

Noninvasive Detection of Prostate Cancer by Quantitative Analysis of Telomerase Activity

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Abstract Purpose: Prostate cancer is the most common male malignancy and the second leading cause of male cancer death; therefore, there is urgent necessity for noninvasive assays for early detection of prostate cancer. Obtaining prostate tumor samples surgically is problematic because the malignancy is heterogeneous and multifocal and early-stage tumors are nonpalpable. In contrast, exfoliated cells represent the cancer status of the entire gland better due to the general tendency of cancer cells to exfoliate into biological fluids. The purpose of this study was to clarify whether quantitative analysis of telomerase activity in exfoliated cells in urine could serve as a reliable molecular marker of prostate malignancy.

Experimental Design: We analyzed prospectively post-prostatic examination – exfoliated cells from the urine of 56 patients undergoing routine prostate screening. Epithelial cells were isolated and enriched by immunomagnetic separation. Telomerase activity was analyzed by quantitative real-time PCR telomeric-repeat amplification protocol assay using Opticon MJ research instrument.

Results: We report now that all prostate cancer patients revealed high levels of telomerase activity thereby showing 100% of the assay sensitivity. In contrast, the majority of patients with clinically confirmed benign prostatic hyperplasia (BPH) did not express any telomerase activity (70% of all BPH patients), most likely presenting cancer-free cases, or expressed low levels of activity (18%). However, about 12% of BPH patients revealed high levels of telomerase activity that potentially can reflect hidden prostate cancer.

Conclusions: We suggest that the quantitative analysis of telomerase activity can be useful for the selection of prostate cancer and cancer-free cases.

Prostate cancer is the most common male malignancy and the second leading cause of male cancer-related death (1, 2). Small prostatic carcinomas were detected in 30% of men ages 30 to 49 years, 40% of males over age 50, and 64% of men ages 60 to 70 years (3–5). For this reason, an annual screening is generally recommended for all men over the age of 50. Screening on such a large scale requires noninvasive methods of detection and the use of reliable, clinically validated markers of prostate cancer. On the other hand, the total number of detected carcinomas exceeds the number of clinically manifested cases; therefore, there is a need for the markers that are suitable not only for distinction between benign and malignant growth but also for the selection of potentially aggressive tumors that require more aggressive treatment.

Prostate cancer screening is currently based on the digital rectal examination (DRE), prostate-specific antigen (PSA) monitoring, and transrectal ultrasound (TRUS) with TRUS-guided biopsy for morphologic examination as the reference standard. However, because prostate cancer is a heterogeneous and multifocal disease with typically low malignant cell infiltration, obtaining the representative tumor samples by biopsy is problematic. None of the standard methods are sufficiently sensitive and specific for prostate cancer, which makes early detection of the disease difficult. Early-stage prostate tumors are nonpalpable by definition, and about one third of all prostate tumors are not accessible to the DRE by location. PSA elevation (the recommended threshold levels for further diagnostic evaluation are ≥ 4.0 ng/mL) is not specific for cancer because PSA is also produced by other tissues and it occurs in men with benign diseases or after physical or surgical manipulation of prostate; in addition, men with prostate cancer often have normal PSA levels (5–7). A TRUS-guided needle biopsy may miss up to 34% of clinically localized prostate cancers, and about 10% to 19% of patients with initially negative needle biopsy were diagnosed with prostate cancer on a second biopsy (8–10). The morphologic manifestation and the expression of some molecular markers in multiple tissue samples obtained from different areas of cancer-bearing prostates vary dramatically (11); thus, each biopsy can miss the presence of prostate cancer, including even the most aggressive foci.

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In contrast to invasive sampling, prostate manipulation during DRE can harvest a representative pool of prostate cancer cells, as was shown earlier (12–14). This is possible because of the increased ability of cancer cells compared with normal cells to exfoliate into biological fluids (15) due to the weakening of cell-to-cell and cell-to-extracellular matrix contacts as a result of up-regulation and activation of extracellular matrix-degrading enzymes (16, 17). Exfoliated prostate cancer cells can be isolated from urine because about 3 to 4 cm of the urethra lies entirely within the prostate and receives the openings of all ducts of the gland. Indeed, previous data have shown not only the presence of viable tumor cells harvested by prostate massage in urine specimens from prostate cancer patients but also a significant correlation of their biological variables with the histopathologic status of the same surgically removed tissues (13, 14). The proportion of prostate cells among epithelial cells present in urine samples is higher in men with prostate cancer than in men who are free of prostate cancer (18). In general, the attempts to detect these cells by routine urine cytology are not expedient due to relatively low number of malignant cells, especially at the early stages of prostate cancer and high false-negative rates (up to 50%) of the method (19–21). In contrast, modern PCR-based molecular methods can detect much lower numbers of malignant cells compared with conventional histologic or cytologic examination. In addition, these methods are free of subjective data interpretation and have a high throughput, thereby providing a reliable basis for noninvasive cancer detection upon the availability of specific molecular markers of cancer.

