Detection of Telomerase Activity Using a Quantitative Assay and Immunohistochemistry Techniques In Urinary Bladder Lesions

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Abstract: Telomerase activity is found in 85%-90% of all human cancers and absent in most of the normal cells and thus represents a potential cancer biomarker. Our aim was assessment of telomerase activity by TRAP method and to detect, localize cellular telomerase expression (hTERT) by immunohistochemistry technique in tissue samples of bladder lesions and to correlate the results with clinicopathological parameters (histopathological grade and stages). Bladder specimens were obtained from 85 patients and 10 cases served as control. Telomerase activity by PCR-ELISA technique was measured using TRAP (telomeric repeat amplification protocol) technique and immunohistochemistry (IHC) method was performed on paraffin sections to detect the protein human telomerase reverse transcriptase (hTERT). The patient group included 75 males and 10 females, fifty nine of our patients (59/85) have proven to be associated with schistosomiasis. TRAP activity was detected in 48/60 bladder cancer cases (80%), non schistosomal urothelial carcinoma (non Sch. urothelial carcinoma) (71.4%), Sch. urothelial carcinoma (75%) while were (100%) in squamous cell carcinoma (SQCC). TRAP activity was positive in 100% of high grade urothelial carcinoma compared to low grade urothelial carcinoma 50% and 92% positive in invasive tumors compared to superficial tumors 50%. hTERT protein expression was detected in (86.6%) of bladder cancer cases; non Sch. urothelial carcinoma (80.9%), Sch. urothelial carcinoma (87.5%) while in SQCC it was (93.3%). In addition; hTERT was positive in 100% of high grade and invasive urothelial carcinoma compared to low grade 70.8% and superficial 65% urothelial carcinoma. We conclude that both TRAP and hTERT protein increased in SQCC and urothelial carcinoma than in non cancerous cases and also was higher in SQCC than urothelial carcinoma. Both methods may be used for the detection of telomerase activity bladder cancer. TRAP activity could be a promising diagnostic marker in addition to other histological parameters. The significant value of TRAP in cancer bladder is not only based on its diagnostic validity but also on its poor prognostic impact in those patients thus widening the range of application of this technique in future.

Key words: TRAP; telomerase reverse transcriptase (hTERT) enzyme; urothelial carcinoma; sqcc; schistosomiasis.

Sch: schistosomal

INTRODUCTION

Schistosomiasis ranks as one of the major health problems in Egypt and schistosoma associated bladder cancer (SABC) is the most common malignant neoplasm among male Egyptians (El Bolkainy et al., 1981). A causal relationship between schistosomiasis and bladder cancer was suggested because of the higher rate of bladder cancer in areas with endemic schistosomiasis (Rosin et al., 1994). Schistosomiasis infested bladders frequently show squamous metaplasia and dysplasia of the transitional epithelium (Kitinya et al., 1986). The molecular mechanism underlying these changes and their relationship to the development of bladder cancer are still poorly understood. Malignant transformation and tumor progression are multistage processes requiring
the accumulation of several genetic alterations, mainly the activation of one or more genes. The type and number of genes involved in this multistep process seem to determine the biological properties of the tumor (Orlando et al., 1993).

Telomerase is considered an important tumor marker for detecting bladder cancer since telomerase is found at high levels in 90% of cancer cells (Sanchini et al., 2005). Three major subunits comprising the human telomerase complex have been identified including a ribosomal RNA component (hTR) that serves as a template for telomere repeat synthesis, a protein component known as hTERT (human telomerase reverse transcriptase) that is responsible for the enzymatic activity of telomerase enzyme and telomerase enzyme associated protein1 (TP1) with unclear function (Muller, 2002). Telomerase activity has been detected in the majority of malignant tumor specimens tested (Shay, 2005). The enzyme is undetectable in normal somatic cells; therefore, detection of telomerase activity in human tissue samples has value for the recognition of malignant cells in clinical specimens (Urquidi et al., 2000).

