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ORIGINAL ARTICLE

Evaluation of Urinary Human Telomerase Reverse Transcriptase mRNA and Scatter Factor Protein as Urine Markers for Diagnosis of Bladder Cancer

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SUMMARY

Background: **Expression of the human telomerase reverse transcriptase (hTERT) gene, which codes for the catalytic subunit of telomerase is considered an important tumor marker used for bladder cancer detection being found in the majority of cancer cells. Scatter Factor (SF) is a secretory protein produced by fibroblasts and smooth muscles and induces scattering of the epithelial cells. The aim of the current study was to evaluate the potential usefulness of hTERT and SF measurement as urinary markers for bladder cancer diagnosis.**

Methods: **Voided urine specimens were collected from patients with histologically confirmed bladder urothelial carcinoma (malignant group: n = 60), urological patients without urothelial carcinoma (benign group: n = 25), and healthy volunteers (control group: n = 20). All cases underwent urine cytology, serological schistosomiasis antibody assay and detection of urinary hTERT mRNA using RT-PCR and SF using ELISA.**

Results: **Positivity rate of hTERT mRNA was markedly higher in malignant versus benign or control cases (86.67%, 8%, and 0%, respectively, p-value < 0.001). Combining hTERT and cytology increased the sensitivity of cytology to 95%. According to a cut-off value of urinary SF (≥ 410 ng/mg protein), 57 (95%) of the patients with bladder carcinoma, 10 (40%) with benign lesions, and non of the control individuals were positive and the difference between the 3 groups was statistically significant (p < 0.001). The sensitivity of cytology was increased to 98.33% when combined with the SF assay. When associating the two urinary markers with different clinicopathological factors of the bladder cancer group, only SF exerted a significantly higher positivity rate at the invasive stage (100%) than the superficial stage (88.46%) as well as in transitional cell carcinoma (100%) than squamous cell carcinoma type (87.5%).**

Conclusions: **hTERT and SF can be considered potential useful markers for detection of bladder cancer.**
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KEY WORDS

hTERT, bladder cancer, RT PCR, scatter factor

INTRODUCTION

Bladder cancer is the fourth most prevalent cancer in males and the eighth most prevalent cancer in females and has a considerable impact on health economics. The cost per patient with bladder cancer from diagnosis to death is the highest of all cancers. In terms of total medical care expenditures, it was the fifth most expensive cancer in 2005 accounting for 4 billion dollars [1]. Bladder carcinoma is the most prevalent malignant disease among Egyptians and other Africans where schistosomiasis is endemic. At the National Cancer Institute (NCI), Cairo, Egypt, bladder cancer constitutes 20 - 30% of all cancers. The incidence is particularly high in the Nile river valley [2].

Bladder cancer is a heterogeneous disease with an unpredictable clinical course [3]. Non transitional cell carcinomas behave more aggressively and in general, are less responsive to treatments other than surgery [4]. In general, the prognosis of patients and the choice of.

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treatments depend on the aggressiveness and grade of the tumor. The survival disparity between early and late stage detection is marked. Early localized tumors have a 5 year survival rate of 94%, while regional or distant disease has rates of 49% and 6%, respectively. The disease exhibits a high rate of recurrence of up to 70% in all affected individuals. Therefore, even successfully treated patients require life-long follow up and monitoring [5]. That is why a marker which is preferably non invasive and is relatively low in cost is highly required. The gold standard for the detection of bladder cancer is cystoscopy but it is invasive, requires operative procedures, and causes significant patient discomfort. The standard non invasive technique used for the detection of bladder cancer is cytology but it is used as an additive diagnostic tool to cystoscopy due to its low sensitivity [6].

Telomeres are responsible for protecting the chromosomes during cell division. Accordingly, activation of telomerase will lead to cellular immortality and oncogenesis [7]. Three major subunits comprising the human telomerase complex have been identified, a ribosomal RNA component (hTR) that serves as a template for telomere repeat synthesis, a protein component known as telomerase reverse transcriptase (hTERT) that is responsible for the enzymatic activity of telomerase enzyme and telomerase enzyme associated proteins with an unclear function [8]. Eissa et al. [9] had reasoned that the enzyme telomerase may offer new diagnostic opportunities because telomerase activation is the hallmark of carcinogenesis in different tumors. Scatter Factor has long been recognized as being one of the most powerful angiogenic factors as it up-regulates the expression of vascular endothelial growth factor (VEGF) [10] and down-regulates the expression of thrombospondin-1 (TSP1) in the same cell type [11]. This along with its morphogenic ability [12] makes SF common in cancerous tissues and, therefore, a useful tumor marker. The aim of the present study was to evaluate the potential usefulness of hTERT mRNA and SF protein as urinary markers for the diagnosis of bladder cancer.