Recent widespread interest in telomerase was initiated by the discovery that unlimited proliferation in most cancer and immortal cells is highly dependent on the activity of this ribonucleoprotein enzyme complex (22). Telomerase has the essential biological function of protecting DNA from degradation by catalyzing the elongation of telomeres by addition of noncoding repeated TTAGGG sequences to the ends of chromosomes (23). In contrast to almost 100 proposed molecular markers that can be applied only to a single or several types of cancer, telomerase is unique due to its almost universal expression in a vast majority of tumor tissues and its general lack of expression in normal somatic tissues, with the exception of highly proliferative tissues, germ line, and stem cells (24, 25). Telomerase activity may serve as a useful marker for early cancer detection because in many cancer types it appears early in the preneoplasia state (26). The diagnostic and prognostic utility of telomerase has been widely studied. There is increasing evidence that stronger telomerase activity with higher rate of detection correlates with poorly differentiated cancers and higher Gleason scores (26–29). It was suggested that the levels of telomerase activity might predict clinical outcome because high levels usually correlate with poor prognosis and higher cancer aggressiveness (30). The value of telomerase as a diagnostic marker for one particular urologic malignancy, bladder cancer, has been addressed in a large number of studies (reviewed in refs. 21, 31). Recent comparative analysis of various screening methods has shown that telomerase activity has the highest combination of sensitivity and specificity for cancer diagnosis reaching sensitivity of up to 90% and clinical specificity for cancer of 94% to 100% (21, 32–35). It is important to note that the sensitivity of telomerase assays at the early stages of cancer development is

significantly higher than the sensitivity of cytology-based assays, 75% versus 8%, respectively (36).

Telomerase is strongly associated with prostate cancer showing activation in up to 93% of cases and in all cell lines derived from human prostate cancer (27, 28, 37–39). In contrast, no telomerase activity was detected in normal uncultured prostate tissues. However, conflicting data were reported about telomerase activity in the adjacent noncancerous tissues. When a malignancy is present, the adjacent tissues may be contaminated by neighboring cancer cells and may show telomerase activity (25). Thus, it was shown that about 10% of samples from benign prostatic hyperplasia (BPH) are telomerase positive (27, 37); however, if the prostate is confirmed to be cancer-free, BPH tissues are telomerase negative (37, 38, 40). Presently, differential detection of benign and malignant prostatic diseases is one of the most challenging problems in the field and it requires clinical validation of molecular markers suitable for noninvasive screening for prostate cancer. One of the most promising candidates for this role is telomerase. However, only limited data exist about the correlation of telomerase activity in exfoliated cells in urine with prostate clinicopathology (35, 41, 42). In addition, accurate quantitative methods of telomerase activity measurements are required for reliable evaluation of the clinical utility of telomerase. Therefore, the major goal of this study was to clarify whether the quantitative analysis of telomerase activity in naturally voided urine specimens collected after DRE could serve as a reliable molecular marker of prostate malignancy. As an attempt to avoid false-positive and false-negative measurements of telomerase activity, we used immunomagnetic isolation of epithelial cells and highly sensitive quantitative real-time PCR telomeric-repeat amplification protocol (TRAP) assay. In addition, to avoid possible overestimation of the clinical utility of telomerase assay, we analyzed all samples prospectively, without prior knowledge of the patients' clinicopathologic status.

Materials and Methods

Patients. Patients undergoing routine medical examination of the prostate at the Department of Urology, State University of New York at Stony Brook and willing to participate in this study by signing the Institutional Review Board–approved consent form were recruited by participating urologists. Careful thorough palpation and examination of the prostate done by urologist during DRE to evaluate its size and presence of tumor was considered as a prostate massage. Naturally voided urine specimens were collected prospectively, immediately after medical examination, without prior knowledge of the patient's clinicopathologic data. The clinicopathologic status of each patient was assessed with the following standard procedures widely used for prostate cancer screening: clinical medical history and physical examination, DRE, serum PSA levels measurement, urine cytology, TRUS examination, and TRUS-guided biopsy for morphologic examination as a reference test. Histomorphologic grading was done in accordance to the Gleason system and staging of prostate cancer was defined according to the international tumor-node-metastasis classification. In all cases, results of standard pathologic study and quantitative measurements of telomerase activity by real-time PCR TRAP assay were determined independently, in a double-blind manner.