Several methods have been developed for the detection of telomerase activity. The telomeric repeats amplification protocol (TRAP) assay is the most widely used method for monitoring telomerase activity (Kim et al., 1994). The TRAP assay is highly sensitive and specific for telomerase activity in tumor samples but it has several practical limitations. One limitation is that it is time and labor intensive, another limitation is that because telomerase contains an RNA component, the activity is labile and easily destroyed by “RNAase”. (Wu et al., 2000) and the TRAP assay also offers no information at the cellular level (Sugino et al., 1997).

Expression of hTERT mRNA is very closely associated with telomerase activity in human tumors (Fan et al., 2005) and more recently quantitative measurement of hTERT mRNA using real-time quantitative RT-PCR which became a new, useful tool (Oh et al., 2006). However, this approach also does not offer any information at the level of the individual cell and so correlative comparison of molecular data with cellular morphology is not attainable. Therefore, immunohistochemical (IHC) methods of hTERT protein evaluation, which can both detect and localize cellular telomerase expression in human tissue, would be optimal for the differential diagnosis of cellular material (Khalbuss and Goodison, 2006). Improving the diagnostic criteria and the identification of new molecular markers is one of the most challenging goals for the early detection of schistosoma associated bladder cancer (SABC).

The aim of this work was assessment of telomerase activity by TRAP method and to detect, localize cellular telomerase expression (hTERT) by immunohistochemistry technique in tissue samples of bladder lesions and to correlate the results with clinicopathological parameters (histopathological grade and stages).

MATERIALS AND METHODS

This study enrolled 85 patients admitted to the urology department at Theodor Bilharz Research Institute (TBRI) Hospital during the period May, 2005 to May, 2007 with bladder lesions. Fifty nine (59/85) of them associated with schistosomiasis (schistosomal infestation was diagnosed by identifying the bilharzial ova in urine and tissue samples or detecting schistosomal antibodies in serum using ELISA technique). They included 75 males and 10 females, the patients’ age varied from 20 to 70 years with a mean age of 45.5 ± 6.21 years. Ten patients subjected to TUR prostate after taking their consent, served as normal controls. Tumor specimens were taken by cystoscopy (Transurethral resection biopsies, TUR) and cystectomy. Only cystoscopic biopsies containing muscle tissue were included, so that muscle invasion by the tumor could be assessed. The study protocol was approved by the institutional committee for the protection of human subjects and conformed to the guidelines of the 1975 Declaration of Helsinki. Patients were subjected to full clinical examination, routine laboratory investigations, complete urine analysis, abdominal & pelvic ultrasonography, general and abdominal examination, digital rectal examination (DRE), bimanual examination under anesthesia, plain x-ray of the urinary tract, intravenous urography (IVU), cystoscopy & transurethral resection (TUR) biopsies were taken from apparent growths.

Histopathological Study:

Tissues were fixed in 10% buffered formalin, paraffin embedded and processed routinely. Hematoxylin and Eosin stained slides were used to evaluate the pathological diagnosis of all bladder lesions, and to assess urothelial carcinoma cases for pathological grades and stages (Eble et al., 2004). Accordingly samples were classified into 6 groups. Group (1): chronic non-specific cystitis (5 cases), group (2): chronic schistosomal cystitis (10 cases), group (3): schistosomal cystitis with urothelial dysplasia (10 cases), group (4): non schistosomal associated urothelial carcinoma (21 cases), group (5): schistosomal associated urothelial carcinoma (24 cases) and group (6): schistosomal associated SQCC (15 cases).
Assessment of Telomerase Activity by TRAP:
Sections from each tissue specimens were homogenized with 200 μl of cold lysis buffer. After 30 minutes incubation on ice, the lysates were centrifuged at 100,000 Xg for 30 minutes at 4 C. The supernatant was carefully removed and its protein concentration was determined according to the method of Bradford, 1976. Approximately 10 μg total proteins were used from each lysates to quantify telomerase activity by the polymerase chain reaction (PCR) - based Telomeric repeat amplification protocol (TRAP) assay as described by Kim et al., 1994. The assay (Telo-TAGGG Telomerase PCR ELISA plus) kit was supplied by Roche (Roche Diagnostics, Mannheim, Germany). In this two step assay process, the telomerase in the sample adds telomeric repeats (TTAGGG) to the 3' end of the biotin labeled synthetic P1-TS primer. These elongation products, as well as the internal standard (IS) in the same reaction vessel, were amplified by PCR using the primers P1-TS and P2. The PCR products were split into two aliquots, denatured and hybridized to the streptavidin coated plates, with a digoxigenin (DIG)- labeled detection probes (P3T, P3IS for the sample and internal standard respectively) complementary to telomeric repeat sequence. The immobilized amplicons were detected using antidigoxigenin antibody conjugated to horseradish peroxidase (Anti-DIG–HRP) and the sensitive peroxidase substrate TMB (tetramethyl benzidine). The absorbance at 450 nm was determined. To confirm product specificity, a negative control was performed for each sample by heat inactivation of telomerase at 85°C for 10 min. The relative telomerase activity (RTA) in each sample was determined in relation to IS and the control (provided within the kit) readings using the formula provided by the manufacturers.

