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Detection of human telomerase reverse transcriptase messenger RNA in urine of high risk patients as a non invasive molecular diagnostic tool for urinary bladder cancer in Egyptians

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ABSTRACT

Introduction and Objectives: Bladder cancer is an important national health problem as it is the leading cancer in men in Egypt. Cystoscopy and biopsy, currently remains the gold standard procedure for diagnosis, yet, it is invasive and costly. Urinary cytopathology remains to be the only non-invasive alternative method for diagnosis. Although it is tumour specific, yet it has a poor sensitivity, especially for low grade tumours. Detection of Telomerase enzyme in exfoliated urinary cells is a potentially good molecular diagnostic marker in bladder cancer, since the catalytic subunit of this enzyme (hTERT) proved to be essential for cellular immortality and oncogenesis.

Subjects and Methods: The study comprised 39 patients (36 with urothelial carcinomas and 3 cases were squamous cell carcinoma) with bladder cancer and 22 non cancer control (including 14 patients with benign urological disorders and 8 healthy volunteers). The urine sample was split into two aliquots one was used to undertake RNA extraction and hTERT/GAPDH RT-PCR semi-quantitative assay and the second for cytological examination. Cystoscopy was considered the reference standard for the identification of bladder cancer.

Results: The hTERT/GAPDH RT-PCR test showed significantly higher diagnostic sensitivity than cytology (84% Vs. 75% $p<0.008$) for confirmed UCC, particularly for low grade non-muscle invasive UCC (82% Vs. 64% $p<0.005$). On combining the two tests a sensitivity of 95% was obtained. A positive hTERT expression was detected 4-5 months earlier than cystoscopic evidence of recurrence in 2 patients during their follow up.

Conclusion: In this pilot study, detection of hTERT expression in urine has shown to be a more sensitive marker for diagnosis of bladder cancer than cytology. The combination of urinary hTERT mRNA with cytological testing augments the sensitivity for the non-invasive early diagnosis of bladder cancer. This finding warrants further extended study to validate the potential role of hTERT expression as a diagnostic non invasive tool for high risk patients and detection of recurrence in bladder cancer in Egypt.

Key Words:

Bladder cancer, telomerase, hTERT RT-PCR, urine cytology.

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INTRODUCTION

Bladder cancer is an important national health problem. It is the leading cause of cancer in Egyptian males[1,2]. Bladder cancer has a high recurrence rate (70%), with 20-30% progressing to invasive disease[3,4]. Hence, optimal management of this disease requires early detection of both primary tumour as well as recurrences.

Cystoscopy and biopsy, currently remains the gold standard procedure for diagnosis, yet, it is uncomfortably invasive, costly and sophisticated[5,6]. Urinary cytopathology has remained the only alternative non-invasive method for examining patients presenting with hematuria, for primary bladder cancer, or recurrence. Although cytology is tumour specific, its value as a diagnostic test is quite limited due to its poor sensitivity, especially for low grade tumours (22-62%) in which the morphological features of the neoplastic cells are indistinguishable from the normal urothelial cells[7-10]. It is also highly dependent upon proper sampling and is open to subjective interpretation.[11]

Bladder cancer is one of the most amenable carcinomas for tumour marker development as many of them are either secreted in urine, or are tumour cell-associated, which can be detected by analyzing exfoliated cells in urine specimens. The impetus for developing new bladder tumour markers comes from the idea that an accurate biomarker can reduce the number of cystoscopies performed each year and thus, cut down the frequency of this invasive and costly procedure.

Many new urine-based tests for bladder cancer have been developed including fluorescent in situ hybridization (FISH)[12], immunocytology (ImmunoCyt™)[13], survivin[14], cytokeratins (19, CYFRA 21-1)[15], flow cytometry and Quanticyt™[15], bladder tumour-associated antigen (BTA) test[16], nuclear matrix protein 22 (NMP22) test[16]. Among them, BTAstat, BTA-trak, NMP22, ImmunoCyt and FISH (UroVysion) have been approved by the food and drug association (FDA)[17,18]. Among the markers proposed to improve diagnostic sensitivity in urine, increasing attention has been focused on the role of Telomerase enzyme in exfoliated urinary cells as a potential candidate for bladder cancer detection[19,20]. Telomerase (an enzyme first detected in human cancer cell lines in 1994)[21] is a unique cellular reverse transcriptase (RT) that functions to elongate telomeric ends of chromosomes, thus compensating for progressive erosion of telomeric sequences inherent to DNA replication.[5,22]