Isolation of exfoliated cells. Immediately after collection, urine specimens were put on ice and centrifuged as soon as possible (not later than a 20-minute interval) at 3,000 rpm, 4°C, for 7 minutes. Pelleted exfoliated cells were washed twice, first in 10 mL of cold PBS (pH 7.4)

followed by 2 mL of PBS containing 100 units/mL of RNase and protease inhibitor cocktail (SUPERase-In; Ambion, Palo Alto, CA).

Immunomagnetic cell sorting. To enrich and isolate malignant and normal epithelial cells from other cells in urine sediment, we used colloidal superparamagnetic microbeads conjugated with monoclonal antibodies against human epithelial antigen and the magnetic cell sorting device (miniMACS; Miltenyi Biotec, Auburn, CA) as recommended by the manufacturer. Briefly, washed and pelleted exfoliated cells were resuspended in 400 μ L ice-cold buffer and incubated with 50 μ L of human epithelial antigen microbeads and 50 μ L of blocking reagent at 4°C to 6°C on shaker followed by washing with 5 mL cold PBS containing 2 mmol/L EDTA, 0.5% bovine serum albumin, and 100 units/mL of SUPERase. Analyzed sample was placed into miniMACS separation column and the outcoming buffer with *negatively* selected cells was discarded. Then, miniMACS column was removed from magnetic device, washed with 1 mL PBS containing SUPERase, and *positively* selected by immunomagnetic beads epithelial cells were collected for further processing.

Protein extraction. Cells were pelleted again at 3,000 rpm, 4°C, for 7 minutes and resuspended in 25 to 50 μ L of ice-cold CHAPS lysis buffer (Celliance, Norcross, GA) containing 100 units/mL of SUPERase followed by an incubation on ice for 30 minutes. Lysates were then centrifuged at high speed (16,000 \times g for 20 minutes at 4°C). The aliquots of supernatant fluid was aliquoted and stored at -80°C.

Prostate cancer cell lines. Human prostate cancer cells, PC-3, were obtained from the State University of New York cell culture/hybridoma facility, where they were routinely cultured. Cells were harvested using trypsin-EDTA (Sigma, St. Louis, MO) and processed as it was described for exfoliated cells. Same protein extract from PC-3 cells was used in each real-time PCR run as a positive control and as a standard. Serial dilutions of protein extracts from these telomerase-positive cells, equivalent to 10⁵, 10⁴, 10³, 10², 10, and 1 cell, were used for standardization and relative quantification of telomerase activity in clinical samples by previously described real-time quantitative TRAP assays (42). Modified highly tumorigenic prostate cancer PC3-P and PC3-MM2 cells were kindly provided by Dr. I. Fidler (M.D. Anderson Cancer Center, Houston, TX). The protein concentration was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Real-time quantitative telomerase-repeat amplification protocol assay. Real-time PCR analysis of telomerase activity was carried out in a 96-well plate using the Opticon MJ Research instrument and optimized standard SYBR Green protocol. A fluorescence dye SYBR Green is capable of binding with exceptionally high affinity to the double-stranded amplicons and generating fluorescence signals after each PCR cycle. Collected fluorescence signals were analyzed with the Opticon software. The TS and ACX primers (TS, 5'-AATCCGTCGAGCAGAGTT-3'; ACX, 5'-GCGCGCTTACCCTTACCCTTACCCTAACC-3') were synthesized by Invitrogen Life Technologies (San Diego, CA). Stock solution of each primer (1 μ g/ μ L) was aliquoted and kept at -20°C. Total reaction volume was 20 μ L per well, containing 10 μ L of 2 \times SYBR Green Master Mix (Qiagen, Chatsworth, CA), 0.1 μ g of each primer, 2 μ L of protein extract, and 7.8 μ L of RNase-free water. The reaction mixture was first incubated at 25°C for 20 minutes to allow the telomerase presented in the protein extract to elongate the TS primer by adding TTAGGG repeat sequences. The PCR was then started at 95°C for 15 minutes (hot start) to activate the AmpliTaq polymerase, followed by a 40-cycle amplification (denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 60 seconds, and plate reading at 60°C for 10 seconds). Fluorescence signals produced by binding of SYBR Green to new double-stranded amplicons were collected and analyzed after each PCR cycle with Opticon software. All samples were run in duplicates. Reaction mixture with 2 μ L of CHAPS lysis buffer instead of protein extract (no target) was used as a negative control. Serial dilutions of the protein extracts from known number of prostate cancer PC-3 cells were used for construction of the standard curve for relative quantification of telomerase activity in patients samples and as a positive control. Relative telomerase activity

