Comparison of Telomerase Activity in Bladder Carcinoma and Exfoliated Cells Collected in Urine and Bladder Washings, Using a Quantitative Assay

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ABSTRACT

Telomerase activity was measured with a quantitative assay, based on a modification of telomeric repeat amplification protocol method, in bladder cancers and apparently normal mucosa in 33 patients. In the same patients, the enzyme was also measured in exfoliated cells collected both with voided urine and bladder washings. Results obtained in urine were compared with those from 20 healthy subjects.

Telomerase activity was present in 31 (94%) bladder cancer tissues and in 23 (72%) apparently normal mucosa samples. However, the levels of enzyme activity were significantly higher in cancer tissues in comparison with normal mucosa (mean ± SD, 47.3 ± 23.2 and 14.9 ± 6.1 ng DNA/μg protein, respectively; \( P < 0.0001 \)). Telomerase activity in bladder cancer tissues was not related to tumor stage and grade. Enzyme activity was present in 27 urine samples and in 27 (82%) bladder washings collected from cancer patients. We did not find correlation between the activity in urine and washings, and their mean levels were not different (22.2 ± 10.1 and 20.7 ± 8.0, respectively). Telomerase activity in bladder cancer tissues was correlated to its activity in urine (\( r = 0.650, P < 0.001 \)) and in bladder washings (\( r = 0.410, P < 0.05 \)). Only 2 of 20 urine samples from control subjects were found to express telomerase activity at a very low level.

This was the first attempt to correlate telomerase activity in exfoliated cells from urine and bladder washings with the activity in corresponding bladder cancers. According to these results we postulate that telomerase activity in urine sediment reflects the activity in bladder cancers better than bladder washings and, for its easy collection, is to be preferred as diagnostic marker in this tumor.

INTRODUCTION

Because of the strong tendency of bladder cancer to recur, the monitoring of recurrences in asymptomatic patients has a particular importance. Simple and noninvasive methods for the detection of urothelial malignancies could represent an important tool in follow-up of bladder carcinomas. Urine cytology is a simple and specific procedure, but its sensitivity is insufficient, especially for the detection of well differentiated tumors (1).

Because tumor progression is believed to occur through the multistep accumulation of genetic alterations, a number of specific genes, implicated in bladder tumorigenesis, have been studied in voided urine as molecular diagnostic markers. The identification of p53 mutations in exfoliated cancer cells has been reported (2). Similarly, mutations of H-ras gene were found in 47% of patients with bladder cancer (3). Recently, abnormal CD44 gene activity was found in exfoliated cells in urine samples from 91% of patients with bladder cancer (4).

Some other urinary tumor markers have been proposed for the detection of bladder cancer: the bladder-associated antigen BTA (5); nuclear matrix protein NMP22 (6); fibrin/fibrinogen degradation product FDP (7); and chemiluminescent hemoglobin assay for the detection of hematuria/hemoglobinuria (8), which revealed a variable grade of sensitivity and specificity in detecting bladder cancer. Finally, the detection of telomerase activity in voided urine or bladder washings was recently proposed as a valuable tool in the diagnosis of bladder carcinoma (9 –23), even if most of these studies evaluated only the presence/absence of telomerase activity as a diagnostic marker. It remains to be clearly elucidated whether quantitative differences of telomerase activity in bladder tissues and exfoliated cells can improve the clinical relevance of this molecular marker.

Human telomeres are repetitive sequences of the TTAGGG motif, located at the very ends of chromosomes (24, 25). Their function seems to be the stabilization of chromosomes and prevention of DNA degradation, as well as to provide a signal of cellular senescence (26 –28). Telomerase is a ribonucleoprotein complex that catalyzes the addition of telomeric repeats to the 3‘-end of chromosome DNA, thereby preventing the loss of telomeric sequences at each cell division and contributing to the maintenance of cell immortality and to the uncontrolled growth of cancer cells, by elongating telomere ends (29).

Because of this central role in carcinogenesis and for the high frequency of its activation in human cancers, telomerase has been expected to be a new and promising marker for cancer diagnosis and therapy (30 –34). A major improvement for the possible evaluation of the clinical significance of telomerase
assay was the development of TRAP assay (34). Recently, we presented a modification of the TRAP assay (35) based on the use of a sensitive fluorescent dye (36) that allows a quantitative evaluation of telomerase activity in cellular extracts.