MATERIALS AND METHODS

Patient population

Blood and voided urine samples were taken from 85 patients who presented with either hematuria or a bladder mass to the National Cancer Institute, Cairo, Egypt. After cystoscopy examination and biopsy, 60 cases proved to be bladder cancer and 25 cases were benign bladder lesions. In addition, 20 healthy volunteers were enrolled in the study. Informed consent was obtained from all subjects with approval granted by Ain-shams Research and Ethics Committee (Ain-shams Faculty of Medicine federal number IRB00006444) prior to sample collection. Accordingly, the 105 cases were classified into 3 groups: A bladder cancer group n = 60 including 48 (80%) males and 12 (20%) females with a mean age 58.12 ± 10.17 years (range 42 to 78), a benign group with non malignant urological lesions n = 25 including 16 (64%) male and 9 (36%) female with a mean age of 43.23 ± 15.22 years (range 18 to 69) and a control group of healthy individuals n = 20 including 6 (60%) male and 4 (40%) female with a mean age of 36.6 ± 12.12 years (range 20 to 58). Exclusion criteria for the cases include: patients who had a history of another urological malignancy (kidney-ureter-urethra), recent urological operative interference or pelvic malignancy. Tumors were staged according to the TNM classification of IUA CC criteria [13] and graded according to WHO criteria [14].

Blood Sample Processing

5 mL of blood sample was withdrawn from each of the studied individuals. Each blood sample was centrifuged at 700 × g for 5 minutes for separation of the serum. The obtained sera were stored immediately at -80ºC for the assay of bilharzial antibodies.

Urine sample Processing

The voided urine samples were obtained immediately before cystoscopy, centrifuged at 2500 - 4000 × g for 15 - 20 minutes. Supernatants were concentrated 30-fold using Amicon YM-20 (20 KDs) ultrafiltration membranes then divided into aliquots, stored at -80ºC until assay of protein and SF [15].

The pellets were then washed twice with phosphate buffered saline to remove any urine residues. A small part of the pellet was fixed on a slide for cytological examination and the rest was mixed with 500 µL of a protease inhibitor cocktail and stored at -80ºC immediately for the telomerase RT PCR [16,17].

RNA Extraction

RNA extraction was done using purescript RNA extraction kit (Gentra System, Minneapolis, MN, USA). Up to 1 x 10^7 cells were disrupted and homogenized with the lysis buffer (citric acid, EDTA, and sodium dodecylsulfate (SDS)). One volume of 70% ethanol was added to the lysate and up to 700 µL of sample were applied to the RNeasy Mini spin column. High quality RNA was eluted in ribonuclease-free water.

RT-PCR for hTERT mRNA

Total RNA (5 µg) was subjected to first strand cDNA synthesis using Maloney murine leukemia virus RT and oligo primer pd $(T)_{12–18}$ for 1 hour at 42°C. RT-PCR was performed using Ready-to-Go™ RT-PCR beads. The cDNA was then amplified to detect hTERT using hTERT primers (sense 5′ -ACTTTGTCAAGGTGGAT GTGACGG- 3′ and antisense 5′ -AAGAAATCATCCA CCAAACGCAGG-3′ [18]. PCR conditions for hTERT were optimized in a Hybaid thermocycler for 35 cycles of denaturation at 95°C for 5 minutes, annealing at 62°C for 30 seconds, extension at 72°C for 30 seconds and final extension for 10 minutes. The product for hTER was separated on 2% agarose gel and visualized by ethidium

Table 1. The concentration of SF (ng/mg protein) in the study groups.

SF	Malignant	Benign	Control	Chi-square	p-value
Mean Rank	68.63	47.28	13.27	50.73	< 0.001
Median	750	330	185	-	-
Range	400 - 45000	180 - 1820	40 - 387	-	-

Table 2. hTERT mRNA, SF and urine cytology in study groups.