in patient samples was calculated based on the threshold cycle (C_t). The measurements of fluorescence signals at the early phase of exponential amplification allows comparative analysis of samples with different initial amount of target sequences and ensures the accuracy of the quantification because at this point the accumulation of inhibitory PCR products and the limitation of the reaction products are unlikely to occur.

Statistical analysis. Telomerase activity oriented toward detection of prostate cancer was compared with the standard diagnostic criteria based on clinicopathologic variables. The levels of telomerase activity in patient samples (integrated fluorescence per PCR cycle) were normalized to the levels of activity in the known number of prostate cancer PC-3 cells using a standard curve and Opticon MJ software. The cut point for telomerase assessment was determined to be 100 cell equivalent based on ROC curves. Sensitivities, specificities, positive and negative predictive values, overall percent agreements, and kappa statistics were estimated based on this cut point. All of the above statistics were also estimated for standard PSA measurements. The widely accepted PSA cut point of 4.0 ng/mL was used. Pearson test was done to evaluate correlations between levels of telomerase activity and the clinicopathologic variables in prostate cancer patients and patients with nonmalignant prostate diseases. Sensitivity, specificity, and the predictive values of our measurements was estimated by comparing levels of telomerase activity with the presence or absence of cancer, as well as with the presence or absence of nonmalignant disease, via standard clinicopathologic tests. Kappa statistics were used to assess this association. The Gleason score and presence or absence of telomerase activity was compared using the Mann-Whitney test. P s < 0.05 were considered statistically significant.

Results

Quantitative analysis of telomerase activity. For each experiment, under optimized conditions, we set up standard curves for serial dilutions of telomerase-competent prostate cancer PC-3 cell extracts, equivalent to different cell count (from 100,000 cells to single cells). Linearity and accuracy of real-time quantitative TRAP assay were tested using the same samples (serially diluted extracts of PC-3 cells) analyzed in 12 separate experiments in duplicates that have shown high reproducibility and precision (a systematic error was 0.121 PCR cycle; P < 0.001). The limits of sensitivity for the Opticon MJ Research were determined as about 10 PC-3 cells; thus, all measurements of the telomerase activity in patient samples were considered as negative if after normalization to the PC-3 cell equivalent the numbers were <10.

Subsequent review of the coded medical records revealed nine patients with clinically confirmed prostate cancer, one patient with atypical cells suspicious for prostate cancer, two patients with high-grade prostatic intraepithelial neoplasia and 44 patients with no clinical evidence of malignant disease (BPH). Table 1 represents a summary of all cases with standard pathologic evaluation, telomerase activity, and PSA levels measurements. Median patient age was 61.3 years, ranging from 41 to 82 years. After immunomagnetic separation of the epithelial pool of exfoliated cells (that may or may not contain prostate cancer cells) from the rest of urinary cellular content, high levels of telomerase activity equivalent to the average 4,242 cells (range, 100-19,489 PC-3 cells) were detected in all patients with clinically localized prostate cancer ($n = 9$; Table 1). Two of two patients with high-grade prostatic intraepithelial neoplasia and one of one patient with atypical cells suspicious for prostate cancer also revealed relatively high levels of

