A prospective evaluation of the diagnostic and potential prognostic utility of urinary human telomerase reverse transcriptase mRNA in patients with bladder cancer

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Human telomerase reverse transcriptase (hTERT) mRNA expression has been considered a surrogate marker for telomerase activity based on its parallel detection in urological malignancies, including transitional cell carcinoma (TCC) of the bladder. The objective of this study was to prospectively evaluate the diagnostic performance of urine hTERT mRNA marker and urine cytology in the detection of bladder cancer.

The multiplex hTERT/GAPDH (glyceraldehyde-3-phosphate dehydrogenase) reverse transcription polymerase chain reaction (RT-PCR) assay was employed to assess hTERT mRNA expression in urine sediments from 43 patients with clinically apparent TCC undergoing transurethral resection. Tumor grade and pathological stage were determined. The results of urine cytology were compared with urine hTERT mRNA expression. The control group consisted of 46 age-matched healthy volunteers without known urinary tract disease.

The sensitivity of hTERT mRNA expression marker in the detection of bladder cancer was significantly better than urine cytology (95% versus 65%, $p<0.001$). The hTERT mRNA was detected with high sensitivity in both low and high grade tumors, and in superficial and invasive phenotypes. No correlation was seen between hTERT mRNA and the histopathological grade and stage. The specificity of urinary hTERT mRNA marker was 93.5%. The detection of hTERT mRNA expression in urine was a highly sensitive marker for the diagnosis of TCC of the bladder in this study. This urine-based marker shows promise as a non-invasive adjunct to cystoscopy in patients undergoing bladder tumor surveillance.

Key Words: bladder cancer, telomerase, urine markers

Introduction

Transitional cell carcinoma of the bladder is the fifth most common malignancy in North America.[1] The majority of newly diagnosed transitional cell tumors present as superficial disease, confined to the urothelium and submucosal tissues. Recurrence rates are high however, and the risk of progression mandates patients undergo rigorous follow up with lifelong surveillance.[2] Cystoscopy, the most sensitive diagnostic test currently available, is not without its limitations. Papillary transitional cell carcinoma lesions are easily identified, however the detection of carcinoma in situ can be more problematic. Although the widespread availability of flexible instrumentation has reduced patient discomfort, cystoscopy remains

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an invasive procedure.

Urine cytology is also employed in an ancillary role in the surveillance of patients with transitional cell carcinoma. The test is based on the concept first described by Papanicolau that exfoliation of malignant urothelial cells can be detected in the voided urine of affected patients.[3] Expression of the malignant phenotype occurs as a result of cell dedifferentiation. As cells become more anaplastic, there is a loss of cell adhesion molecules resulting in an impairment in the adherence to the cellular matrix and to adjacent cells.[4] As such, malignant cell sloughing can then occur. Urine cytology is based on the qualitative microscopic evaluation of the morphological characteristics of exfoliated urothelial cells. The main clinical utility of using urine cytology in patients on a surveillance protocol with known bladder cancer, is to aid in the detection of high-grade, non-visible lesions (carcinoma in situ). Although efficient in detecting the presence of high grade lesions, urine cytology is plagued by a low sensitivity for low grade, low stage tumors resulting in an overall sensitivity of only 40%-60%.[5] Both intra- and inter-observer variability can affect test results.

The limitations associated with cystoscopy and urine cytology lead to the development and investigation of a number of urine based tests in an attempt to provide a non-invasive and sensitive method of bladder tumor detection. Human telomerase is a unique cellular reverse transcriptase (RNA-directed DNA polymerase) that functions to elongate telomeric ends of chromosomes, thereby compensating for the progressive loss of telomeric sequences that occur as the normal consequence of DNA replication.[6] Normal human somatic cells lose 50-100 base pairs (bp) of their telomeric sequences with each cell division.[6] Once a critical chromosome length is reached, cell division ceases and the cell exits the cell cycle and becomes senescent.[6] Telomerase is normally repressed in most somatic tissues. Expression at low levels is found in germ cells, stem cells and highly proliferative tissues.[7] Reactivation or up-regulation leads to cellular immortalization and is a critical early step in the development of malignancy. Telomerase activity has been detected in many human cancers including bladder, prostate and renal malignancies.[8,9]