Immunohistochemistry of hTERT Antibody:
Five-μm bladder sections were collected from formalin-fixed, paraffin-blocks on microscopic slides coated with 3-amino propyl Triethoxy Silane (Sigma). The specificity of the antibody has been reported in previous reports (lezkowski et al., 2002). Antibody-mediated detection of hTERT was performed using the standard streptavidin-biotin peroxidase complex method. Following deparaffinization and rehydration, antigen retrieval was performed by microwaving in 10m M citrate buffer, pH 6.0. Non-specific antibody binding was prevented by pre-incubation with 100-μl blocking serum for 30 min at room temperature. Sections were incubated overnight with the primary antibody, anti-human telomerase reverse transcriptase (hTERT) monoclonal antibody (NCL-hTERT; Novacastra Laboratories Ltd, UK) at the optimal working dilution of 1:25. After thorough washings in blocking buffer, the bound antibodies were detected with biotinylated second antibody, followed by streptavidin alkaline phosphatase conjugate. Use substrate chromagen mixture (Histostain – SAP Kit, Alkaline phosphatase red, Zymed Lab-Sa system, USA) and sections were counterstained with Mayer's hematoxylin before mounting. Positive control was done using normal colon (hTERT-positive at the base of the crypts). Negative control slides included a blank control and omission of primary antibody. Positive and negative controls were stained in the same settings (Fullen et al., 2005).

Assessment of hTERT Immunostaining:
The hTERT stained slide was examined for immunostaining of hTERT as nuclear +_ nucleolar urothelial cell positivity (red color). Urothelial cells in entire sections were examined in ten consecutive fields under light microscopy at magnifications x 400 with the highest expression and the percentage was calculated from their mean. A negative staining was defined as the absence of cells expressing the marker (0 %) according to (Khalbuss and Goodison, 2006).

Statistical Analysis:
SPSS for windows, version 9.0 computer program was used for statistical analysis. Comparison between means of different groups was done using one way ANOVA. Comparison between percent of positive cases was calculated by Chi-square test. A "p" value of less than 0.05 was considered statistically significant. Pearson Correlation coefficient "r" was used to measure the relationship between two variables.

RESULTS AND DISCUSSION

Results:
Telomerase Activity by TRAP:
Application of the TRAP detection system to 85 cases with various bladder lesions displayed telomerase activity in 62.4% (53/85). Non malignant tissue samples were devoid of any telomerase activity. The investigation of 60 bladder carcinoma tissue samples (groups 4, 5, 6) led to the detection of telomerase activity in 48 cases (80%); Telomerase activity was detected in 15/ 21 (71.4%) in group 4, 18/24 (75%) in
group 5 and (100%) in SQCC group 6. There was no statistical difference between group 4 and group 5. Five cases showed positive telomerase activity out of 10 cases with schistosomal cystitis with dysplasia with a statistically significant difference compared to both control (p<0.001) and non schistosomal cystitis group (P<0.001). Moreover TRAP was 100% positive in SQCC compared to urothelial carcinoma group both non schistosomal associated (P<0.01) and schistosomal associated (P<0.01) (Table 1). In addition, TRAP activity was 100% positive in high grade urothelial carcinoma with Chi square P<0.05 compared to low grade urothelial carcinoma 50% and 92% positive in invasive tumors with Pearson Chi square test (P<0.01) compared to superficial tumors 50% (Table 2).