Table 1. Summary of all cases for telomerase activity and total serum PSA levels

Case	Pathology	Telomerase activity		PSA (ng/mL)	
		0-100	≥100	≤4	≥4
1	Prostate cancer	—	100	—	6.3
2	Prostate cancer	—	225	—	74.1
3	Prostate cancer	—	235	—	4.2
4	Prostate cancer	—	450	—	21.2
5	Prostate cancer	—	732	—	5.0
6	Prostate cancer	—	3870	—	4.1
7	Prostate cancer	—	5830	—	8.76
8	Prostate cancer	—	7247	3.49	—
9	Prostate cancer	—	19489	—	13.25
10	HG PIN	—	157	—	4.6
11	HG PIN	—	202	—	4.0
12	Atypical Cells	—	214	2.66	—
13	BPH	0	—	2.8	—
14	BPH	0	—	—	7.0
15	BPH	0	—	—	7.0
16	BPH	0	—	NA	NA
17	BPH	0	—	—	8.0
18	BPH	0	—	0.47	—
19	BPH	0	—	1.36	—
20	BPH	0	—	0.97	—
21	BPH	0	—	1.88	—
22	BPH	0	—	0.75	—
23	BPH	0	—	1.22	—
24	BPH	0	—	1.30	—
25	BPH	0	—	1.98	—
26	BPH	0	—	1.48	—
27	BPH	0	—	1.76	—
28	BPH	0	—	—	10.08
29	BPH	0	—	0.31	—
30	BPH	0	—	2.13	—

Table 1. Summary of all cases for telomerase activity and total serum PSA levels (Cont'd)

Case	Pathology	Telomerase activity		PSA (ng/mL)	
		0-100	≥100	≤4	≥4
31	BPH	0	—	—	8.39
32	BPH	0	—	0.43	—
33	BPH	0	—	0.14	—
34	BPH	0	—	1.47	—
35	BPH	0	—	2.52	—
36	BPH	0	—	1.33	—
37	BPH	0	—	1.21	—
38	BPH	0	—	1.57	—
39	BPH	0	—	1.22	—
40	BPH	0	—	1.43	—
41	BPH	0	—	0.33	—
42	BPH	0	—	0.38	—
43	BPH	0	—	—	11.3
44	BPH	0 (55)	—	—	6.7
45	BPH	0 (13)	—	1.21	—
46	BPH	0 (19)	—	1.27	—
47	BPH	0 (26)	—	0.96	—
48	BPH	0 (32)	—	0.69	—
49	BPH	0 (53)	—	0.48	—
50	BPH	0 (88)	—	2.57	—
51	BPH	0 (89)	—	—	12.1
52	BPH	—	164	—	8
53	BPH	—	173	1.55	—
54	BPH	—	176	1.15	—
55	BPH	—	431	—	4.7
56	BPH	—	616	1.71	—

Abbreviations: HG PIN, high-grade prostatic intraepithelial neoplasia; NA, not applicable.

telomerase activity (average 330 cell equivalent for high-grade prostatic intraepithelial neoplasia and 214 for atypia). Figure 1 shows a representative real-time PCR TRAP analysis of telomerase activity in patient with clinically confirmed prostate cancer. Amplification plots revealed a cell number-dependent amplification of telomeric repeats on serial dilutions of PC-3 cells. Possible artifacts (such as primer-dimer amplification) were monitored by using *no template control* by addition of the lysis buffer instead of the protein extract to the reaction mixture. At described conditions, the primer-dimer amplification did not occur because fluorescence signals in negative controls and highly diluted samples were always below the amplification threshold (*dotted line, left*).

The cohort of patients diagnosed with BPH ($n = 44$) revealed three different subgroups based on the levels of telomerase activity normalized to the number of PC-3 cells (see Table 1; Fig. 2, *right*). The first largest group (31 of 44 cases, ~70%) did not show any amplification of the telomeric repeats that was considered as an absence of telomerase activity. The second group (8 of 44, ~18%) expressed low levels of telomerase activity equivalent to the average 46 PC-3 cells (range, 13-89 cell equivalent). The third group (5 of 44, ~12%) showed

relatively high levels of telomerase activity at average 312 PC-3 cells (range, 164-616 cells), comparable with those of patients with clinically localized prostate cancer. One patient diagnosed with BPH and inflammation had relatively high levels of telomerase activity; in about 1 year, he was diagnosed with prostate cancer (Gleason score 7).

Statistical analysis. We have evaluated the *sensitivity* (percent of the telomerase-positive cases among cases with clinically localized prostate cancer), *specificity* (percent of the telomerase-negative cases among clinically defined nonmalignant cases), *positive predictive value* (a probability to have clinically localized prostate cancer if telomerase activity is positive), and *negative predictive value* (a probability to be clinically free of prostate cancer if telomerase activity is negative). To evaluate these variables, we have excluded two cases with high-grade prostatic intraepithelial neoplasia and one case with atypical cells as cases with clinically indefinite diagnosis (neither normal nor cancer). We used a standard cut-off level of 4.0 ng/mL for serum PSA. Diagnostic performance of the telomerase activity was analyzed for cut-off level of 100 PC-3 cell equivalent. Therefore, after exclusion of three cases with clinically indefinite diagnosis, we had nine cases with clinically manifested