The Telomerase enzyme is composed of 3 main parts: an enzymatic human Telomerase reverse transcriptase (hTERT) catalytic subunit, an RNA component (hTR or hTERC): Human Telomerase RNA and Telomerase associated protein (TP1/TLP1)[23,24]. Telomerase activity has been detected in about 85% of human cancer samples and is associated with cell immortalization and the acquisition of malignancy, whereas the majority of normal tissues have low or no Telomerase activity[25,26]. Human Telomerase reverse transcriptase (hTERT) catalytic subunit proved essential for cellular immortality and oncogenesis; hence, its detection provides a potential new cancer diagnostic possibility. In the present study, we sought to investigate

the potential of clinical usage of hTERT mRNA in urine sediments as a diagnostic marker for bladder cancer.

SUBJECTS AND METHODS

This prospective study was conducted on (50-100ml) morning voided urine samples obtained from 39 patients with bladder cancer (31 were newly diagnosed cases of bladder cancer and 8 were follow-up patients). Fourteen patients with urological disorders not related to bladder cancer of matched age and sex (as control cases). All were collected from the Urology Department, Faculty of Medicine, Alexandria University; in the period between January 2007 and March 2008. The control group also included 8 apparently healthy volunteers of matched age and sex. The study was approved by the University of Alexandria, Faculty of Medicine Ethics Review Board and informed consent was obtained from all patients who agreed to participate.

Cystoscopic biopsy was used as the reference standard for the identification of bladder cancer. All urologic samples were coded and the results of the RT-PCR assay were interpreted blind of the cytologic, or histologic diagnosis. Histopathological classification of the urothelial tumours was done according to the WHO/ISUP consensus classification 2004.[27,28]

Urine Cytology:
An aliquot of the sedimented urinary cell pellet was utilized for cytological examination which was performed using Papanicolaou and Hematoxylin and Eosin stained cytospin preparations.

RNA Extraction:
Another aliquot was utilized for fresh RNA extraction and RT-PCR based technology. Total RNA was prepared using the RNeasy minikit for animal cell RNA extraction (Qiagen, GmbH Germany) according to manufacturer's recommendations. All RNA exractions were performed in a designated sterile laminar-flow hood with RNase-free labware. The integrity of the extracted RNA was confirmed by running on 1% agarose gel and detection of the 18S and 28S ribosomal subunits as bright, discrete bands[29]. Samples with poor RNA quality were excluded from the study.

Multiplex hTERT/GAPDH RT-PCR:
A two-step RT PCR assay was performed using Ready-To-Go RT-PCR Beads (Amersham Biosciences-UK Ltd.), which entailed first strand complementary DNA synthesis (cDNA) by addition of random hexamer primers, and template RNA (according to kit instructions); followed by specific primer amplification in a multiplex reaction. Specific primers were added in a concentration of 25 picomoles for hTERT primers (5'-CGGAAGAGTGTCTGGAGCAA-3', 5'-GGATGAAGCGGAGTCTGGA-3') (9) and 2.5 picomoles for GAPDH primers (5'-TGGGATGGACTGTGGTCATGAG-3', 5'-ACTGGCGTCTTCACCACCATGG-3') (VBC-GENOMICS Bioscience Research Gmbh) in a 50 µl reaction. Each PCR batch included a sample without RNA as a negative control. Reactions were subjected to 35 PCR cycles of 94ºC for 60 seconds, 58ºC for 60 seconds and 72ºC for 60 seconds followed by 7 minutes extension step at 72ºC (Thermal Cycler, Biocycler

TC-S, V. GmbH, Austria. HVD-Life Sciences). The PCR products were analyzed by electrophoresis on an ethidium bromide stained 2.5% agarose gel in TBE buffer (Figure. 1). A sample was considered positive for the presence of hTERT mRNA based on the detection of a specific 145-bp amplicon. A sample was considered negative based on the absence of a 145-bp amplicon and the presence of a GAPDH-specific 250-bp amplicon.

