Relevance of Urine Telomerase in the Diagnosis of Bladder Cancer

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The incidence of human bladder cancer has greatly increased over the last few decades, with more than 60,000 new cases diagnosed each year in the United States alone, and now represents the 4th most common malignancy in men and the 10th most common in women. According to the latest reports from the National Cancer Institute, the incidence of this pathology is higher in industrialized than in developing countries.

Bladder cancer is 3 times more common among men than women, and the incidence increases with age. Approximately 80% of newly diagnosed individuals are aged 60 years or older. At present, about 20% of patients die each year, but when the disease is diagnosed and treated in the early stage, the chances of survival are good, thus highlighting the importance of a timely and accurate diagnosis.

More than 90% of newly diagnosed bladder cancers are transitional-cell carcinomas. Approximately 75% of patients present with superficial cancer, 20% with invasive disease, and the remaining 5% with metastatic disease at first diagnosis. Established approaches for detecting bladder cancer include urine cytology and cystoscopy, used singly or in sequence. However, the invasiveness and relatively high cost of cystoscopic examination and the limited sensitivity of urinary cytology, especially for low-grade superficial lesions, make it of the utmost importance to develop a noninvasive, reliable, and simple test to increase the rate of detection of bladder cancer. Among the markers investigated for this purpose, an important role has been played by telomerase activity in voided urine or bladder washings, determined by the telomeric repeat amplification protocol (TRAP) assay. Initially, studies dealt with qualitative determinations. To obtain a more accurate and reliable estimate of telomerase activity levels, a quantitative TRAP assay was developed, based on the exponential amplification of the

Context The identification of new molecular markers is one of the most challenging goals for the early detection of bladder cancer because available noninvasive methods have neither sufficient sensitivity nor specificity to be acceptable for routine use.

Objective To develop a relatively simple, inexpensive, and accurate test that measures telomerase activity in voided urine to apply to large-scale screening programs for bladder cancer detection.

Design, Setting, and Participants Case-control study conducted in 218 men (84 healthy individuals and 134 patients at first diagnosis of histologically confirmed bladder cancer), frequency matched by age and recruited between March 2003 and November 2004 in Italy. Urine telomerase activity was determined using a highly sensitive telomeric repeat amplification protocol (TRAP) assay. Urine samples were processed for cytological diagnosis and TRAP assay. The diagnosis of bladder cancer was based on biopsic and cystoscopic examinations. The performance of the TRAP assay to detect urine telomerase activity was compared with urine cytology as an aid to early cancer detection. Quantification of urine telomerase activity was conducted in a blinded manner.

Main Outcome Measure Sensitivity and specificity of TRAP to detect bladder cancer.

Results Using a 50 arbitrary enzymatic unit cutoff value, we validated the results obtained in the pilot study. In the overall series, sensitivity was 90% (95% confidence interval [CI], 83%-94%) and specificity was 88% (95% CI, 79%-93%). Specificity increased to 94% (95% CI, 85%-98%) for individuals aged 75 years or younger. The same predictive capacity of telomerase activity levels was observed for patients with low-grade tumors or with negative cytology results.

Conclusions The present validation study demonstrated the ability of urine telomerase activity levels to accurately detect the presence of bladder tumors in men. This test represents a potentially useful noninvasive diagnostic innovation for bladder cancer detection in high-risk groups such as habitual smokers or in symptomatic patients.
primer-telomeric repeats generated in the telomerase reaction.\textsuperscript{12-13} Using this assay, telomerase activity has been detected in almost all superficial urothelial cell carcinomas, but not in healthy urothelium.\textsuperscript{16} We used the TRAP assay with the internal standard developed by Wright et al\textsuperscript{17} and added a reference curve to obtain more accurate and reproducible results.\textsuperscript{18}

The promising results from our pilot study\textsuperscript{19} prompted us to carry out a case-control study, prospectively planned and performed blindly on urine from male individuals to validate the 50 arbitrary enzymatic units (AEUs) that emerged as the best cutoff and to define the diagnostic accuracy of different telomerase activity cutoff values in terms of sensitivity and specificity.

**METHODS**

**Case Series**

The study was conducted in 218 men (Figure 1), of whom 84 were healthy individuals and 134 were patients at first diagnosis of bladder cancer, frequency matched by age (≤75 years and >75 years). Median age was 62.4 years (range, 22-98 years) in healthy individuals and 69.8 years (range, 33-88 years) in patients.

Healthy individuals were recruited from hospital laboratory staff and geriatric wards, and none had been previously clinically diagnosed with any type of cancer or with inflammatory pathologies of the urogenital tract.

Patients were prospectively enrolled from the Urology Departments of Pierantoni-Morgagni Hospital (Forlì) and Infermi Hospital (Rimini) between March 2003 and November 2004. All patients underwent cystoscopy as a reference standard for bladder cancer detection, and all tumors or suspicious lesions were resected. Patients who had undergone previous treatment were excluded.