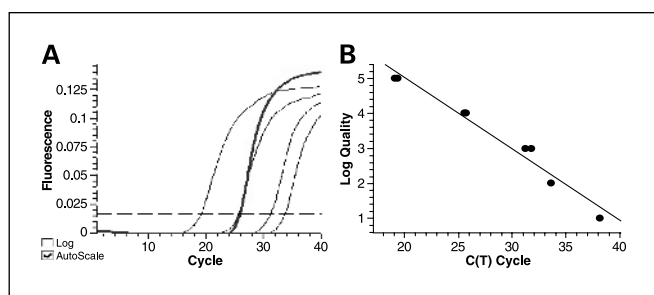


Fig. 1. Analysis of telomerase activity in urine of prostate cancer patient by real-time PCR. Epithelial pool of exfoliated cells harvested by prostate massage was immunomagnetically enriched and isolated from urine of patient with prostate cancer (Gleason score 7; T1cNxMx). Amplification curves (A, family of thin lines) from serially diluted protein extracts of 100,000 PC-3 cells served as a *standard curve* (B) for relative quantification of telomerase activity and as a positive control. The lack of the amplification in highly diluted samples (<10 cells), as well as in samples with omitted protein extracts (negative control) reflected the absence of artifacts, including primer dimers formation. In this case, relative telomerase activity automatically calculated by Opticon MJ Research software was equivalent to the activity of ~10,000 PC-3 cells. Note that all samples can be compared with each other in a linear range above the threshold cycle (C_t , dotted line).

prostate cancer and 44 BPH cases. Because all cancer cases were telomerase positive, a sensitivity of this assay was calculated as 100% (9 of 9). An assay specificity with cut-off level of 100 cell equivalent was 88.6% (39 telomerase-negative cases of 44 BPH patients). Positive predictive value was 64.3% (nine clinically localized prostate cancer among 14 cases with high telomerase activity). A negative predictive value, was calculated as 100% because all telomerase-negative cases were clinically normal (BPH, 39 of 39). Analysis of the PSA levels had shown 88.9% sensitivity for prostate cancer (8 of 9); specificity 76.7% (33 cases with normal PSA of 43 BPH patients); positive predictive value was 50% (among 18 cases with elevated PSA levels, nine were diagnosed with prostate cancer) and negative predictive value was 97% (among 34 cases with normal PSA levels, 33 patients were clinically normal). These data were summarized in Table 2. For telomerase activity, Pearson correlation coefficient was 0.504 ($P < 0.001$); $\kappa = 0.726$ ($P < 0.001$). For serum PSA levels, Pearson correlation coefficient was 0.437 ($P < 0.001$); $\kappa = 0.47$ ($P < 0.001$). Because all prostate cancer cases in this study had high Gleason scores (range, 6-9) and high levels of telomerase activity, but cellular equivalents of relative telomerase activity were variable, no correlation was detected between the levels of telomerase activity and Gleason score.

Discussion

Our study had principal differences with similar recently published studies (35, 41, 42). First, we collected post-prostatic massage exfoliated cells prospectively, without prior knowledge of clinicopathologic data. Second, we isolated an epithelial pool of exfoliated cells by immunomagnetic cell sorting to exclude possible contribution of other telomerase-competent cells, such as activated lymphocytes or other proliferating cells that might be present in urine. Because tumor cells may be present in low numbers and because the enzymatic activity of telomerase and the PCR efficiency might be affected by the presence of inhibitors in urine, we combined the standardized sample processing with highly sensitive measurements of telomerase activity by quantitative real-time PCR. In contrast

to previously published studies (35, 41, 42) that have reported telomerase activity in exfoliated cells in prostatic fluid and urine in 90%, 91%, and 58% of prostate cancer patients, respectively, in the present study, all patients with clinically localized prostate cancer have shown relatively high levels of telomerase activity that corresponds to 100% of the assay sensitivity. These data are in agreement with basic knowledge that tumor cells exfoliate early during carcinogenesis, *before* the primary tumor can be detected by standard clinical tests (*CAP Today Archive*, July 1998). The fact that prostate cancer cells do exfoliate into voided urine was confirmed by early studies (12–14) that showed the presence of viable cancer cells of prostatic origin in post-massage samples. In the present study, higher than previously described rates of the detection of telomerase activity in prostate cancer cases can be explained by the use of the real-time PCR technology compared with conventional TRAP assays in all previous reports. Although TRAP assay (24) is the most common strategy for detecting telomerase activity, for prostate cancer, which is characterized by low tumor cell infiltration, the number of exfoliated cancer cells might be insufficient for detection by conventional TRAP techniques that require at least 250 to 5,000 tumor cells (43). In contrast to the end-point PCR amplification, the real-time quantitative TRAP assay allows comparative analysis of samples with different initial amounts of target sequences, because fluorescence signals are measured at the early phase of exponential amplification when reaction products are not limited and the accumulation of inhibitory PCR products is unlikely to occur (44). In addition, RTQ-TRAP assay has several other advantages, such as the exclusion of the time- and resources-consuming post-PCR steps, reliable measurements of telomerase activity in the linear range down to low number of cell equivalents, no interference of primer-dimer artifacts, and relatively high throughput.