In transitional carcinoma of the bladder, telomerase activity has been detected in 90% of bladder tumor tissue samples.[8] Several investigators have evaluated telomerase activity in exfoliated urothelial cells as a potentially non-invasive urine test with reported sensitivities ranging from 0-86%.[8-10] This discrepancy has been attributed to technical issues related to the various assays and the instability of the enzyme during prolonged exposure to the urinary environment which contains proteases, RNAses, urea and acidic pH.[11]

Early attempts to detect telomerase activity in in vivo tumors required 10^7 to 10^8 cells.[12] The sensitivity of telomerase enzyme detection was significantly improved with the development of a PCR-based TRAP[1] assay.[13] The TRAP assay provided a very sensitive tool to detect telomerase activity in cellular extracts obtained through homogenization of tumor biopsy specimens.

Most recent publications describing the effectiveness of telomerase as a urine marker have employed the TRAP assay to measure human telomerase activity.[8] Human telomerase exists as a complex of at least two subunits essential for its activity: an RNA component encoded by the human telomerase RNA (hTR) gene[14] and the protein subunit human telomerase reverse transcriptase, specified by the hTERT gene,[14,15] The RNA component, hTR serves as the template for telomeric repeat synthesis.[14] The protein subunit, hTERT contains the enzyme's catalytic component that functions analogous to the reverse transcriptase enzyme found in retroviruses.[15,16] While hTR is expressed regardless of telomerase activity, hTERT on the other hand has been found to correlate with telomerase activity in malignant tumors including bladder cancer.[17,18]

The present study assessed the clinical value of a sensitive multiplex hTERT/GAPDH RT-PCR assay in detecting hTERT mRNA expression in exfoliated urothelial cells from urine specimens of patients with bladder cancer.

Materials and methods

Patients and specimens

This prospective study analyzed the expression of hTERT mRNA in cystoscopy urine specimens from 43 patients with a presumptive clinical diagnosis of TCC of the bladder. This cohort included patients with a well-established history of previous bladder tumors as well as patients with their first tumor. No patient had undergone intravesical chemotherapy or immunotherapy within one year of inclusion in the study. All patients had a sterile preoperative urine culture at the time of transurethral resection. All patients underwent cystoscopy and transurethral resection of visible tumors. Resected tissues were subjected to histopathological assessment. Tumor staging was carried out using the TNM pathological staging system[19] and graded according to the guidelines set by the World Health Organization.[20] Forty-six asymptomatic healthy, age-matched volunteers were also recruited to serve as

the control population. In addition, nine patients clinically suspected of having TCC but subsequently found to be negative for malignancy by histopathological assessment were included. The study was approved by the University of Western Ontario's Health Sciences Review Ethics Board.

Specimen collection and processing

Cystoscopy urine specimens were obtained, following the insertion of a rigid cystoscope using minimal irrigation. The urine was drained into a sterile basin through the sheath of the cystoscope into 50 ml centrifuge tubes, mixed to ensure equal cell distribution, and divided into two equal portions. Specimen processing commenced within 3 hours of sample collection. Total RNA was subsequently extracted from one portion of sedimented urothelial cells and analyzed for hTERT expression. Urine for cytology was submitted from the second portion of the urine sample and subjected to standard cytologic assessment.

Total RNA extraction

For total RNA extraction, the urothelial cells were washed in 10 ml ice-cold HEPES buffer (10mM HEPES-KOH pH 7.5; 1.5mM $MgCl_2$; 10 mM KCl; and 1mM DTT) to remove red blood cell contamination present in some urine specimens. Otherwise, the cells were washed in 1 ml of ice-cold HEPES buffer and resuspended in either 500µl of RLT buffer (QIAGEN, Mississauga, ON) or TRIzol (Invitrogen), and stored at -80°C until further processing.