We present here the results of this quantitative assay in bladder cancers and in adjacent normal tissues, as well as in exfoliated urothelial cells, collected in urine and bladder washings of the same patients to explore the quantitative relationships among these samples and their possible relationships with bladder cancer telomerase activity.

MATERIALS AND METHODS

Samples Collection and Extraction. Urinary exfoliated cells were obtained from 33 patients with bladder cancer and 20 control subjects (10 males and 10 females). In the same patients, tissue samples of bladder cancer (n = 33) were collected by transurethral resection and immediately frozen in liquid nitrogen. In 32 of them, apparently normal mucosa samples were collected in duplicate with a cold cup rigid biopsy forceps, for pathological evaluation and telomerase assay, respectively. In all these samples, the presence of malignancy was excluded. Patients were classified according to tumor stage [pTa (n = 10), pT1 (n = 16), pT2 (n = 6), and pT3 (n = 1)] and tumor grade [G1 (n = 11), G2 (n = 5), and G3 (n = 17)]. In all patients and controls, exfoliated cells were collected from 50-ml samples of voided urine. In cancer patients, bladder washings were obtained transurethrally before tumor resection and in the same morning of urine collection by catheterization and irrigation with 50 ml of saline and barbotage with a Toomey syringe. Urine and bladder washings were centrifuged at 10,000 × g for 10 min, within 15 min of collection. The treatment of these samples immediately after collection reduces the denaturing effects of RNases and proteases that can be present in urine (37). Cell pellets were washed twice with PBS and extracted as described previously (35, 38, 39) in 200 μl of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate lysis buffer. After 30 min of incubation on ice, the lysates were centrifuged at 16,000 × g for 20 min at 4°C, and the supernatant was rapidly frozen and stored at −80°C until the assay. Tissue samples were homogenized in 200 μl of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate buffer, and supernatants were stored as above.

Telomerase Assay. Telomerase activity was assayed in duplicate by a modification of conventional TRAP assay in protein extracts from exfoliated cells or tissues, as described previously (35). Our method is based on the use of a sensitive fluorochrome that selectively binds double-stranded DNA. Because telomerase generates double-stranded DNA from a protein extract and the amount of newly synthesized DNA is proportional to telomerase activity (40), the measurement of the DNA concentration in post-PCR samples can be considered quantitatively related to telomerase activity.

Samples from the same patient were measured simultaneously. Each specimen was also measured after pretreatment with 0.5 μg of assay RNase (Boehringer Mannheim Italia, Milan, Italy) for 30 min at 37°C to inactivate telomerase. We measured protein concentration in each extract by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Sample extracts containing 6 μg of protein were assayed in 47.2 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4.5 mM MgCl₂, 1 mM each dNTP, 20 pmol TAG-U primer (41), and 0.5 μM T4 gene 32 protein (Boehringer Mannheim Italia). After a 60-min incubation at 30°C for telomerase-mediated extension of TAG-U primer, the reaction mixture was heated at 90°C for 3 min and then subjected to 60 PCR cycles.

The abbreviation used is: TRAP, telomeric repeat amplification protocol.
mg protein. A protein extract of the human prostate ng DNA/calculate as the mean of duplicates and expressed in terms of amount obtained in the same specimen after RNase treat-
ment, as reported previously (41). Telomerase activity was generated by dilutions of a normal control DNA (from 0 – 100 each sample was calculated by extrapolation on a standard curve n
m
5
n
tissue; apparently normal mucosa; U, exfoliated cells in urine; W, exfoliated cells in bladder washings; +, positive control, 1 µg of protein extract from MCF7 human breast cancer line; −, the same sample of positive control treated with RNase.

RESULTS

Fig. 3 Telomerase activity detected by conventional TRAP assay in samples collected from two patients with bladder carcinoma. T, tumor tissue; n, apparently normal mucosa; U, exfoliated cells in urine; W, exfoliated cells in bladder washings; +, positive control, 1 µg of protein extract from MCF7 human breast cancer line; −, the same sample of positive control treated with RNase.