Groups	hTERT m RNA No Post (%)	SF No Post (%)	Cytology No Post (%)
Malignant	52 (86.67)	57 (95)	28 (46.67)
Benign	2 (8)	10 (40)	0
Normal	0	0	0
Chi-square	69.88	66.67	25.87
p-value	< 0.001		

Table 3. hTERT mRNA, SF and urine cytology in relation to Clinicopathological Features of bladder cancer group.

Groups	hTERT mRNA No. pos. (%)	SF No. pos. (%)	Cytology No. pos. (%)
Gender Male (48) Female (12)	 42 (87.50%) 10 (83.33%)	 45 (93.75%) 12 (100%)	 23 (47.92%) 5 (41.67%)
Bilharziasis ve (14) +ve (46)	 12 (85.71%) 40 (86.96%)	 14 (100%) 21 (87.50%)	 15 (51.7%) 8 (47.1%)
Pathology Transitional cell carcinoma (36) Squamous cell carcinoma (24)	 33 (91.67%) 20 (83.33%)	 36 (100%)* 21 (87.50%)	 15 (51.7%) 8 (47.1%)
* p-value = 0.042			
Grade Low grade (1 + 2) (24) High grade (3) (36)	 21 (87.50%) 31 (86.11%)	 23 (95.83%) 36 (100%)	 13 (54.17%) 5 (41.67%)
Stage Early stage (0 + 1) (26) Late stage (2 + 3) (34)	 23 (88.46%) 29 (85.29%)	 23 (88.46%)* 34 (100%)	 11 (42.31%) 17 (50%)
* p-value = 0.049			
Cytology -ve (32) +ve (28)	 29 (90.63%) 23 (82.14%)	 32 (100%) 27 (96.64%)	 - -

*** significant p-value (< 0.05).**

bromide staining GAPDH was used as a housekeeping gene. Its primer pair was sense 5′ -GAGTCAACGGAT TTGGTCGT- 3′ and antisense 5′ -TTGAT TTTGGAG GGATCTCG- 3′ [19]. The GAPDH product was shown at 637 bp (Figure 1, 2).

Scatter Factor ELISA

Urinary level of SF was measured using ELISA (sandwich technique). The Quantikine human growth factor (SF) immunoassay Kit was used. SF was bound by the immobilized antibody.

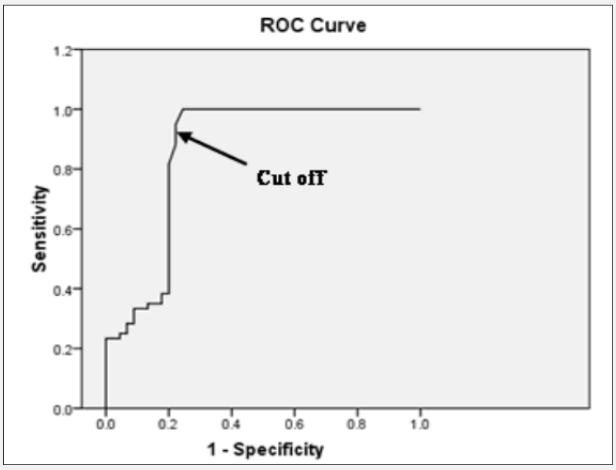

Figure 3. The ROC curve analysis for the SF in the malignant group versus the benign and normal control groups as non-malignant group to calculate the best cut-off value. Area under the curve was 0.857. The arrow denotes the cut-off point at 410 ng/mg protein at which SF sensitivity was 95% and specificity was 77.78%.

After washing away any unbound antibody, an enzyme-linked polyclonal antibody was added and after incubation the substrate was added and finally a stopping solution. The optical density was determined using a microplate reader Titertek (Labsystems, ICN Biomedicals) Multiskan Plus 311 BO set to 450 nm. SF values were normalized to protein and measured by the Bradford method [20].

Qualitative Detection of Bilharzial Antibodies in Serum

The indirect hemagglutination test was performed using the Cellognost Schistosomiasis H kit (Dade Behring, Marburg, Germany).

Statistical Analysis

The nonparametric Mann-Whitney sum rank U and Kruskal-Wallis (x2) tests were used for the comparison of the variables between groups. The positivity rates were compared by chi-square test. The level of significance was determined to be less than 0.05. The threshold value for optimal sensitivity and specificity was determined by receiver operating characteristic (ROC) curve which was constructed by calculating the true-positive fraction (sensitivity %) and false-positive fraction (100-specificity %) of survivin at several cut-off points [21]. All analysis were performed using the Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL, USA).