RNA concentrations were determined using the fluorometric assay, RiboGreen™ (Molecular Probes, Eugene, OR) as previously described.[21] All procedures requiring the manipulation of RNA were conducted in a designated and sterile laminar flow hood utilizing disposable RNase-free labware.

Multiplex hTERT/GAPDH RT-PCR assay

RNA preparations were pre-treated with DNase I prior to reverse transcription to eliminate DNA contamination. Complementary DNA (cDNA) was synthesized in a 25 µl reaction containing heat denatured RNA (70°C, 12 min), 50mM Tris-HCl (pH7.3), 75mM KCl, 3mM MgCl2, 0.25mM dNTP (Roche Diagnostics), 5mM random hexamers, 8.0 mM dithiothreitol (Invitrogen, Burlington ON), 10U of SUPERase-IN RNase Inhibitor (Ambion, Austin TX) and 200 units of SuperScript II RNase H-Reverse Transcriptase (Invitrogen) at incubation conditions recommended by the suppliers. The expression of hTERT mRNA and the housekeeping transcript, GAPDH were analyzed in a multiplex PCR reaction previously validated in our laboratory.[22]

Determination of detection sensitivity of the hTERT/GAPDH RT-PCR assay

To evaluate the ability of the assay to detect the minimum copy number of hTERT mRNA, total RNA equivalents of bladder cancer cells were used. The telomerase positive bladder cancer cell line RT-4 obtained from the American Type Culture Collection (Manassas,VA) was propagated in RPMI (Invitrogen) with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) in 5% CO_2 at 37°C. The cells were counted using a Brightline haemocytometer (Horsham, PA) and a Leica compound microscope (Richmond Hill, ON) and adjusted to $1x10^6$ cells. The total RNA per RT-4 cell was estimated using the equation: (total RNA extracted) ÷ ($1x10^6$ cells) and calculated at 0.02ng/cell. RNA equivalents ranging from 12.5 to $5x10^4$ cells were subjected to reverse transcription and 2.5 to 10^4 RNA cell equivalents (representing 1/5 volumes of RT reactions) were then amplified using the hTERT/GAPDH PCR assay.

Statistical analysis

All urine specimens were coded and results of the hTERT/GAPDH RT-PCR assay were interpreted without knowledge of the cytologic or histologic diagnoses. Histopathologic diagnosis was used as the "gold standard" for presence or absence of bladder cancer. The sensitivity of the RT-PCR assay was defined as the frequency of samples correctly identified as malignant by the assay among the total number of patients with histologic diagnosis of bladder cancer. The specificity was calculated as the frequency of samples correctly identified as benign by the assay among the total number of participants without bladder cancer. McNemar's chi-square test was used to determine the significance of differences in the sensitivity of the hTERT/GAPDH RT-PCR, and urine cytology.

Results

Patient characteristics

This prospective study involved 33 males and 10 females who were clinically suspected of having transitional cell carcinoma (TCC) of the bladder based on cystoscopic evidence obtained at the time of initial investigation or on clinical follow-up for the surveillance of recurrent disease. The mean age of these patients was 71.5 years (range 51 to 90 years).

The distribution of pathological grade and stage is presented in Table 1. The control group consisted of 46 healthy volunteers (mean age 57.2, range 21 to 84).

