Original article:

Quantitative analysis of telomerase activity in exfoliated human urothelial cells of transitional cell carcinoma of urinary bladder

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Abstract:

Introduction: The present study was planned to evaluate the clinical utility of urinary telomerase activity in transitional cell carcinoma (TCC) of urinary bladder and provides a non-invasive diagnostic test for the detection and monitoring of urinary bladder cancer (UBC), especially low-grade tumors and recurrent bladder tumors.

Methodology: Quantitative analysis of telomerase activity was evaluated using the SYBR Green real-time quantitative telomeric repeat amplification protocol (TRAP) in exfoliated cells of urine obtained from 148 patients with UBC, 37 patients with benign prostatic hyperplasia (BPH), and 30 healthy subjects. The quantitative levels of urinary telomerase activity were evaluated with comparison of standard curve obtained from serial dilutions of telomerase-positive telomerase substrate oligonucleotide (TSR) template.

Results: Telomerase activity was found to be positive in 72.0% (67 of 93) of non-muscle-invasive bladder cancer (NMIBC) and 83.6% (46 of 55) of muscle-invasive bladder cancer (MIBC) patients. Thus overall 76.3% (113 of 148) UBC patients were found to be positive for telomerase activity expression. Urinary telomerase activity was detected in 75.3% (52 of 69) patients with low grade and, 77.2% (61 of 79) with high grade TCC of bladder tumors respectively. Telomerase activity was detected in significantly higher number of primary UBC patients as compared to recurrent UBC patients (83.2% vs. 16.8%, p=0.004).

Conclusions: Quantitative analysis of telomerase activity in exfoliated urothelial cells using SYBR Green real-time quantitative TRAP could be a minimally invasive and useful method for detecting UBC.

Keywords: Telomerase, Quantitative assay, exfoliated urothelial cells, Urinary bladder cancer, Real-Time PCR

Introduction:

Telomerase is a ribonucleoprotein enzyme that adds TTAGGG repeats to telomeres and maintains telomere length.(1) It is necessary for the immortalization of eukaryotic cells and once immortalized cells maintain high telomerase activity.(1) Urinary telomerase activity was evaluated in various human malignancies, including UBC.(2-10) Telomerase activity is generally undetectable among somatic cells; however its expression was detected in some normal tissues,(5, 11), benign diseased tissues(3, 6) and bladder mucosa(7, 10) at low level derived from recent published reports. The most common methods for detecting telomerase activity are TRAP.(2) In traditional TRAP assay,
signal intensities differed in urinary exfoliated urothelial cells from patients with UBC and some false-positive results were obtained in patients with no malignancy. At present, the TRAP assay is widely used for telomerase activity assessment. However, like in other conventional PCR methods, inherent problems exist in the current TRAP assay. For example, the limited dynamic range, as well as end-point detection of the PCR product, makes the accurate measurement of telomerase activity difficult. Moreover, the post-PCR processing is time-consuming and adds further variables during analysis of the PCR products.

Recently Real-time quantitative TRAP (RTQ-TRAP) method was introduced, which is capable of binding to the double-stranded amplicons and generating fluorescence signals in a PCR reaction, allows the amount of PCR products to be determined based on the fluorescence produced during the extension step of each cycle in a closed tube.(12)

The aim of our study was to develop non-invasive methods to diagnose UBC. Furthermore, objective of this study is to evaluate telomerase activity quantitatively using SYBR Green real-time quantitative PCR method in exfoliated urothelial cells from patients with UBC and see its clinical utility in diagnosis of UBC patients.

**Methodology:**

**Exfoliated urothelial cells:**

Exfoliated urothelial cell samples from 148 patients with histologically proven UBC (mean age 55.81± 12.02 years) were collected from naturally voided urine specimens. Exfoliated urothelial samples for the negative control were obtained from 50 ml of spontaneously voided urine from 37 subjects with BPH (mean age 57.02± 13.63 years) and from 30 healthy volunteers (mean age 29.53 ± 6.34 years). Briefly, voided urine specimens were centrifuged as soon as possible in 50 ml conical tubes at 900g for 10 minutes at 4°C, washed once in phosphate-buffered saline (PBS), and centrifuged at 2500g for 5 minutes at 4°C.(1) Samples were then suspended in 100 µl of ice-cold TRAP lysis buffer, incubated on ice for 30 minutes, and centrifuged at 10,000g for 20 minutes at 4°C. The supernatants were removed and stored at -80°C. Protein concentrations of each cell extracts were measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and diluted with lysis buffer to adjust the protein concentration to 0.1 µg/µl. All subjects have provided signed informed consent and the study were approved by institutional ethics committee.

**Urine Cytology:**

Each urine specimens was centrifuged for 5-10 min at 2000 rpm and each urine sediment was transferred onto a glass slide forming two smears then quickly immersed in 95% ethanol for fixation, then stained with modified Papanicolaou stain [Papanicolaou and Marshall, 1945].