of 95°C for 30 s, 64°C for 30 s, 72°C for 30 s, followed by 72°C for 10 min, after the addition of 2.8 µl of a second reaction mixture containing 20 pmol CTA-R primer (41), and 0.3 µl of 5 units/µl Taq Gold (Perkin-Elmer Corp., Foster City, CA). After PCR, we mixed 10 µl of each amplification product with 490 µl of TE buffer [10 mM Tris-Cl and 1 mM EDTA (pH 7.5)] and 500 µl of ultra-sensitive fluorescent dye PicoGreen (Molecular Probes Inc., Eugene, OR; 1:1000 diluted stock solution). Fluorescence was recorded in a spectrofluorophotometer RF-540 (Shimadzu, Kyoto, Japan) using standard wavelengths (ex-
citation = 480 nm, emission = 520 nm). DNA concentration in each sample was calculated by extrapolation on a standard curve generated by dilutions of a normal control DNA (from 0 – 100 ng/ml). DNA concentration was obtained by subtracting the DNA amount obtained in the same specimen after RNase treat-
ment, as reported previously (41). Telomerase activity was calculated as the mean of duplicates and expressed in terms of ng DNA/µg protein. A protein extract of the human prostate cancer cell line LNCaP was also added as positive control. A subset of samples (12 bladder cancers with corresponding normal mucosa and exfoliated cells collected with urine and bladder washes) was also evaluated to confirm the presence of telomerase activity by the conventional TRAP assay (34) with autoradiographic revelation of radiolabeled PCR products.

An estimated methodological cutoff was calculated in di-
lution experiments as the lowest amount of telomerase activity measurable with our assay procedure, both with protein extracts from bladder cancers and urinary sediment. The sensitivity of the assay was fixed at 4.5 ng DNA/µg protein.

always <20% for cancer and normal bladder tissues, as well as for exfoliated cells, collected with urine and bladder washes. Telomerase activity was variably expressed in 31 of 33 (94%) bladder cancer tissues (range, 11.4 – 97.5; mean ± SD, 47.3 ± 23.2 ng DNA/µg protein). In the same patients we found detectable telomerase activity in 22 of 32 (69%) apparently normal mucosa, but the levels of enzyme activity in these samples were significantly lower (range, 8.7 – 29.3; mean ± SD, 14.9 ± 6.1 ng DNA/µg protein; P < 0.0001) in comparison with corresponding cancer tissues, as reported in Fig. 1. A significant relationship was found between telomerase activity in cancers and corresponding apparently normal tissues (r = 0.579, P < 0.01; Fig. 2). Telomerase activity in bladder cancer tissues was not related to tumor stage and grade (data not shown). The presence of telomerase activity was also con-
firmed, in a subset of samples (12 bladder cancers and corre-
spanding normal mucosa), by using the conventional TRAP assay (see Fig. 3).

Telomerase in Urine and Bladder Washings. To test possible differences in exfoliated cell recovery by urine and bladder washing collection, we compared the amount of cellular proteins extracted from the same patient with both procedures. We found a higher quantity of total proteins (and consequently a higher number of cells) in samples collected by bladder washings (120.5 ± 219.3 µg/µl sample extract) in comparison with urine-derived exfoliated cells (43.5 ± 89.6 µg/µl; P < 0.004).

In Fig. 4, we report the results obtained in exfoliated bladder cells collected with voided urine and bladder washings from cancer patients. We found that telomerase activity was present in 27 of 33 urine samples (22.2 ± 10.1). Similar levels (20.7 ± 8.0) were found in 27 of 33 bladder washings collected from the same patients. In particular, we found that telomerase activity was simultaneously present in urine and bladder washings in 24 of 33 patients. The levels of telomerase activity in urine samples and in corresponding bladder washings were not

Fig. 4 Telomerase activity detected in exfoliated cells collected in urine and bladder washings from bladder cancer patients. Telomerase activity was undetectable in six bladder cancers and in six normal mucosa samples. The difference is not statistically significant.
correlated \((r = 0.361)\). Among normal control subjects, telomerase activity was detectable in urine sediments only in two subjects, whereas the remaining samples were under the detection limit of the assay. However, in these two subjects the level of telomerase activity (10.3 and 8.6, respectively) was below the range of cancer patients.

We also found that the level of telomerase activity in bladder cancer tissues was highly correlated to its activity in urine \((r = 0.650, P < 0.001)\) and in bladder washings \((r = 0.410, P < 0.05, \text{Fig. 5})\). No relationship was found between telomerase activity in exfoliated cells collected in urine or bladder washings with tumor grade and stage (data not shown).

The presence of telomerase activity in urine and bladder washings \((n = 12)\) was confirmed by using the conventional TRAP assay (see Fig. 3).