RESULTS

The concentration of SF in urine supernatant of the malignant, benign, and control cases are shown in (Table 1). The Receiver Operating Characteristics (ROC) curve (Figure 3) was used to determine the threshold value for optimal sensitivity and specificity, which was constructed by calculating the true positive fraction (sensitivity percent) and the false positive fraction (100-specificity) of SF at several cut-off points. Accordingly, the best cut-off value (by considering both benign and control as non-malignant group) was ≥ 410 ng/mg protein.

hTERT by RT-PCR and SF by ELISA in 105 samples revealed positivity in 86.67% and 95%, respectively, of the malignant group and in 8% and 40% of the benign group, respectively, and not detected in normal individuals, p-value < 0.001 (Table 2). On associating the two urinary markers with different clinicopathological factors of the bladder cancer group, only SF exerted a significantly higher positivity rate in the invasive stage (100%) than superficial stage (88.46%) p = 0.042 and also higher positivity in transitional cell carcinoma (100%) than squamous cell carcinoma type (87.5%), p = 0.049 (Table 3).

Sensitivity and specificity for each urinary marker as well as their combination were tested for detection of bladder cancer. Both sensitivity (95%) and specificity (77.78%) of urinary SF was the highest. The combined use of both markers together revealed a sensitivity of 98.33% and specificity of 77.78%. Urine cytology revealed the lowest sensitivity (46.67%). The 46.67% sensitivity of urine cytology improved when combined

with hTERT mRNA (95%) while the best sensitivity (98.33%) for urine cytology was achieved when combined with SF (Table 4).

DISCUSSION

Urine tumor markers have been proposed for use as diagnostic aids in patients who present with haematuria, as prognostic indicators of disease recurrence and survival, and as early detectors of recurrent disease in the monitored patients [22].

Quantitative analysis of SF by enzyme linked immunosorbent assay (ELISA) was performed in concentrated supernatant of voided urine from malignant, benign, and healthy normal groups. Urinary SF levels (pg) were found to be influenced by hydration status and urine output, thus normalized to urinary protein content (mg) was done for the accuracy of the test. Normalization to total protein was found to be better than creatinine as it is less influenced by hematuria, a condition commonly found in bladder cancer patients. [23] The performance of SF by ELISA has been validated and was proved to be reliable for application in minimal amounts of voided urine samples [15].

In our study, the mean rank of SF was compared between the different studied groups and was found to be 68.63 in the malignant, 47.28 in the benign, and 13.27 in the control group. There was a 1.45-fold increase in the level of SF in the malignant group as compared to the benign and a 5.17-fold increase as compared to the control group. The difference between these values was statistically significant ($p < 0.001$). This suggests the functional role of SF as an angiogenic factor essential for the survival of tumor cells.

The Receiver Operating Characteristics (ROC) curve was used to determine the best cut-off value for SF considering both benign and control groups as a non malignant group. The cut-off was found to be ≥ 410 ng/mg of protein, the sensitivity was 95% and the positive and negative predictive values were 85.07% and 92.11%, respectively.

Applying this cut-off, the positivity rate of the SF was significantly higher in the malignant group (95%) than in the benign (40%) and control (0%) groups. This difference was statistically significant ($p < 0.001$). Ten patients with bilharzial cystitis in the benign group had false positive results. This may be attributed to the release of inflammatory mediators such as interleukins as a response to the continuous irritation due to cystitis or bilharzial ulcers since interleukins induce the release of SF. Also, Rosen et al. [24] stated that some patients of his control group suffering from cystitis showed elevated levels of SF but accounted for no significant difference.

On correlating the positivity rate of SF with the studied clinicopathological factors, no significant correlation was found regarding either age or sex of the patients ($p > 0.05$). Strong correlation was found between the level of SF and the pathological type and stage of the tumor. The SF level was higher in TCC than in SCC and in late stages than in early stages. Thus SF could be a useful marker for tumor aggressiveness. This agrees with Wang et al., [25] who found significant correlation between SF serum level and tumor muscle invasion. They contributed the enhancement of bladder cancer cell invasion to the cross-talk with fibroblast by way of humoral factors, including the SF. Also in colorectal carcinoma, Kammula et al., [26] found that SF gene expression in biopsy specimens appears to be a viable molecular marker of invasion and metastasis, which could be incorporated in the staging of the disease.