### Table 1: Telomerase TRAP and hTERT expression in tissue sections and distribution of positive cases in different studied bladder lesions.

<table>
<thead>
<tr>
<th>Histopathological diagnosis</th>
<th>Positive Telomerase TRAP cases</th>
<th>Positive hTERT in bladder tissue sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>Mean ± S.D</td>
</tr>
<tr>
<td>Control (10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 1(n=5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 2 (n=10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 3 (n=10)</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Group 4 (n=23)</td>
<td>15</td>
<td>(71.4)</td>
</tr>
<tr>
<td>Group 5 (n=24)</td>
<td>18</td>
<td>(75)</td>
</tr>
<tr>
<td>Group 6 (n=15)</td>
<td>15</td>
<td>(100)</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD


* Significant difference versus control group at p < 0.001.
* Significant difference versus group 1 at p < 0.001.
* Significant difference versus group 2 at p < 0.001.
* Significant difference versus group 6 at p < 0.05.
* Significant difference versus group 4 at p < 0.05.

Pearson Chi Square: * Significant difference of group 6 vs. group 4 or group 6 vs. group 5 at p < 0.01, ^ Significant difference of group 6 vs. group 4 at p < 0.05

### Table 2: Telomerase TRAP and hTERT expression in tissue and distribution of positive cases in different histopathological grades and stages of TCC.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Positive Telomerase TRAP cases</th>
<th>Telomerase TRAP ng/ml (cutoff=0.3)</th>
<th>Positive hTERT cases</th>
<th>hTERT in bladder tissue sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>Mean ± S.D</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Histopathological Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade urothelial carcinoma (24)</td>
<td>12</td>
<td>50</td>
<td>0.99±0.50</td>
<td>17</td>
</tr>
<tr>
<td>High Grade urothelial carcinoma C (21)</td>
<td>21</td>
<td>100*</td>
<td>2.14±0.30</td>
<td>21</td>
</tr>
</tbody>
</table>

* Significant difference versus low grade at p < 0.01.
* Significant difference versus superficial tumor at p < 0.01

Pearson Chi Square: * Significant difference of low vs. high grade tumors or superficial vs. Invasive tumor at p<0.05

### Immunohistochemical Studies of hTERT:

Expression of hTERT was clearly evident as nuclear staining ± nucleolar positivity in our cases, (Fig.1). In telomerase-positive tumors not all the tumor cells showed hTERT immunoreactivity. A significantly heterogeneous hTERT protein expression was observed in human tumor tissues. Chronic inflammatory cells as lymphocytes showed positive stain for hTERT protein. Localization of hTERT in tissue section of the 85 cases with various bladder lesions showed hTERT positivity in 65.9% (56/85). Non malignant tissue samples were devoid of any hTERT expression. The investigation of 60 bladder carcinoma tissue samples (groups 4, 5, 6) led to the detection of hTERT in 52 cases (86.3%); hTERT was detected in 80.9%, 87.5% and 93.3% in groups 4, 5, 6 respectively. Four cases showed positive telomerase activity out of 10 cases of schistosomal cystitis with dysplasia with a statistically significant difference compared to both control (p<0.001) and non schistosomal cystitis group (P<0.001). The highest positive telomerase cells expression was found in SQCC with statistically significant difference compared to non schistosomal urothelial carcinoma (p< 0.01) (Table 1).
Table 3: Correlation of Telomerase TRAP and hTERT expression to Schistosomal infestation