Semiquantitative analysis of RT-PCR results:

Semiquantitative analysis using "Gel-Pro Analyzer" version 4 (Media Cybernetics, USA) was performed. This method employs using the housekeeping gene 'GAPDH' as an internal positive control in each sample[30,31] to normalize hTERT expression for sample-to-sample differences in RNA input, RNA quality and RT-PCR assay efficiency. The expression of hTERT mRNA relative to GAPDH mRNA was determined as a calculated ratio between the amounts of hTERT relative to that of GAPDH within the same lane.

Statistical Analysis:

The sensitivity of the RT-PCR assay and cytology in urologic specimens was defined as: The frequency of samples correctly identified as malignant by the assay among the total number of patients with histologic confirmation of bladder cancer. The specificity was calculated as: The frequency of samples correctly identified as non-malignant by the assay among the total number of participants without histologically proven bladder cancer. Mc Nemar's chi-square test was used to determine the significance of differences in the sensitivity of hTERT/GAPDH RT-PCR and urine cytology. Reciever Operating Curve (ROC) analysis using semiquantitative data was done to set a cut-off point to discriminate malignant from non-malignant cases.

RESULTS

Clinicopathologic characteristics of the bladder cancer patients:

In the malignant study group, mean age of the patients was 61.7±10.3 years, with a peak age incidence during the seventh decade. Thirty-four cases were males and five were females. The study included 36 cases of urothelial cell carcinoma (92.3%) and 3 cases with squamous cell carcinoma (7.7%). The urothelial tumours were classified according to WHO/ISUP consensus classification[27]: one case (3%) was a papillary urothelial neoplasm of low malignant potential (PUNLMP), 10 cases (27%) were low grade papillary urothelial carcinoma, 11 cases (30%) were high grade papillary urothelial carcinoma and 12 cases (32%) were high grade invasive (non papillary) urothelial carcinoma. Cases were subsequently staged according to TNM system[28]; 43% were non-muscle invasive tumours and 57% were muscle invasive tumours.

Overall Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Value of hTERT/GAPDH RT-PCR:

The multiplex RT-PCR assay for hTERT expression in voided urine specimens showed an overall sensitivity of 83.8% and a specificity of 72.7% (Table 1).

The hTERT mRNA expression was detected in urologic specimens from 26 of 31 (83.8%) patients with histopathologic confirmation of bladder cancer and 7 of 8 (87.5%) of follow-up patients,

with clinically diagnosed non-muscle invasive bladder cancer. False negative results were scored in 5 patients with histologically confirmed bladder cancer. All of those cases showed evidence of intense inflammatory infiltrate in the tumour histologically, as well as by urine cytology.

Positive hTERT mRNA expression was detected in urine samples from 2 patients with previous history of bladder cancer who were cancer-free by cystoscopy and upper tract imaging at the time of urine sample collection (Figure. 2). The urine analysis and cultures indicated that the patients did not have urinary tract infections. Clinical follow-up of the patients confirmed that the patients developed recurrence 5 and 4 months, respectively, following the hTERT/GAPDH RT-PCR assay positivity.

Based on Mc Nemar's chi-square paired comparison of the 37 bladder cancer cases with pathologic confirmation, the hTERT/GAPDH RT-PCR test demonstrated significantly higher sensitivity than the conventional urine cytology (83.8 % vs. 75.7%, $p<0.0089$).

The positive predictive value of the test defined as the probability that a positive hTERT/GAPDH RT-PCR assay is true-positive compared with the final pathologic diagnosis was 83.8% in this study.

The negative predictive value, calculated as the probability that a negative hTERT/GAPDH RT-PCR assay is true-negative compared with the final pathologic diagnosis, was 72.7 %.

Semiquantitative Analysis of hTERT mRNA in urine samples:

Semiquantitative analysis of hTERT expression relative to GAPDH house keeping gene using "Gel-Pro Analyzer" version 4 (Media Cybernetics, USA) was performed. The mean ration of hTERT/GAPDH was observed to be higher in the malignant group relative to the benign cases. The difference between the mean ratio for benign cases (0.30 ± 0.35) and malignant cases (0.66 ± 0.46) was statistically significant using the Mann Whitney test, $p< 0.0001$ (Table 2).