Superficial tumors usually have a papillary morphology with a well-organized vascular tree whereas invasive or high grade and stage tumors are generally solid with a disorganized vasculature and areas of necrosis. Jemal et al. [27] suggested that there may be two different angiogenic pathways in bladder cancer and that each is associated with a different tumor morphology and behavior. Approximately 70% of superficial and low grade and stage tumors are thought to have mutations of chromosome 9 whereas mutations on chromosome 10 and 11 are more usual in invasive cancers. [28] The two different genetic pathways are associated with two different angiogenic pathways which explain the over-expression and stage-dependence of SF.

In our study, the sensitivity of cytology in the studied group was 46.67%. On combining both SF and cytology the sensitivity increased to 98.33% and the accuracy to 86.67%.

Studies have pointed out that high hTERT mRNA expression is associated with malignancy in many tumor histotypes and has shown great potential for early cancer detection in body fluids [29,30]. Also, it had been reported by others that hTERT mRNA expression was a more sensitive marker of telomerase function than assessment of telomerase activity by TRAP assay [31]. Eissa et al. [9] compared three methods for detecting telomerase in the urine samples; measuring the telomerase activity via the telomerase repeat amplification protocol (TRAP) assay, detecting the mRNA of the catalytic subunit of telomerase by RT-PCR, and real time PCR. Based on the results of this comparative study, we selected the RT-PCR technique to detect hTERT mR NA in urine samples.

Using RT-PCR, hTERT mRNA expression in urine was determined by comparing the expression of the target gene hTERT with that of the housekeeping gene GAP DH, which was thought to be suitable to normalize expression. Our results revealed a marked increase in the positivity rate of urine hTERT in the malignant vs. the benign group (86.7% vs. 8%) while no normal urine samples showed positive hTERT mRNA ($p < 0.001$). Sanchini et al. [7] and Gelmini et al. [29] found telomerase sensitivities of 82% and 90%, respectively, using the TRAP assay. Also, Brems-Eskildsen et al. [32] reported a sensitivity of 63% for the hTERT assay using RT-PCR and a highly significant difference in the posi-

tivity among bladder cancer and non malignant cases. In the current study, a conflict appeared between the results obtained from cytology and those obtained from RT-PCR when cytology was negative and the telomerase activity was positive. This may be due to the presence of a small number of cancer cells in the malignant sample which may be insufficient for cytological evaluation but detectable by the RT-PCR.

In the benign group, the presence of hTERT mRNA in 8% of cases may have been a predictor of premalignant lesions that would become evident in the future since telomerase has been referred to as a marker of cell proliferation rather than of malignancy. Also, in some cases cytological examination was positive while the telomerase gave negative results. This was prevalent among high-grade tumors and can be attributed to the degradation of telomerase in the urinary sediment sample. Thus, the use of the hTERT assay would be more useful in detecting low-grade tumors.

There was no correlation between the hTERT and any of the studied clinicopathological factors; thus, hTERT can be used for the detection of any stage or grade of bladder cancer. Bowles et al. [33] found that hTERT mRNA expression correlates positively with tumor stage and grade. On combining hTERT with cytology, the sensitivity was increased to 95% and the accuracy from 69.52% to 95.24%. Having a specificity of 95.5%, hTERT can be used as a tumor marker with cytology to improve its sensitivity.

When a urine molecular marker is used for bladder cancer diagnostic assessment it should have high sensitivity and PPV [34]. Accordingly urinary hTERT mRNA and SF are significantly superior to urine cytology (sensitivity: 86.67%, 95.5%, and 46.67%, respectively). SF detected by ELISA is considered the best individual test while the combination of SF with urine cytology is the best combined test increasing sensitivity of urine cytology up to 98.33%.

The limitation of our study was the relatively small sample size. The present study could have yielded more consistent results if a larger number of patients had been included and a follow-up had been done.

CONCLUSION

hTERT and SF are useful markers for the detection of bladder cancer, increasing the sensitivity of urine cytology.

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Declaration of Interest:
All authors declare that there is no conflict of interest.

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