The final diagnosis of bladder cancer was based on histologic examination. Histologic type and tumor cell differentiation were determined according to World Health Organization criteria. Fifteen (11%) tumors were well differentiated (G1), 55 (41%) were moderately differentiated (G2), and 57 (42%) were poorly differentiated (G3). There was 1 carcinoma in situ. Grading was not available for 6 patients.

Demographic data and medical history were collected at study entry. The local ethics committee reviewed and approved the study protocol for each center, and all participants provided written informed consent.

**Urine Collection**

Urine samples from both healthy individuals and patients were processed for cytological diagnosis and TRAP assay. Each patient evaluated for bladder cancer provided a voided urine sample immediately before cystoscopy.

**Cytology**

Cytological examination was performed in all the urine samples from healthy individuals (n=84) and in 103 of the 134 bladder cancer patients analyzed with TRAP assay. Forty-eight (46.6%) patients had positive cytology, 40 (38.8%) had negative cytology, 8 (7.8%) patients with suspicious cytology findings had evidence of bladder cancer at histologic examination, and 7 (6.8%) had nonassessable cytology because of a lack of exfoliated cells. The cytological examination was unavailable for 31 patients because they bypassed this preliminary urine evaluation and directly underwent cystoscopy.

**TRAP Assay**

Cell extract preparation and TRAP assay were carried out as previously described.\textsuperscript{18,19} Cells were pelleted by centrifugation (850g for 10 minutes at 4°C) within 1 to 3 hours of urine sample collection, washed once in phosphate-buffered saline, resedimented by centrifugation (2300g for 5 minutes at 4°C), and stored at −80°C until use (a maximum of 12 months). The pelleted cells were resuspended in 200 µL of lysis reagent\textsuperscript{19} and left on ice for 30 minutes.

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**Figure 1. Study Flow Diagram**

**URINE TELOMERASE IN THE DIAGNOSIS OF BLADDER CANCER**

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(Reprinted) JAMA, October 26, 2005—Vol 294, No. 16 2053
formed in duplicate, and when varia-
tions the value of MCR and expressed in
the ratio of TRAP ladder/ITAS per cell
sample is presented as the percentage of
telomerase activity per cell for each
150–base pair ITAS peak. The relative
each sample were also normalized to the
tection of telomerase activity, the areas of
different peaks was calculated. To obtain
samples prospectively, without previous
knowledge of the patient’s clinico-
pathologic status.

The threshold value for optimal
sensitivity and specificity was deter-
mined using a receiver operating char-
acteristic (ROC) curve,20 constructed
using the median test. For all tests, a
two-sided P=.05 was regarded as signif-
ificant. Data analyses were performed
with SAS release 8.0 (SAS Institute
Inc, Cary, NC).

All statistical analyses were per-
formed at the Unit of Biostatistics and
Clinical Trials of Istituto Oncologico
Romagnolo, Forli, Italy.

RESULTS

The median telomerase activity value
in urine was 27 AEUs (range, 0–88) in
healthy individuals and 112 AEUs
(range, 30–382) in patients. We did not
observe any patients with a telomer-
ase activity value lower than 30 AEUs
or any healthy individuals with a telom-
erase activity value higher than 90
AEUs. Moreover, in patients with nega-
tive or positive cytology, the median
telomerase activity values in urine were
99 (range, 38–265) and 134 (range, 37–
253) AEUs, respectively.

As primary end point, we validated
the results obtained in the pilot study
using a 50-AEU cutoff value. In the
overall series, 90% (95% CI, 83%–
94%) sensitivity and 88% (95% CI, 79%–93%) specificity were observed.

As secondary end point, the diag-
nostic relevance of urine telomerase ac-
tivity was analyzed for the overall se-
ties and for the subgroups of individuals
75 years or younger and older than 75
years. The ROC curve analysis pro-
vides a graphic demonstration of the
sensitivity and specificity of telomere-
ase activity in the overall series and the
even higher specificity in the sub-
group of individuals 75 years or younger (FIGURE 2).

In particular, sensitivity in the over-
all series ranged from 61% to 100% and
specificity from 54% to 100% accord-
ing to the different AEU cutoff values
(TABLE 1). As shown in Figure 2, a simi-
lar sensitivity and an even higher speci-
ficity (94%) (95% CI, 85%–98%) was
obtained in the subgroup of individu-
als 75 years or younger.

Although an increase in urine telom-
erase activity levels was observed from
histologic grades 1 to 3, it did not reach
statistical significance (TABLE 2).