ROC curves were used to to assess the accuracy of hTERT/GAPDH ratio in diagnosing malignant cases. The area under the curve (AUC) can range from 0-1. Diagnostic tests with AUC that approach 1 indicate a perfect discriminator. In this study the positivity lies within the presence of malignancy and the negativity being its absence. The results showed an AUC of 0.84 ± 0.06, which was significantly better than indifference $p<0.0001$ (Figure. 3). Using the ROC curve a cut-off point of 0.07 AU was proposed, with a sensitivity of 97.3%. By elimination of the confounding factor of inflammation the sensitivity of the test using the 0.07AU cut-off point increased from 97.3% to 100% and the specificity from 66% to 68%.

Comparison between the performance of RT-PCR assay and Conventional Cytopathology:

The sensitivity of the multiplex RT-PCR assay (83.8%) was significantly higher than that for conventional cytology

(75.7%) (p=0.0089) (Table 3). However, the specificity of the assay was lower 72.7% as opposed to 100%.

When combining RT-PCR and urine cytology, the sensitivity highly increased to reach 95%. However the specificity remained constant at 72.7%. The positive predictive value rose to 86%, as well as the negative predictive value 89%.

Effect of Tumour Grade on the Efficiency of hTERT expression and Conventional Cytology:

The efficiency of RT-PCR assay in low grade UCC (81.8%) was not much lower than that in high grade UCC (91.3%) possibly indicating that increased Telomerase expression occurs early in bladder cancer tumourigenesis. In contrast, urine cytology revealed a poorer sensitivity in comparison to hTERT/GAPDH assay particularly in low grade UCC (Figure. 4).

Table 1: Relation between hTERT and histopathology (the gold standard).

PCR hTERT	Gold standard (histopathology)		Total
	Positive for malignancy	Negative for malignancy♠	
Positive	31	6	37
Negative	6	16	22
Total	37	22	59
Chi square	**18.84**		
P value	**<0.0001***		
Sensitivity	31 / 37= 83.8		
Specificity	16 / 22= 72.7		
PPV	31 / 37= 83.8		
NPV	16 / 22= 72.7		

***:** Statistically significant at P≤0.05.

♠: Cases with benign urological disorders and healthy volunteers did not have cystoscopic biopsy, but were included in 'negative for malignancy' Group.

Abb.: PPV: Positive predictive value, NPV: Negative predictive value.

Table 2: Semi-Quantitative hTERT/GAPDH measurement in malignant and benign urologic cases.

	BENIGN CASES (16)	MALIGNANT CASES (37)
Range	0 -1	0.05- 2.60
Mean ± SD	0.30 ± 0.35	0.66 ± 0.46
Z of Man Whitney test	4.50 P<0.0001*	

Table 3: Comparison between the performance of the non invasive diagnostic modalities (hTERT RT PCR assay and cytopathology) of bladder cancer in voided urine specimens.

Parameters	Sensitivity	Specificity	PPV[1]	NPV[2*]
Telomerase RT-PCR	83.8*	72.7	79.5	83.8
Cytology	75.7*	100	100	72.7
Cytology + RT PCR in urine (either)	94.9	72.7	86	89

Chi square test comparing between sensitivity of cytology and RT-PCR for hTERT was 6.85 with a p value* 0.0089. Abbreviation:1:PPV: Positive predictive value, 2:NPV: Negative predictive value