Detection of hTERT mRNA in exfoliated urothelial cells

The multiplex hTERT/GAPDH RT-PCR assay had an excellent detection sensitivity evidenced by its ability to detect as few as 5 RT-4 bladder cancer cells expressing hTERT mRNA Figure 1. The hTERT mRNA marker was detected in cells sedimented from 41/43 urologic specimens obtained from patients with a histopathological diagnosis of TCC of the bladder, accounting for an overall clinical sensitivity of 95.3% Table 2. The hTERT mRNA marker was detected in the urological specimens obtained from patients with lesions of all stages and grades. The hTERT mRNA marker expression was observed in specimens with a mean concentration of total RNA of 202 ng/ml (range 1.7 to 1,554 ng/ml). The multiplex hTERT/GAPDH RT-PCR test successfully detected malignancy in the urological specimens of 18/20 (90%) individuals diagnosed with superficial pTa lesions Table 2. The two missed cases were from one primary and one recurrent tumor both histopathologically determined to be pTa Grade I lesions. Each of these specimens appeared to have inadequate content of total RNA ($\leq$1.0 ng/μl) which may have contributed to the false negative result.

The specificity of the urine-based multiplex hTERT/GAPDH RT-PCR assay was 93.5% accounting for a false-positive test in 3 of the 46 healthy volunteers Table 2. Of the three false positive results observed in the control group, one patient had a history of recurrent urinary tract infections while no clinical information was available for the two other hTERT mRNA-positive volunteers. The hTERT mRNA marker expression was also observed in 7/9 individuals with a previous history of bladder cancer and cancer-free status established by histopathology and upper tract imaging. In 3 of 9 hTERT mRNA-positive, cancer-free individuals, histopathological assessment revealed conditions associated with inflammation (inflamed fibromuscular tissue, patchy lymphocytic infiltrate, and inflammatory urothelial atypia). Four of 9 hTERT mRNA-positive individuals had benign lesions described by the pathologist as hyperplasia, metaplasia and dysplasia. In these four cases, there was no evidence of tumor infiltrating lymphocytes, known to express low levels of hTERT mRNA.[23] In addition, urinalysis confirmed the absence of urinary tract infection. In follow-up, 3 of these 4 patients developed a recurrence of their bladder cancer (within 3-6 months).

TABLE 1. **Histopathological distribution of stage and grade of bladder tumors**

Grade	Stage				
	pTis	pTa	pT1	pT1/Tis	pT2-T3
I		8			
II		12	3		
III			7	2	9
Total	2	20	10	2	9

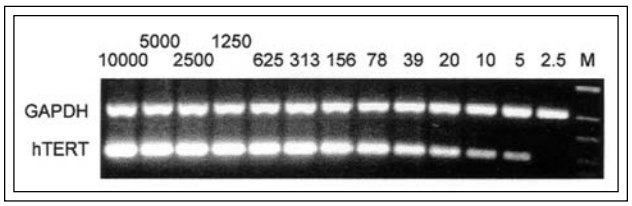

Figure 1. Detection of sensitivity of the multiplex hTERT/GAPDH RT-PCR assay.
The hTERT transcript indicated by a 145 bp cDNA band was detected in 0.10 ng of total RNA representing approximately 5 RT-4 bladder cancer cells using the multiplex hTERT/GAPDH RT-PCR assay. A 249 bp DNA band indicated detectable GAPDH expression in each of the 13 reactions containing cDNA generated from total RNA extracted from 2.5 to 10,000 RT-4 cells (0.05 to 200 ng). Lane M, molecular weights marker.

Urine cytology

Urologic specimens were assessed in parallel by cytology. Urine cytology was successful in identifying 28 of the 43 patient specimens analyzed as positive for malignancy accounting for an overall clinical sensitivity of 65% Table 2. Urine cytology sensitivity increased from 50% for patients with early grade I lesions to 72% in samples obtained from patients with grade III TCC Table 2.