The sensitivity of urine telomerase ac-
tivity in detecting bladder tumors
was similar in the subgroups of patients with different tumor grades at
all AEU cutoff values. In particular, at
50 AEUs the sensitivity was 93%, 87%,
and 89% for grades 1, 2, and 3, respec-
tively (TABLE 3).

COMMENT

Telomerase has been investigated as a
potentially useful biomarker for early
cancer detection21–23 and prognosis24
and for monitoring residual disease.21
Elevated levels of telomerase expres-
sion, in particular of the human telom-
erase reverse transcriptase catalytic sub-
unit, have been observed in almost all
human tumor histotypes, including
bladder cancer. In contrast, telomere-

Cell lysates were centrifuged (10 000 g
for 20 minutes at 4°C), and the super-
natant extracts were stored at −80°C. Al-
quots of each urine sample containing
1 µg of protein lysate were used for the
TRAP assay. Telomerase products were
evaluated on fluorescence electrophero-
grams, and the area underlying the dif-
frent peaks was calculated. To obtain
semi-quantitative levels of telomerase ac-
tivity, an internal telomerase assay stan-
dard (ITAS; 25 attograms17), amplified
by the same 2 primers used for the
telomerase activity assay, was included
in the TRAP buffer. Protein concentra-
tions corresponding to 10, 30, 100, 300,
1000, and 3000 cells of a human blad-
der cancer line (MCR)18 were analyzed
in each assay and used as the reference
curve. To obtain quantitative evalua-
tions of telomerase activity, the areas of
each sample were also normalized to the
150–base pair ITAS peak. The relative
telomerase activity per cell for each
sample is presented as the percentage of
the ratio of TRAP ladder/ITAS per cell
vs the value of MCR and expressed in
AEUs. All experiments were per-
fomed in duplicate, and when varia-
tions were greater than 15%, observed
in about 10% of cases, a third analysis
was performed. Telomerase activity was
expressed as a continuous variable in all
analyses.

Statistical Analysis

The population size was defined on the
basis of results from the previous pilot
study18 in which we obtained 93% sen-
tivity and 90% specificity using the 50-
AEU cutoff value for the subgroup of
male individuals. In fact, for the 84
healthy individuals and 134 bladder
cancer patients of the present study, we
predicted the 95% confidence interval
(CI) to be ±5% with respect to the single
estimated value for sensitivity and speci-
ficity. To avoid bias in the clinical util-
y of the TRAP assay, we analyzed all
samples prospectively, without previous
knowledge of the patient’s clinico-

Figure 2. Receiver Operating Characteristic
Curve of Telomerase Activity

For the overall series, the area under curve is 0.951
(95% confidence interval [CI], 0.925–0.976) and for
individuals aged ≤75 years, 0.968 (95% CI, 0.942–
0.993). Points are marked to demonstrate the sensi-
tivity and 1–specificity of urine cytology and of the
telomeric repeat amplification protocol (TRAP) test at
cutoff points of 40, 50, 60, and 70 arbitrary enzym-
atic units (AEUs).
ase activity has not been detected in most of the somatic cells. 31-33

We confirmed the high sensitivity and specificity of urine telomerase levels, in particular of the 50-AEU cutoff observed in our pilot study, in detecting bladder cancer. 18

Moreover, we observed that sensitivity is not age dependent, whereas specificity is higher in individuals younger than 75 years.

The test we developed requires a small amount of urine; is noninvasive, inexpensive, and easy to perform; and permits a quantitative evaluation of telomerase activity in urine. Furthermore, it is objective, reproducible, and specific and is not reliant on the expertise of the cytopathologist. 8 Another important advantage of this test is its ability to also identify low-grade tumors, which often escape detection during cytologic examination.

However, notwithstanding the validated optimal diagnostic accuracy of the test, it is not recommended for use in routine screening programs because of the low incidence of bladder cancer, and should be aimed at high-risk subgroups. Specifically, smokers have about a 3-fold increased risk of developing bladder cancer compared with nonsmokers. It might be even more advantageous in terms of cost/benefit to use the TRAP assay in selected individuals who present with hematuria.

For this subgroup, the incidence of bladder cancer is about 10% to 15% and the sensitivity of urinary cytology is only 30% to 50%. 31-33 Although cystoscopy is the gold standard for the diagnosis of bladder cancer because of its 90% sensitivity, the invasiveness and low specificity of the procedure in about 90% sensitivity, the invasiveness and low specificity of the procedure in bladder cancer is about 10% to 15% and the low incidence of bladder cancer, and should be aimed at high-risk subgroups. Further prospective studies on larger patient populations are needed to assess the diagnostic role of urinary telomerase, to define the ability of this assay to detect low-grade tumors, and to forecast clinical relapse.