<table>
<thead>
<tr>
<th></th>
<th>R= Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TRAP # Schistosomal infestation</td>
<td>r= 0.433</td>
</tr>
<tr>
<td>2</td>
<td>hTERT expression # Schistosomal infestation</td>
<td>r= 0.708</td>
</tr>
<tr>
<td>3</td>
<td>hTERT expression # TRAP.</td>
<td>r=0.72</td>
</tr>
</tbody>
</table>

# = versus

Fig. 1: A control case showing negative expression of hTERT antibody (Immunostain Alkaline Phosphatase, x100)

In addition, hTERT protein was 100% positive in high grade urothelial carcinoma with Chi square P<0.05 compared to low grade urothelial carcinoma and 100% positive in invasive tumors with Pearson Chi square (P<0.05) compared to superficial tumors ((Table 2, Fig.2 & 3 & 4). The hTERT immunostaining in fixed tissues was concordant with telomerase activity by TRAP activity (Table 3).

Discussion:
Telomerase is an enzyme that can reconstitute the ends of chromosomes after cell division and thus circumvent the damage that occurs in normal adult somatic cells during successive mitotic cycles (Erdem et al., 2003).
In this study, telomerase activity detected by TRAP in tissue samples derived from bladder carcinomas of various tumor stages and degrees of differentiation was increased compared to samples of benign lesions and the control group. TRAP was positive in 80% of malignant samples (71.4%, 75%, and 100%) in groups 4, 5, 6 respectively. These results are in agreement with earlier studies of bladder tumors, Yoshida and Toge, 2004 and Eissa et al., 2007 and El Gendy et al., 2006 who reported the activity of the enzyme in more than 70% and 75% and 90% respectively of bladder cancer by TRAP method.

No TRAP activity was detected in either the control or cystitis groups. These results are in accordance with Dettlaff-Pokora et al., 2005. Contrary to our findings, low levels of telomerase activity was identified in a small number of cases with a histological normal urothelial tissue immediately adjacent to confirmed tumors. This was probably due to the presence of individual tumor cells or precursors that had escaped detection with conventional light microscopy. In addition, the pathological review of positive benign cases revealed that they
contained extensive lymphoid infiltrates with germinal centers and these cells were the source of TRAP activity (Asaad et al., 2006).

The absence of telomerase activity in some tumors may be due to the presence of a telomerase inhibitor. An inhibitory effect on telomerase activity has also been detected in other types of telomerase negative tumors including squamous cell carcinoma of larynx (Hohaus et al., 1996), colorectal and gastric tumors (Hiyama et al., 1995). Another explanation of some negative malignant cases may be due to the fact that some neoplasms have another mechanism for telomerase maintenance called ALT (alternative telomere maintenance). This is evidenced by the presence of elongated telomeres in the absence of telomerase activity (Reddel and Bryan, 2003). Another explanation is that some neoplasms may not require the reactivation of telomerase even when they are clinically detectable (Asaad et al., 2006).

The higher specificity of our results compared to those of Erdem et al., 2004 & Morsi et al. 2006 who both used urine samples could be attributed to a more specific tissue specimen used in this study. They both explained the false negative results obtained by the presence of gross hematuria or inflammation which was found to reduce the specificity of telomerase activity.

In this study, cases with schistosomal cystitis with dysplasia showed 50% positivity for TRAP activity with a statistically significant difference compared to both control and non schistosomal cystitis group (p<0.001). TRAP could therefore be an earlier marker of malignancy than are microscopic and macroscopic morphological changes in cells undergoing malignant transformation. Yoshida et al., 1997 also described 2 cases of dysplastic bladder with positive telomerase activity. The authors hypothesized that telomerase might be activated in the early stages of urological carcinogenesis as reported previously in colorectal carcinomas (Tahara et al., 1995). Hence TRAP activity could be a promising diagnostic marker in addition to other histological parameters.

