer cell lines, T24, RT4, and KK47, derived from bladder cancers were also examined. Expression of cytokeratin-19 was confirmed in all samples tested. TRAP assay was performed for 10 urinary sediments from patients with bladder cancers, using 5 μg of protein, but telomerase activity was detected in only one case. RT-PCR analysis revealed that 25 of 33 urinary sediments from the patients with bladder cancer, including 2 samples from carcinoma *in situ* patients, expressed hTERT mRNA, whereas only 1 of 26 sediments from patients without urothelial cancer did. Three cancer cell lines exhibited strong hTERT mRNA expression (Fig. 1). The sensitivity for screening of bladder cancer was therefore 76%, whereas the specificity was 96%. One false-positive sample was obtained from the patient with amyloidosis of the bladder. No significant correlation was found between the expression of hTERT mRNA and clinicopathological features of tumors (Table 2).

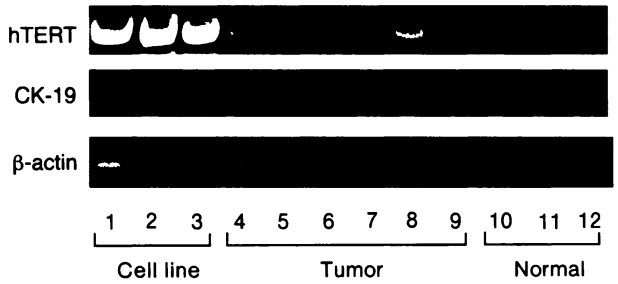

Fig. 1 Representative results of RT-PCR analysis for the expression of hTERT in urine samples. Expression of hTERT mRNA was examined by RT-PCR. *Lane 1*, T24 cells; *Lane 2*, RT4 cells; *Lane 3*, KK47 cells; *Lanes 4–9*, urine samples from patients with bladder cancer; *Lane 10–12*, urine samples from patients without bladder cancer. *CK-19*, cytokeratin-19.

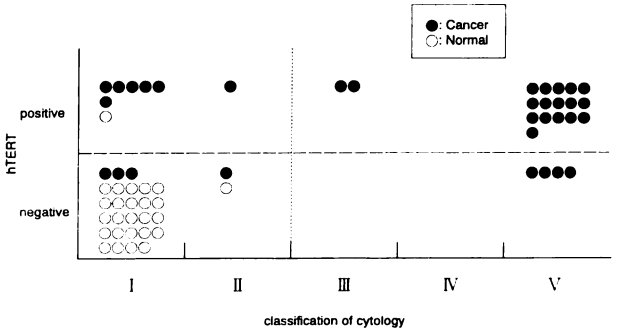

Fig. 2 Correlation of the results between expression of hTERT in urine samples and urinary cytology. Urine cytology was classified according to the Papanicolaou's classification. *Closed* and *open circles*, bladder cancer and normal cases, respectively, which were histologically confirmed.

Table 2 Expression of hTERT mRNA in urine samples and urinary cytology

	Cancer	Normal
hTERT		
+	25	1
−	8	25
Cytology		
III–V (positive)	22	0
I–II (negative)	11	26

Urinary cytology was positive (class III–V) for 22 of 33 samples from the patients with bladder cancer but for none from patients without bladder lesions (sensitivity, 67%; specificity, 100%). The results of RT-PCR for hTERT expression and cytology were then compared (Fig. 2). Eighteen of 22 (82%) samples from the patients with positive urinary cytology (class III–V) expressed hTERT mRNA, whereas 29 of 37 from those with negative cytology (class I or II) was negative for hTERT, indicating a significant correlation between hTERT mRNA expression and urinary cytology. However, some cases of discordance were found. Interestingly, 7 of 11 (64%) cytologically negative bladder cancers expressed hTERT mRNA (Table 2).

DISCUSSION

The expression of hTERT is specifically observed at high levels in telomerase-positive cancer cell lines and clinical cancer tissues including bladder cancers (15–18). We reported previously that hTERT mRNA was frequently detected in bladder cancer tissues and was strongly associated with telomerase activity (18). These findings prompted us to examine whether detection of hTERT mRNA is a useful diagnostic tool for the screening of bladder cancer. hTERT mRNA was detected in ~80% of urinary sediment samples from the patients with bladder cancer but rarely in those without urothelial cancer, indicating that the expression of hTERT is frequently detected in urothelial cancer patients and is useful for screening for bladder cancer. The sensitivity of the hTERT detection assay was higher than that of urinary cytology. We found some cases of discordance, in which cytology was negative but hTERT was positive. Some samples contain very few cancer cells, which may be insufficient for cytological evaluation but detectable by RT-PCR assay. Another explanation for this discrepancy is that cytological testing may underdiagnose low-grade tumors because of difficulty in detecting well-differentiated cancer cells. In fact, all of the cytologically negative cases that expressed hTERT showed G_1 and G_2 cancers in these cases. In addition, whereas the sensitivity of cytological detection was 44% for G_1 cancer, that of hTERT was 67%, being much higher than cytology. Thus, RT-PCR assay for hTERT detection may be advantageous, particularly for the screening of low-grade cancers. However, we also found some discordancies in which cytology was positive but hTERT was negative. This type of discordance was prevalent among high-grade cancers. One possible explanation for these discrepancies is simply the degradation of RNA in urinary sediment samples. In contrast to low-grade cancers, high-grade cancer cells have severe morphological abnormalities that may increase the sensitivity of cytological diagnosis. Taken together, these findings suggest that the combination of cytological examination with the detection of hTERT expression may improve the sensitivity and specificity of screening for bladder cancer.

Using the TRAP assay, telomerase activity has been demonstrated in urinary samples from bladder cancer patients. Kinoshita *et al.* (21) detected telomerase activity in 55% of 50 ml of naturally voided urines and in 84% of 50 ml of washing fluids from patients with bladder cancer. Yoshida *et al.* (22) and Kavaler *et al.* (23) also detected telomerase activity in respectively 62 and 85% of naturally voided urines. However, Linn *et al.* (24) did not detect telomerase activity in any urine sample from 11 patients with bladder cancer. We detected telomerase activity by TRAP assay in urinary sediment in only 1 of 10 cases of bladder cancer, consistent with the latter report. We do not know at present why these discrepancies in findings arise. In the TRAP assay, it is difficult to determine the positivity of samples with low telomerase activity, because faint or weak signals sometimes cannot be distinguished from nonspecific signals. In addition, assay sensitivity and specificity vary among researchers because of differences between the assay systems: radioisotope or nonradioisotope; presence of internal standard; purification or not of telomerase-extension products before PCR, all of

which may affect the results of TRAP assay. In our assays, detection of hTERT expression was more sensitive than the TRAP assay for screening for bladder cancer.

In conclusion, hTERT mRNA was detected frequently in urinary sediments from bladder cancer but not in those from normal bladders. RT-PCR assay for detection of hTERT in urine samles may therefore be a useful screening method for bladder cancer. Combination with cytological testing increases sensitivity and specificity and may contribute to early detection of bladder cancer.

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