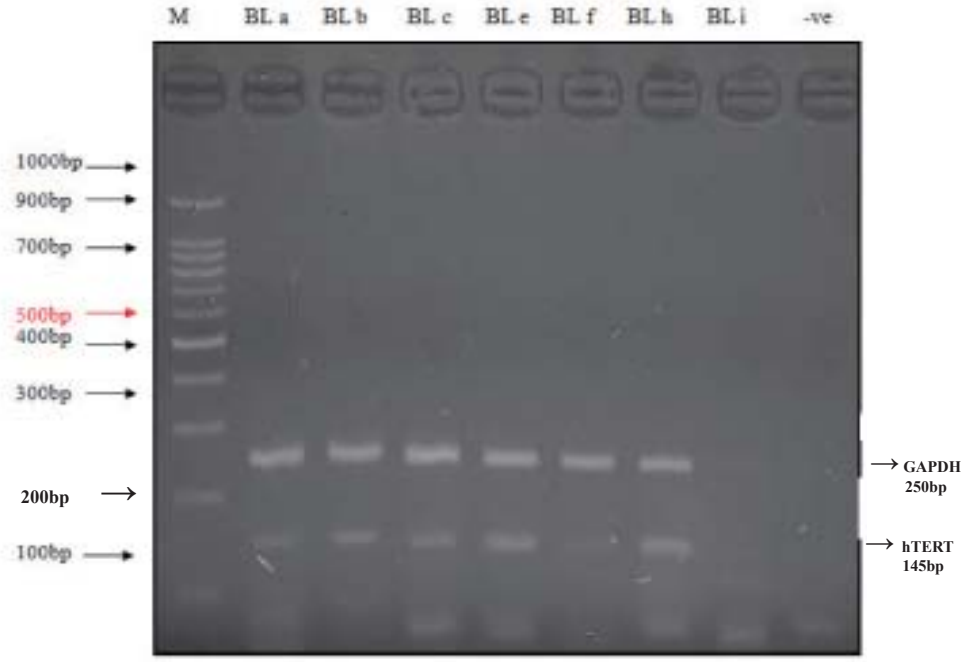

Fig. 1: Multiplex PCR product. Lane M shows the standard 100bp molecular marker. Samples coded BLa – BLh show 2 amplicons, one at 145bp representing hTERT gene the other at 250bp representing the GAPDH gene. Sample BLi did not show GAPDH gene and hence was excluded from the study. A negative control sample was included in all PCR reactions to exclude contamination.