Telomerase activity measured by TRAP method was higher in SQCC than urothelial carcinoma with statistical significant difference, which could be explained, by different biological behavior or different assay methods or SQCC is a malignant tumor with poorer prognosis and more aggressiveness. Telomerase activity measured by TRAP was also higher in urothelial carcinoma associated with schistosomiasis than those without infestation this is in agreement with previous reports by (El Gendy et al., 2006).

We found that the higher the tumor grade the higher the telomerase activity level, in this study, TRAP activity was statistically significantly increased in high grade urothelial carcinoma compared to low grade urothelial carcinoma (p<0.01), this is in agreement with the results reported by El Gendy et al., 2006 and was also significantly increased in invasive tumors compared to superficial tumors (p<0.01). This denotes that the significant value of TRAP in cancer bladder is not only based on its diagnostic validity but also on its poor prognostic impact in those patients and widening the range of application of this technique in future.

Our study has shown that through the application of a monoclonal antibody, hTERT can reliably be detected in paraffin-embedded tumor specimens. In agreement with the few previous reports describing hTERT protein localization, we found hTERT to be localized to the nucleolus (Mavrommatis et al., 2005) and nucleus (Yan et al., 2004), it is logical that hTERT is localized predominantly in the nucleus, the site of nucleoprotein complex assembly (Urquidi et al., 2000). We did not observed hTERT expression in all benign lesions; this is in agreement with the results of Khalbuss and Goodison, 2006. Our findings are in agreement with the previous telomerase-based studies in urological cancers, in that telomerase expression correlates with malignancy, but the use of immunohistochemistry adds specific advantages in the context of diagnosis. Although assays such as the TRAP assay or mRNA measurement can be designed to be semi or fully quantitative, they do not provide information regarding the source of the protein. Furthermore, the technical sensitivity of molecular assays may result in mislabeling samples which have only a few positive cells in a background of negative ones. With slide-based immunodetection, a single positive cell can be identified regardless of background content. These considerations are particularly relevant in samples where few cells may be present; the immunohistochemical detection of hTERT provides additional information upon which the observer can act.

In this study it was found four cases showing positive expression of telomerase out of 10 cases with schistosomal cystitis with dysplasia with a statistically significant difference compared to both control (p<0.001) and non schistosomal cystitis group (P<0.001). Also we detected 93.3% of SQCC and 87.5% of schistosomal associated urothelial carcinoma cases expressing hTERT in their nuclei, we observed that telomerase expression is increased in schistosomal associated bladder cancer cases than non schistosomal associated urothelial carcinoma but the difference did not reach the statistical significant level and this is in agreement with previous reports (Eissa et al., 2003), which could be explained, by different biological behavior or different assay methods or may be due to that schistosomiasis infested bladders frequently show squamous metaplasia and
dysplasia of the transitional epithelium and bladder SQCC is a malignant tumor with poorer prognosis and more aggressiveness than urothelial carcinoma (El Bolkainy et al., 1981, Kitinya et al., 1986). We found also that hTERT expression was positive in 100 % of high grade TCC with Chi square P<0.05 compared to low grade urothelial carcinoma. Also it was positive 100% in invasive tumors with Pearson Chi square test (P<0.05) compared to superficial tumors.

We conclude that hTERT protein can be detected by IHC in fixed human tissues, but the choice of the antibody, tissue processing, and reaction conditions are critical, the use of immunohistochemical detection of the telomerase component hTERT may significantly improve diagnostic accuracy. Further larger studies with more number of patients are still needed to determine its potential value for early detection and possible use as a therapeutic target. hTERT expression positively correlated with urothelial cell neoplasia. Beyond that it may also be a useful marker to identify bladder carcinoma or other lesions that would be amenable to therapies that would involve interference of tumor proliferation through telomerase inhibition.

We conclude that telomerase activity assessed by both TRAP and hTERT protein was higher in SQCC than urothelial carcinoma. Both methods may be used for the detection of telomerase activity in TRAP activity could be a promising diagnostic marker in addition to other histological parameters . The significant value of TRAP in cancer bladder is not only based on its diagnostic validity but also on its poor prognostic impact in those patients thus widening the range of application of this technique in future.

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