**DISCUSSION**

In the clinical evaluation of bladder cancer, the detection of low-grade bladder tumors and the availability of new molecular markers for patient follow-up are the most challenging goal for noninvasive methods. Although cystoscopy remains the cornerstone for bladder malignancies, this invasive procedure is uncomfortable for patients, particularly in their follow-up. On the other hand, urine cytology can provide a useful tool only in high-grade tumors (1) and is particularly affected by subjective interpretations.

Telomerase has been recently proposed as a reliable marker of bladder malignancies because it was detected in about 90% of transitional cell carcinomas (9, 10, 12–16, 18). In addition, the detection of this enzyme in exfoliated cells collected in normally voided urine or in bladder washings obtained during cystoscopy seems to provide a relevant diagnostic tool for bladder cancer. According to recent results, telomerase activity was detected in urine from bladder cancer patients in a variable percentage (10, 13, 16–18), whereas, in only one report, telomerase was found undetectable (14). The detection of telomerase activity in bladder washings was also frequently reported (10, 11, 14, 15).

Independently from the evaluation of the percentage of positive samples in exfoliated cells and their possible relationship with positivity in bladder cancer, few and semiquantitative data on the possible differences of telomerase activity in cancer tissues and exfoliated cells have been reported (16, 17). In addition, it remained to be clarified whether telomerase activity in exfoliated cells can reflect the enzyme activity in corresponding bladder cancer.

Recently, we developed a procedure for the estimation of telomerase activity in human cancers, based on a modification of TRAP assay (35). We have already demonstrated that this assay can evidence marked differences in telomerase activity among different tumors. We also demonstrated that the measurement of telomerase activity with our method can discriminate between cancer and adenoma of human adrenal gland (42).

In this study, we applied this measurement to the detection of telomerase activity in normal and cancer bladder tissues collected from patients during transurethral resection. Our results indicated that telomerase activity is present in 94% of bladder cancers and is irrespective from cancer grade and stage, as reported previously (14). Telomerase activity is also present, even if significantly reduced, in 72% of apparently normal bladder mucosa samples collected from the same patients. The presence of telomerase in normal bladder was already reported (9, 12, 13), and this was interpreted as a generalized activation of bladder mucosa as an early event of cell transformation or as a physiological feature of bladder epithelial cells. Regarding this aspect, it is important to note that we found a significant relationship between telomerase activity in apparently normal mucosa and cancer tissue of the same patient. This finding seems to indicate that, at least in these patients, the activation of telomerase is in some way connected to cancer activity.

We confirmed that telomerase activity is detectable in the
majority of urine from cancer patients (82%) as well as in bladder washings (82%). In 24 of 33 patients (73%) we found that telomerase activity was simultaneously present both in urine and bladder washings. Despite this qualitative concordance, we did not find a significant linear relationship between the levels of telomerase activity in urine and bladder washings. One possible explanation for this finding could be the different mechanism of sample collection of exfoliated cells: bladder washing is a more “aggressive” approach in comparison with normal voided urine collection, as demonstrated also by the higher quantity of total proteins (and consequently by the higher number of cells) collected by bladder washings in comparison with urine-derived exfoliated cells. This finding could also be connected to a different proportion of cancer cells versus normal mucosa cells obtained by the two collection methods.

In addition, we demonstrated that telomerase in urine samples was strictly related to its activity in corresponding cancers, and a lower, but still significant, relationship, was found also between bladder washings and tumors. However, telomerase activity in cancer exfoliated cells was not correlated to the clinical stage and grade.

In consideration of these results, we think that the measurement of telomerase activity in voided urine is a more reliable index of enzyme production in bladder cancer in comparison with bladder washings, with the additional advantage of a simpler and noninvasive collection of samples, making this assay suitable as an easy tool for larger studies to evaluate the clinical role of telomerase in bladder cancer. Such findings provide new evidence that molecular genetic methods for telomerase activity detection have the potential to provide a powerful and noninvasive tool for the early detection and clinical evaluation of human bladder cancer, as also demonstrated by the recent finding of a clinical role of human reverse telomerase reverse transcriptase expression in urinary sediment from bladder cancers (22). In addition, a quantitative evaluation of telomerase activity in exfoliated cells, could be a valuable tool in the prediction of bladder cancer recurrence after transurethral resection, as recently postulated (25). A follow-up study to test this hypothesis is in progress.

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