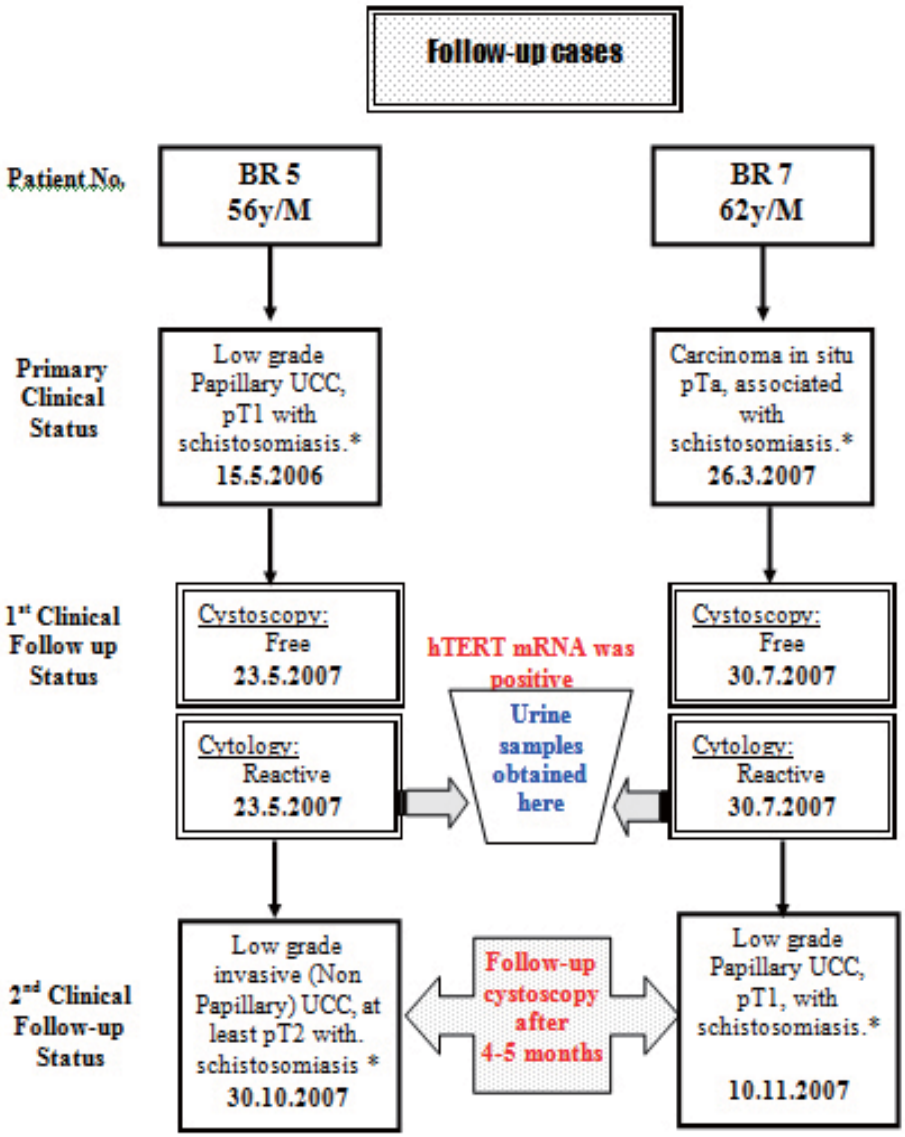

Fig. 2: Flow chart of detection of hTERT mRNA in urinary samples of patients with a history of resected bladder cancer during follow up cystoscopy revealed cancer-free status. All tissue samples indicated by * had histopathologic assessment. Cytology was negative at the time of detection of the Telomerase marker. Samples codes are BR5 and BR7. Abbreviations: M, male; UCC, urothelial cell carcinoma, pTa: Superficial non invasive urothelial carcinoma,
pT1: urothelial carcinoma infiltrating the lamina propria, pT2: Urothelial carcinoma infiltrating the muscularis propria of the bladder wall.

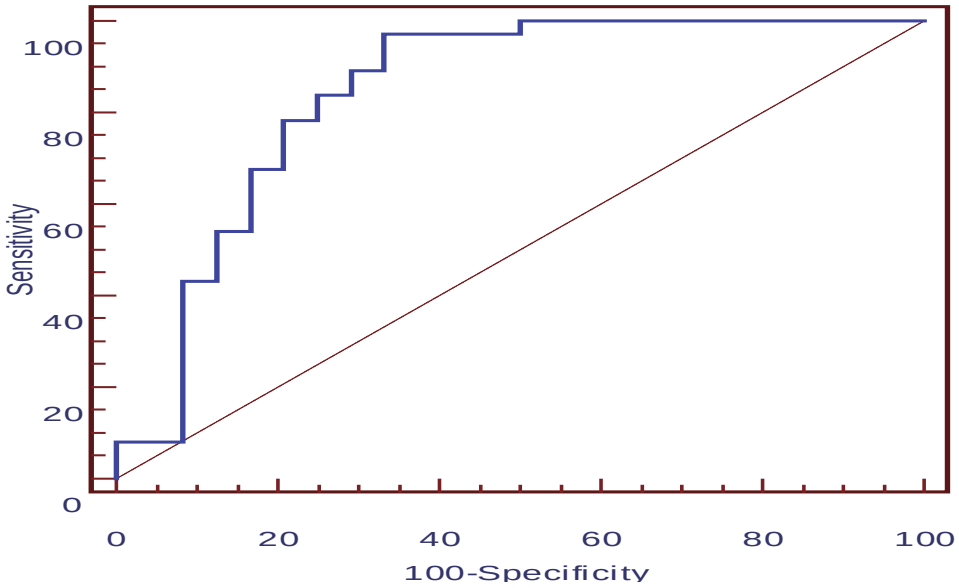

Fig. 3: ROC curve analysis hTERT/GAPDH semiquantitative assay.