Discussion

Our previous investigation demonstrated that the hTERT mRNA expression is a more robust marker in identifying malignant cells exfoliated to urine than telomerase activity.[22] The clinical utility of the hTERT mRNA urine marker determined by the multiplex hTERT/GAPDH RT-PCR assay was compared to urine cytology. This was accomplished in a double-blinded study in which cystoscopy urine specimens

TABLE 2. **Clinical sensitivity of urine hTERT mRNA biomarker and cytology stratified by pathological features of transitional cell carcinoma**

Urine Test	No of Positive / Total No Tested (%)									
	Controls		Stage					Grade		
	HV	NM	pTis	pTa	pT1	pT1/Tis	pT2-T3	I	II	III
hTERT mRNA	3/46 (6.5)	3/5 (60)	2/2 (100)	18/20 (90)	10/10 (100)	2/2 (100)	9/9 (100)	6/8 (75)	15/15 (100)	18/18 (100)
Cytology*	nt	0/5 (0)	2/2 (100)	10/20 (50)	7/10 (70)	2/2 (100)	7/9 (78)	4/8 (50)	8/15 (53)	13/18 (72)

*Cytology results of "Atypia" (n=4) were scored as negative, and "Suspicious" (n=9) were scored as positive for malignant cells.
Abbr.: HV-healthy volunteers; NM-negative for malignancy; nt-not tested

were split into two portions and analyzed by the two techniques. The hTERT mRNA marker successfully detected malignant cells in 41/43 patient urine specimens analyzed representing diagnostic sensitivity of 95%. Cytology predicted the presence of malignancy in 28 of the 43 specimens accounting for an overall sensitivity of 65%. Hence, the sensitivity of the hTERT mRNA expression marker in the detection of bladder cancer was significantly higher than urine cytology (95% versus 65%, $p<0.001$). The mean sensitivity of urine cytology has been reported at 40% to 60% [5] placing the cytology results presented herein at the upper end of the range. The major disadvantage of urine cytology has been its poor sensitivity in detecting low-grade tumors.[24,25] This characteristic shortfall was observed in the current study with cytology detecting malignant cells in 50% of the specimens obtained from patients with superficial (pTa) and low-grade tumors, a finding consistent with that reported in the literature.[5] In contrast, the hTERT mRNA urine marker was more accurate in detecting the low-grade superficial tumors with a sensitivity of 90% for all pTa lesions and 75% for all grade I TCC of the bladder. There was no correlation found in the present study between positive expression of hTERT mRNA in urine sediments and pathologic stage and grade of tumors.

Other investigations have recently evaluated clinical sensitivity and specificity of RT-PCR based detection of hTERT mRNA in urological specimens of patients with bladder cancer. They differ with respect to design of RT-PCR assays, in particular, the selection of PCR primers directed to the amplification of different regions of the hTERT mRNA, as well as the overall number of PCR cycles. Ito et al [26] reported the detection of hTERT mRNA in voided urine samples of 33 patients with carcinoma of the urinary bladder with a sensitivity of 76% and assay specificity of 96%. The false negative results observed by these investigators included two pTA grade II-III, 5 pT1 grade I-III and one pT3 grade II tumors. Similar sensitivity levels for the detection of hTERT mRNA in urine specimens and bladder washes have been described by other groups with assay specifities above 90%.[27-29]

A difference in the clinical sensitivity demonstrated by our RT-PCR assay as compared to those used in the above mentioned studies could be attributed to various detection sensitivity levels of these procedures. Our multiplexed hTERT/GAPDH RT-PCR assay had estimated detection sensitivity of as few as five bladder cancer cells. Another factor, which could potentially account for a more adequate detection of urinary hTERT mRNA has been prompt processing of urologic specimens in our investigation within less than 3 hours thus minimizing the exposure of exfoliated cells to the harsh urological environment. The half-life of the hTERT mRNA transcript was recently estimated to be 2.5 hours.[30]