**SYBR Green real-time telomerase repeat amplification protocol assay:**

The SYBR Green real-time quantitative TRAP assay was performed using Quantitative Telomerase Detection kit (Allied Biotech Inc., Germantown, MD, USA) according to the manufacturer’s protocol, which is based on a PCR-based telomeric repeat amplification protocol. The total volume of the reaction
mixture was 25 µl, which contained 12.5 µl of quantitative telomerase detection (QTD) premix, 11.5 µl of PCR grade water and 1.0 µl of cell extract. About 0.01 µg of protein extract was used for the TRAP assay.

The reaction mixture was first incubated at 25°C for 20 min to allow the telomerase in the protein extracts to elongate the TS primer (5’- AATCCGTCGAGCAGAGTT- 3’) by adding a TTAGGG-repeat sequence. After that, PCR was then performed at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s on a LightCycler 480 Real-Time PCR system (Roche Applied Science, Mannheim, Germany). The amplified fluorescence signal in each urine specimen was measured at the late extension step of each cycle and analysed with detector software. In the negative control, no fluorescent signal was observed. A standard curve was constructed from serial dilutions of telomerase-positive telomerase substrate oligonucleotide (TSR) template (0.5, 0.1, 0.02, 0.004, 0.008, 0.00016, 0.000032 and 0.0000064 µg/ml). The telomerase activity of each urine specimen was calculated based on the standard curve. One unit of relative telomerase activity (RTA) was defined as the activity equivalent to that in 100 molecules of TSR.

Statistical analysis:
Continuous data were explicated as Mean ± SD, while discrete (categorical) in %. Categorical variables were explicated as numbers and percentages. Independent Student’s t test was used to estimate statistical significance between more than two independent groups. The significant difference between the groups was analyzed by Tukey’s post hoc test after adjusting the multiple contrasts for significance. Categorical groups were compared by chi-square (χ²) test. Results were considered statistically significant if p<0.05. Statistical analyses were performed using SPSS 18.0 software. ROC curve was used to estimate sensitivity and specificity of the telomerase activity.

Observations & Results:
A total of 148 UBC patients, 37 subjects with benign prostatic hyperplasia and 30 healthy subjects were included in the present study to evaluate expression of telomerase activity in exfoliated cells of urine using the SYBR Green real-time quantitative telomeric repeat TRAP assay. The clinicopathological characteristics of UBC patients are summarized in Table 1. The majority of UBC patients (77.0%) belonged to higher (>45 years) age group and 91.9% were males. High grade (G2-G3) tumor was present in 53.4% whereas low grade (G1) tumor was present in 46.6% patients. Muscle-invasion (T2-T4) was present in 62.8% patients whereas 37.2% patients had non-muscle-invasive disease (Ta-T1). Further, majority of UBC patients were tobacco users with 48.0% were smokers (bidi + cigarette) and 52.6% chewed tobacco. Among UBC patients, 77.7% were primary patients while 22.3% were recurrent UBC patients.

Expression of telomerase activity:
Telomerase activity was found to be positive in 76.3% (113 of 148) UBC patients. Thus overall 76.3% (113 of 148) UBC patients were found to be positive
for telomerase activity expression. The expression of telomerase activity did not differ significantly (p>0.05) between the NMIBC and MIBC patients (72.0% vs. 83.6% p=0.108). Expression of urinary telomerase activity was detected in 75.3% (52 of 69) with low grade and, 77.2% (61 of 79) with high grade TCC of bladder tumors respectively. The expression of telomerase activity and its association with the clinical characteristics of the UBC patients are summarised in Table 2. Expression of telomerase activity was not found to be significantly (p>0.05) associated with any clinical characteristics of the NMIBC and MIBC patients. Expression of telomerase activity was found to be significantly higher in primary UBC patients as compared to recurrent UBC patients (83.2% vs. 16.8%, p=0.004). Among benign urological patients used as control, telomerase activity was found to be positive in 5/37 subjects. After applying optimal cut-off points using ROC curves we found overall sensitivity and specificity of urinary telomerase activity using TRAP assay were 76.5%, 82.5% respectively. In NMIBC patients 72.0% sensitivity, and 86.4% specificity; in recurrent NMIBC patients 57.5% sensitivity and 86.4% specificity; and in MIBC patients 84.6% sensitivity, and 82.5% specificity were found respectively.

Table 1: Clinical characteristics in non-muscle-invasive and muscle-invasive UBC patients (n=148)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non-muscle-invasive</th>
<th>Muscle-invasive</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤45</td>
<td>20 (21.5%)</td>
<td>14 (25.5%)</td>
<td>0.581</td>
</tr>
<tr>
<td>&gt;45</td>
<td>73 (78.5%)</td>
<td>41 (74.5%)</td>
<td></td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9 (9.7%)</td>
<td>3 (5.5%)</td>
<td>0.363</td>
</tr>
<tr>
<td>Male</td>
<td>84 (90.3%)</td>
<td>52 (94.5%)</td>
<td></td>
</tr>
<tr>
<td>Grade:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (G1)</td>
<td>64 (68.8%)</td>
<td>5 (9.1%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High (G2-G3)</td>
<td>29 (31.2%)</td>
<td>50 (90.9%)</td>
<td></td>
</tr>
<tr>
<td>Stage:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>8 (8.6%)</td>
<td>0 (0.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T1</td>
<td>85 (91.4%)</td>
<td>55 (100%)</td>
<td></td>
</tr>
<tr>
<td>T2-T4</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
<tr>
<td>Smoking:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>62 (66.7%)</td>
<td>15 (27.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yes</td>
<td>31 (33.3%)</td>
<td>40 (72.7%)</td>
<td></td>
</tr>
<tr>
<td>Tobacco:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>62 (66.7%)</td>
<td>29 (52.7%)</td>
<td>0.092</td>
</tr>
<tr>
<td>Yes</td>
<td>31 (33.3%)</td>
<td>26 (47.3%)</td>
<td></td>
</tr>
<tr>
<td>UBC patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>60 (64.5%)</td>
<td>55 (100%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Recurrent</td>
<td>33 (35.5%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
</tbody>
</table>