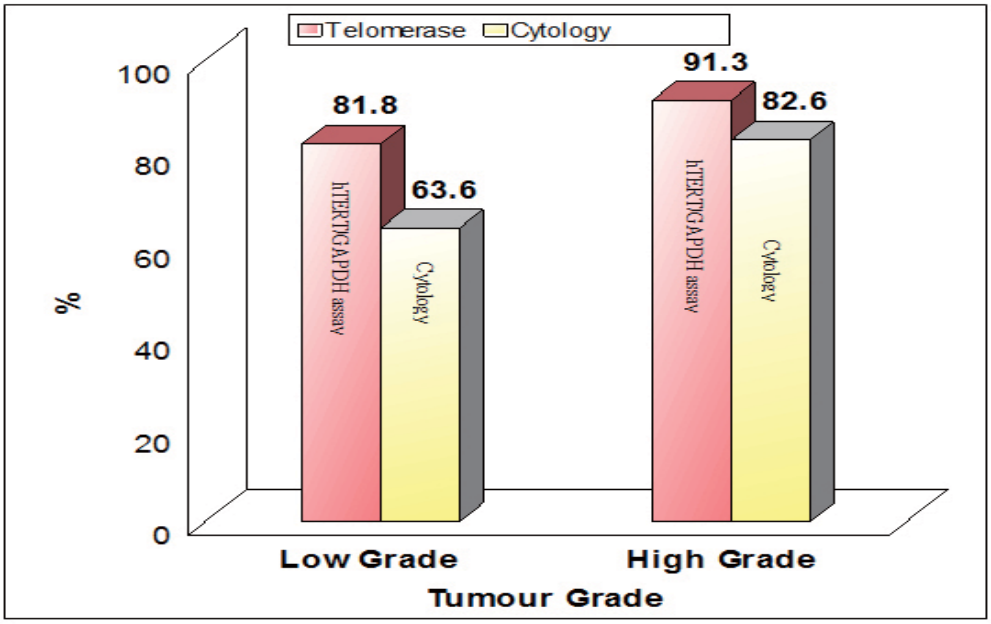

Fig. 4: Comparison between sensitivities of Telomerase and cytology in different tumour grades.

DISCUSSION

There is a pressing need for a non invasive method to diagnose carcinoma of the urinary bladder. To date, however cystoscopy remains the gold standard not only for primary diagnosis of tumours, but is repeated at approximately 3-6 months interval for follow-up of recurrence.

At present, cytology is accepted as the gold standard non-invasive method for screening of bladder carcinoma in both symptomatic patients and the general population, as it is non invasive, safe, and inexpensive.[10,32]

Urine cytologic examination is "morphology-based" and therefore inevitably demonstrates a low sensitivity in low-grade tumours since the cytologic abnormality is slight. It possesses a good overall median specificity of 99% (83–100%) and an acceptable overall median sensitivity of 34% (20%–53%)[33]. In the present study the specificity of urine cytology was 100% and the overall sensitivity was higher than that reported in the literature being 75.7%. This may be attributed to the larger percentage of high grade tumours in our cohort.

Earlier studies reported strong correlation between hTERT expression and detectable Telomerase enzyme[34-36]. Suzuki et al. demonstrated hTERT mRNA expression in 100% of bladder cancer tumour tissue[37]. Ito et al. observed that only the expression of hTERT is specifically associated with more than 90% of urothelial cancers[6]. Limitations of the Telomeric Repeat Amplification Protocol (TRAP) assay used to measure Telomerase enzyme activity including; rapid degradation of the protein enzyme in the hostile urinary environment and the need for large numbers of cells in urine are largely overcome by the use of RT-PCR-based detection of hTERT mRNA. Scientists have claimed that an RT-PCR assay may detect a single positive cell amidst hundred negative cells[38]. In the same context, the work of Muller et al. on bladder carcinoma concluded that RT-PCR detection of hTERT appears to be several orders of magnitude more sensitive than TRAP assay.[39]

The multiplex hTERT/GAPDH RT-PCR applied in this study, correctly predicted bladder cancer in 31 of 37 patients (sensitivity of 83.8%), with a 72.7% specificity and a positive predictive value of 83.8% irrespective of grade or stage of the tumour.

The specificity of the present study (72.7%) was found to be lower than that reported in some earlier studies where the specificity ranged from 90% to 100%[5-7,19,39-41]. However, other studies such as Neves et al.[40] and Morsi et al.[41] reported a lower specificity of 69% and 66%, respectively. The specificity data does not support the use of hTERT mRNA in place of cytology, however the improved sensitivity of the assay suggests its potential utility as an adjunct to cytology.