The specificity of the urine marker hTERT mRNA evaluated in the present study was 93.5% accounting for 3 false-positive results among the 46 healthy volunteers. The hTERT mRNA positive results were also noted among 7/9 urine specimens from patients with clinical suspicion of bladder cancer and exclusion of malignancy by histopathology. False positive telomerase activity results were previously observed in cells sedimented from urological specimens obtained from patients with inflammatory conditions.[31] Similar causes for false positivity were also reported during analysis of telomerase activity in cervical carcinoma,[32] thyroid cancer [33] and liver cancer.[34] The population of cells exfoliated into the urine of patients with inflammation

of the urogenital tract is heterogenous and includes variable numbers of white blood cells (WBC), primarily polymorphonuclear (PMN) cells, with minimal presence of monocytes, eosinophils and lymphocytes.[35] In previous studies hTERT mRNA has not been detected in peripheral blood PMN cells or monocytes by using RT-PCR.[36] Although hTERT mRNA expression has not been assessed in eosinophils, hTERT mRNA expression is likely repressed in these terminally differentiated and not proliferating cells. However, the low levels of hTERT expression have been detected in normal lymphocytes isolated from peripheral blood.[23] This is consistent with reports of upregulation of both hTERT and telomerase activity following the activation of T-cells.[23,37]

An alternative explanation for the source of hTERT mRNA positivity in urine specimens of cancer-free patients comes from reports suggesting that telomerase activity is a marker of proliferation.[38] Kavaler et al[32] detected low levels of telomerase activity in cells sedimented from the urine of patients with benign conditions such as stone disease, urethral stricture, and benign prostatic hyperplasia. Although not a focus of this investigation, clearly further evaluation of these benign entities is warranted to identify potential false positive results.

The 4/9 hTERT mRNA positive specimens were obtained from patients with lesions histopathologically assessed as hyperplasia, metaplasia, and dysplasia. Dysplastic lesions have been argued to be pre-malignant based on histological changes in the microscopic architecture of the bladder epithelium including an increase in cell layers and squamous or glandular differentiation,[39] each characteristic of malignancy. In earlier studies telomerase activity was observed in bladder biopsies of dysplastic lesions.[39-41] The detection of telomerase activity could be interpreted as an early indicator of the malignant potential in these presumptive "benign" lesions. In the present study, clinical follow-up was available for 3 of the 4 individuals with a previous history of bladder cancer, and indicated a recurrence of disease within 3-6 months of an hTERT mRNA positive RT-PCR test. Several recent studies have demonstrated early activation of hTERT transcription and its progressive up-regulation with the progression of the malignancy (from normal tissue, through dysplasia to malignancy) in gastric cancer.[42] Similar observations were also made for early detection of telomerase activity marker in breast cancer[43] and cervical cancer.[44] These results suggest that biochemical changes associated with the acquisition of the malignant phenotype, particularly the re-expression of the telomerase catalytic subunit, may precede observable morphological cancer related changes. Hence, the finding of hTERT mRNA may facilitate the earlier detection of occult disease, thus providing an additional support for the diagnostic utility of this marker.

In conclusion, the urine hTERT mRNA marker proved to be more sensitive in diagnosing the presence of bladder cancer in naturally exfoliated cells than cytology. With a diagnostic sensitivity of 95%, the very sensitive hTERT/GAPDH multiplex RT-PCR test meets patient requirements for a non-invasive test sufficient to screen for recurrent bladder cancer.[45] Due to the potential detection of hTERT mRNA positive lymphocytes, however, the hTERT/GAPDH multiplex RT-PCR test appears to be less valuable as a tool for the initial screening of patients presenting with non-specific signs or symptoms without a previous history of bladder cancer.

The use of bladder wash urine samples was based on the protocol of our previous work.[22] For the assay evaluated in this investigation to be most clinically useful however, further assessment on voided urine samples is necessary and is ongoing. Although not investigated by our laboratory, the potential feasibility of assay automation should be determined.

A multicentred clinical trial evaluating large numbers of patients with all stages of bladder cancer is required to determine whether this assay has a role as a non-invasive adjunct to cystoscopy in the follow up of patients with recurrent bladder cancer.

Urinary hTERT mRNA was unable to predict clinical stage or grade and therefore did not provide useful information regarding prognosis. □

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