Discussion:

Significant efforts have been made to develop reliable and non-invasive diagnostic methods for detecting and predicting biological behavior of UBC. However, to date, none of these methods have proved to be sensitive/specific enough to be accepted as a routine tool in diagnosis of UBC. So, there is a persuasive need for a non-invasive diagnosis. To date, however, cystoscopy remains the gold standard for diagnosis of UBC. This invasive examination is required not only for diagnosis but is repeated at 3-month intervals in follow-up because no other method currently available is adequately sensitive and specific. (13)

Urine cytology is most common non-invasive test, used commonly for the monitoring and detection of UBC. The positive rate of the VUC is unsatisfactory, especially for low grade bladder tumors. (1) Thus a more sensitive and specific non-invasive assay is required for screening of UBC. Kinoshita et al., 1997, detected telomerase activity in 55.0% of 50 ml of naturally voided urine and in 84.0% of 50 ml of washing fluids from patients with UBC. (14) Yoshida et al., 1997 and Kavaler et al., 1998 also detected telomerase activity in respectively 62.0% and 85.0% of naturally voided urine. (9, 15) However, Linn et al., 1997 did not detected telomerase activity in any of the urine samples from 11 patients with UBC. (16) In the present study, we detected telomerase activity in 76.3% (113 of 148) of urinary samples of UBC patients by using SYBR green TRAP assay, consistent with some previous reports by Kavaler et al., 1998; Yoshida et al., 1997. We do not know at present why these discrepancies in findings arise. The sensitivity of the telomerase activity was higher (76.5%) than that of urinary cytology (49.0%). The sensitivity of urinary telomerase activity observed in our study was similar to previous findings in which sensitivities for urine samples have ranged between 0% and 100% [Arai et al., 2000; Biakowska-Hobrzanska et al., 2000; Dalbagni et al., 1997; Gelmini et al., 2000; Kavaler et al., 1998;; Kitsukawa et al., 1999; Linn et al., 1997; Muller et al., 1996, 1998; Rahat et al., 1999; Ramakumar et al., 1999; Yokota et al., 1998; Yoshida et al., 1997]. (1, 9, 15-25) The reasons for these widely divergent findings remain unclear, particularly because methodical differences, such as the treatment of the samples, are not apparent. (26) In our study, expression of urinary telomerase activity was not correlated to the clinical characteristics such as patient’s age, sex, stage, grade, smoking and tobacco chewing, which was similar to previous findings [Lin et al., 1996; Rahat et al., 1999; Gelmini et al., 2000].

We observed some cases of discordance, in which urine cytology was found to be negative but telomerase activity was found to be positive. Some samples contain very few cancer cells, which may be insufficient for cytological examination but detectable by SYBR Green real-time quantitative TRAP assay. Thus, determining urinary telomerase activity may be more advantageous, for the screening of UBC. However, we also found some discordance in which cytology was found to be positive but telomerase was found to be negative. This type of discordance was prevalent among high-grade bladder tumors. One possible explanation for
these discrepancies is simply the degradation of telomerase in urinary sediment samples. Taken together, these findings suggest that the combination of cytological examination with urinary telomerase may improve the sensitivity and specificity of screening for UBC. The quantitative level of telomerase activity reflects the number of cancer cells among exfoliated urothelial cells, because cancer cells were exfoliated into urine more easily in high-grade or aggressive bladder tumors than from low-grade and non-aggressive bladder tumors. In addition, with the TRAP kit, internal control standards demonstrated no inhibition of the PCR amplification. In patients who had a false-positive telomerase results, all had either chronic or severe inflammation; including 2 patients with dysplasia, 3 patients with cystitis. It is possible that in several of these cases, active bladder tissue remodeling is involved. If so, it may explain the presence of telomerase activity in some patients with either severe or chronic inflammation, or dysplasia.

Conclusion:
In conclusion, the combination of such newly available molecular techniques with urine cytology could facilitate improved early diagnosis of UBC. It could be of particular use in the early detection of UBC recurrence after treatment. The determination of Urinary telomerase activity using SYBR Green real-time quantitative PCR is a promising tool for the diagnosis of UBC.

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Conflict of interest: none declared

Reference:


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**Telomerase activity**

![ROC curve for Telomerase activity](attachment: ROC_curve.png)

**Figure 1: ROC curve for Telomerase activity**

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