Author Contributions: Ms Sanchini had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Sanchini, Nanni. Acquisition of data: Sanchini, Gunelli, Fabbri, Sermasi, Bercovich, Ravaiolli, Amadori, Calistri. Analysis and interpretation of data: Sanchini, Bravaccini, Calistri. Drafting of the manuscript: Sanchini, Gunelli, Bercovich, Ravaiolli, Amadori, Calistri. Critical revision of the manuscript for important intellectual content: Sanchini, Nanni, Bravaccini, Fabbri, Sermasi, Amadori, Calistri. Statistical analysis: Sanchini, Nanni. Obtained funding: Sanchini, Amadori, Calistri.

Table 1. Sensitivity and Specificity of Urine Telomerase Activity in the Overall Series and in Individuals Aged ≤75 Years

<table>
<thead>
<tr>
<th>Cutoff, AEU</th>
<th>Overall Series (N = 218)</th>
<th>≤75 y (n = 157)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>54 (43-64)</td>
</tr>
<tr>
<td>40</td>
<td>96 (91-98)</td>
<td>73 (62-81)</td>
</tr>
<tr>
<td>50</td>
<td>90 (83-94)</td>
<td>88 (79-93)</td>
</tr>
<tr>
<td>60</td>
<td>76 (68-83)</td>
<td>90 (82-95)</td>
</tr>
<tr>
<td>70</td>
<td>69 (61-76)</td>
<td>95 (88-98)</td>
</tr>
<tr>
<td>80</td>
<td>63 (54-70)</td>
<td>98 (92-99)</td>
</tr>
<tr>
<td>90</td>
<td>61 (53-69)</td>
<td>100</td>
</tr>
</tbody>
</table>

Abbreviations: AEU, arbitrary enzymatic unit; CI, confidence interval.

Table 2. Relationship Between Telomerase Activity and Histologic Grade

<table>
<thead>
<tr>
<th>Histologic Grade</th>
<th>Patients, No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEU, median (range)</td>
<td>88 (38-382)</td>
<td>100</td>
<td>100</td>
<td>122</td>
</tr>
</tbody>
</table>

Abbreviation: AEU, arbitrary enzymatic units.

*Median test, $\chi^2 = 0.76$, $P = .68$.

Table 3. Sensitivity of Urine Telomerase Activity in Patients With Different Tumor Grades

<table>
<thead>
<tr>
<th>Cutoff, AEU</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 218)</td>
<td>(N = 157)</td>
<td>(N = 157)</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>93 (70-99)</td>
<td>96 (88-99)</td>
<td>96 (86-98)</td>
</tr>
<tr>
<td>50</td>
<td>93 (70-99)</td>
<td>87 (76-94)</td>
<td>89 (79-85)</td>
</tr>
<tr>
<td>60</td>
<td>73 (48-89)</td>
<td>71 (58-81)</td>
<td>79 (67-88)</td>
</tr>
<tr>
<td>70</td>
<td>60 (36-90)</td>
<td>65 (52-77)</td>
<td>75 (63-85)</td>
</tr>
<tr>
<td>80</td>
<td>53 (30-75)</td>
<td>60 (47-72)</td>
<td>68 (56-79)</td>
</tr>
<tr>
<td>90</td>
<td>47 (25-70)</td>
<td>58 (45-70)</td>
<td>68 (56-79)</td>
</tr>
</tbody>
</table>

Abbreviations: AEU, arbitrary enzymatic units; CI, confidence interval.

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Administrative, technical, or material support: Sanchini, Gunelli, Bravaccini, Fabbrì, Sermasi, Bercovich, Ravaoli, Amadori, Calistri.

Study supervision: Sanchini, Calistri.

Financial Disclosures: None reported.

Funding/Support: This work was supported by Istituto Oncologico Romagnolo, Forli and the National Research Council [Consiglio Nazionale delle Ricerche [National Research Council]]–Ministero dell’Istruzione, dell’Università e della Ricerca [Ministry of Education, University and Research] “Oncolegia”: grants 02.00082 ST97 and 03.000073 ST97.

Role of the Sponsors: Istituto Oncologico Romagnolo and the National Research Council supplied the funding but did not participate in the design and conduct of the study, in the collection, management, analysis, and interpretation of the results, or in the preparation, review, or approval of the manuscript.

Independent Statistical Analysis: Independent statistical analysis was performed by Oriana Nanni, MSc, at the Unit of Biostatistics and Clinical Trials of Istituto Oncologico Romagnolo, Forli, Italy.

Acknowledgment: We thank Rosella Silvestrini, PhD, Istituto Oncologico Romagnolo, for her invaluable scientific contribution and Gráinne Tierney, BSc, Division of Geriatrics, Morgagni-Pierantoni Hospital, Forli, for editing the manuscript. We also thank Giuliana Amadori, RN, of the Department of Geriatrics, Morgagni-Pierantoni Hospital, for assistance in sample collection. None of those acknowledged received compensation from the study sponsors.

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