Potential clinical significance of quantitative analysis of telomerase activity. Whereas the quantitative analysis of telomerase activity has shown 100% sensitivity for prostate cancer, its positive predictive value was estimated at only 64.3% (Table 2), due to the fact that we have detected relatively high levels of telomerase activity (comparable with those of prostate cancer patients) in about 12% of BPH patients. Although these findings obviously conflict with standard clinicopathologic data, they are in agreement with the growing number of reports that the presence of telomerase activity in the cytologically benign lesions may be indicative of the existence of hidden cancer or premalignant disease elsewhere in the gland (28, 34,

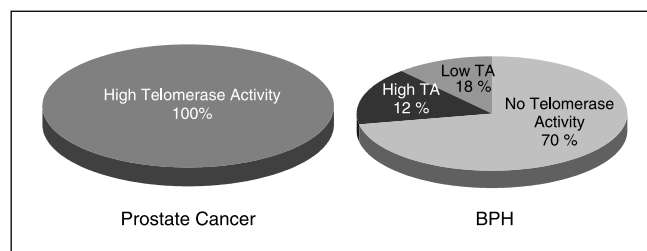


Fig. 2. Differential expression of telomerase activity in prostate cancer and BPH patients. All patients with clinically localized prostate cancer (*left*) had shown relatively high levels of telomerase activity (100% of the assay sensitivity). The cohort of patients with BPH (*right*, no cancer was detected by standard clinical tests) had shown different levels of telomerase activity: about 70% of cases were telomerase negative, 18% demonstrated low levels, and 12% relatively high telomerase activity, comparable with prostate cancer levels.

Table 2. Comparison of the diagnostic performances of telomerase activity and PSA

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Pearson correlation
Telomerase activity	100 (9 of 9)	88.6 (39 of 44)	64.3 (9 of 14)	100 (39 of 39)	0.504 ($P < 0.001$)
PSA	88.9 (8 of 9)	76.7 (33 of 43)	50 (9 of 18)	97 (33 of 34)	0.437 ($P < 0.001$)

35, 38, 40, 45). Previous studies (37, 38, 40) analyzed telomerase activity by conventional TRAP assay in 10, 16, and 46 surgically removed tissues from BPH patients, respectively, and in urine samples from 30 BPH patients in another study (35). In the present study, telomerase activity was analyzed in a double-blind manner in a cohort of 44 patients with independently obtained clinicopathologic data. Because we analyzed a pool of exfoliated cells that presumably represents all regions of the prostate more objectively than a single set of TRUS-guided biopsies, the positive predictive value of telomerase activity should not be expected to show a good agreement with standard clinical tests. In support of this suggestion, a recent study showed that 10% to 15% of patients diagnosed with BPH by standard clinical tests had T1a and T1b carcinomas (40). It was reported that a second set of biopsies revealed prostate cancer in about 19% of patients with initially negative needle biopsy (8, 10). In addition, a 7-year follow-up study had shown that 11% of patients with negative prostate biopsy were subsequently diagnosed with prostate cancer (45). In a recent study, telomerase activity in prostatic fluid was detected by standard TRAP assay in 13% of BPH patients (35). The authors concluded that it represented false-positive results due to the presence of the inflammatory disease in all cases, with detectable foci of lymphocytes concentration. Although we agree that activated lymphocytes express some levels of telomerase activity, such a contribution should not affect our measurements because we used the immunomagnetic separation of the epithelial pool of exfoliated cells. Because several studies have shown the lack of telomerase activity in pure normal samples, without histologic evidence of prostate tumor anywhere within the gland (28, 42), we suggest that 70% of telomerase-negative measurements in our BPH cases reflect the absence of malignancy, which potentially may be an argument for the exclusion of a large population of patients from further harmful diagnostic procedures. One patient with high levels of telomerase activity in the present study, who was previously misdiagnosed with BPH and inflammation, eventually has revealed the prostate cancer with the Gleason score 7. Therefore, although the clinical significance of positive and negative readings of telomerase activity in BPH cases has to be validated during the long-term follow-up with repetitive quantitative measurements of telomerase activity and repetitive accurate clinical tests, current data suggest that high levels of telomerase activity may be a serious argument to suspect prostate cancer even if it is not confirmed by the first set of standard clinical tests.