In two of the follow-up patients (BR5 and BR7, Figure. 2) which have been initially diagnosed as low grade papillary UCC, pT1 lesion and carcinoma in-situ, respectively, (both known to present a high risk for recurrence) the results of the hTERT/GAPDH RT-PCR were positive in the follow-up urine samples. Meanwhile, their cystoscopic and cytologic examinations were negative for malignancy. Both patients developed recurrence within 5 months and

4 months, respectively, of disease-free interval. This finding suggests a role for Telomerase expression in the early detection of recurrence. Hence, molecular changes contributing to cancer recurrence could be highlighted by hTERT/GAPDH RT-PCR assay, prior to any morphologic changes. A similar finding was reported in 3/3 follow-up patients in a study by Bialkowska-Hobrzanska et al.[5] Regarding its potential capability in the prediction of tumour recurrence, RT-PCR assay may be useful prior to the cystoscopy procedure, as it may alter the management strategy of the operating surgeon, especially when no tumour lesion can be identified grossly. Following discussions with urology surgeons through personal contact, the surgeons suggested that extra caution should be taken in the follow-up of these cases and surgeons may accordingly opt to:

A. Take multiple random cold-cup biopsies from the bladder wall,
B. Obtain a barbotage cytology sample,
C. Assess the upper urinary tract to exclude other tumours,
D. Reduce the interval before the following cystoscopic procedure.

In this study we observed a higher diagnostic sensitivity of the hTERT RT-PCR assay (31/37, 83.8%) as compared to the conventional cytology (28/37, 75.7%). We found some cases of discordance, in which cytology was negative but Telomerase expression was positive 5 out of 7 cases (71.4%). These samples contained very few cancer cells, which could have been insufficient for cytological evaluation but detectable by RT-PCR. Follow-up of the cases revealed histological evidence of low grade tumours. Nevertheless, some discordances were observed where cytology was positive but Telomerase was negative (4 out of 6 cases). This type of discordance was prevalent among high-grade cancers. A possible explanation for this discrepancy is simply the degradation of Telomerase in urinary sediment samples in aggressive necrotic tumours. Taken together, these findings suggest that the combination of cytological examination with urinary Telomerase may improve the sensitivity and specificity of non-invasive screening for bladder cancer. In fact, on calculating the combined sensitivity of urinary hTERT mRNA detection and conventional cytopathology, the sensitivity improved to 94.6%.

The specificity of the telomerase expression by RT-PCR is illustrated in patients of the benign group and healthy volunteers. In our results there was a high positive prevalence in those patients, which greatly compromised the specificity to 72.7%. Patients who showed a false-positive Telomerase result, all showed evidence of chronic or severe inflammation as evidenced by cytology and urine analysis.

Using the calculated hTERT/GAPDH ratios for the study groups, a ROC curve was plotted. Using the previously published cut-off value 0.2 AU proposed by Bialowska-Hobrzanska et al.[5] the curve displayed a sensitivity of 86.5% and a specificity of 66.7%. On the other hand, the present study deduced an optimum experimental cut-off value of 0.07AU which displayed a better sensitivity of 97.3% and a specificity of 66.7%. The low specificity of hTERT mRNA is mainly attributed to the high false-positive rate in patients of the benign group, which is most probably due to the presence of Telomerase positive inflammatory cells in urine. In the absence of in-

flammation, whilst using a cut-of value of 0.07, the sensitivity of RT-PCR assay rose up to 100%.

In conclusion, as patient compliance is greater with procedures that are non-invasive, the combination of hTERT/GAPDH assay with cytology could allow the early diagnosis of bladder carcinoma, particularly so, for the early detection of bladder carcinoma recurrence following treatment. It could also be of value in evaluating patients at high risk for developing bladder cancer, including those exposed to occupational carcinogens, Future prospective multi-centre studies conducted with standardized specimen collection and storage procedures are strongly recommended to further validate the clinical usefulness of implementing hTERT RT-PCR assay as a diagnostic and screening method for bladder cancer in voided urine specimens.

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