It has been shown previously that poorly differentiated cancers with higher Gleason scores are generally associated with the higher rate of telomerase detection and stronger telomerase activity (27–29). The measured levels of telomerase activity may potentially predict the clinical outcome

because high telomerase levels usually correlate with poor prognosis and higher aggressiveness (30). It is reasonable to assume that the telomerase activity is proportional to the number of cancer cells in the sample and to their malignant potential. Our recent data have shown that the levels of telomerase activity correlate with invasive potential of prostate cancer cells. Thus, highly invasive cell lines such as PC3-P and PC3-MM2 had dramatically increased telomerase activity compared with the unmodified and less aggressive prostate cancer cell line, PC-3 (Fig. 3, 47). These results provide a basis for potential application of the quantitative analysis of telomerase activity for discrimination between indolent and aggressive prostate carcinomas. In addition, the quantitative analysis of telomerase activity in urine represents no potential risks and requires no additional costs for sampling; therefore, it can be safely used repeatedly.

Because prostate fluids and urine share the common anatomic duct, the urethra, there is a possibility of the diagnostic bias in case if prostate cancer coincides with other urological malignancies. For example, it was reported that about 3.2% to 3.4% of prostate cancer cases coincide with bladder cancer and that 25% to 70% of bladder cancer cases coincide with prostate cancer (reviewed in ref. 48). Theoretically, cancer cells of other urological malignancies may contribute to the measured levels of telomerase activity, but any potential decrease in the assay's specificity is expected to be minor, due to the significant prevalence of prostate cancer compared with other urological cancers. In the present study, the possibility of coincidental presence of prostate cancer with the high-grade bladder cancer was largely eliminated by

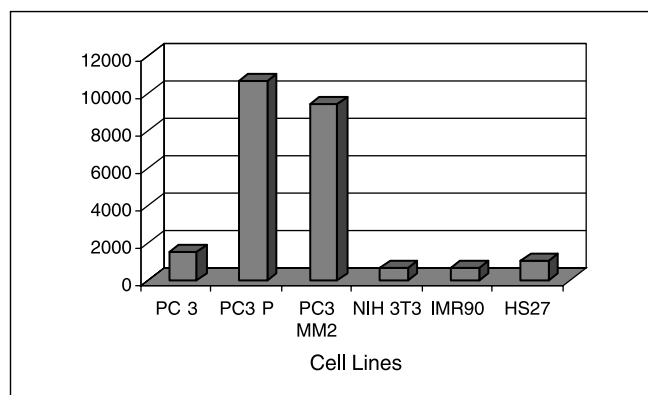


Fig. 3. Comparative analysis of telomerase activity in cells with different invasive potential. Highest levels of activity were detected in modified cells with high invasiveness (PC3 P and PC3 MM2) compared with prostate cancer PC-3 cells and several normal cultured cells (NIH 3T3, IMR 90, and HS27). Same protein concentration from different cell lines was analyzed by real-time PCR using serial dilutions of a known number of PC-3 cells as a standard.

monitoring all the samples with the standard urine cytology. In our opinion, early detection of cancer cells of any origin in urine is beneficial for the patient because it will indicate the need for further diagnostic tests focused specifically on urological malignancies.

In conclusion, our data indicate that high levels of telomerase activity in immunomagnetically separated epithelial cells from post-DRE urine specimens strongly suggest the presence of prostate cancer; in contrast, the absence of telomerase activity may be indicative of the lack of prostate

cancer. We also suggest that differential levels of telomerase activity potentially may be used for selection of nonpalpable and potentially aggressive carcinomas that currently have no clinical manifestation. Clinical significance of low levels of telomerase activity in BPH patients needs to be further investigated. It might reflect the presence of indolent noninvasive prostate cancer or early stage of the disease; however, further studies and long-term clinical follow up are necessary to correlate the quantitative analysis of telomerase activity with clinicopathologic data.

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