Original Article

Telomere instability in papillary bladder urothelial carcinomas: Comparison with grading and risk of recurrence

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ABSTRACT

Introduction: Shortening of telomere is associated with cellular senescence and cancer. This study aims to investigate the relationship between tumor grade and recurrence in relation to telomere length (TL), telomerase activity (TA) and telomere-binding proteins expression (TBPs) in patients with non-muscle invasive bladder cancer (NMIBC).

Materials and Methods: Tumor/healthy tissues were collected from 58 patients (35 with and 23 without NMIBC). Cystoscopy was performed at 3, 6 and 12 months to determine recurrence. Tumor grades and recurrence were correlated with TL, TA and TBPs using the Kruskal–Wallis non-parametric test. Results were considered significant at $P < 0.05$.

Results: Histological evaluation indicated 15 patients (42.9%) with high-grade (HG) and 20 patients (57.1%) with low-grade (LG) NMIBC. TL, TA and TBPs were found to be significantly different in tumors as compared with controls. A significant ($p < 0.05$) difference in the expression of TBPs was observed in the disease-free mucosa of cancer patients as compared with HG and LG tumors. In the follow-up, a total of 11 tumor recurrences were observed; among these eight recurrences were observed in patients with HG tumors and three in patients with LG tumors. TL, Human telomerase reverse transcriptase (hTERT) (that represents TA) and poly (ADP-ribose) polymerase 1 (PARP-1) in tumor samples and telomeric repeat binding factors TRF1, TRF2 and tankyrase (TANK) in normal mucosa obtained from the tumor group were respectively found to exhibit a positive and negative association with the risk of recurrence.

Conclusions: Our study demonstrates that TL, TA and TBPs are altered in tumors and non-cancerous mucosa in patients with papillary urothelial NMIBC. Further studies are warranted to identify their suitability as a potential biomarker.

Key words: Bladder cancer, non-muscle invasive bladder cancer, telomerase, telomere, telomere-binding proteins, urothelial carcinomas

INTRODUCTION

Telomeres are repetitions of short (six to eight base pairs) non-coding DNA sequences that extend for approximately 10-15 kb, located at the ends of chromosomes. Telomeres function as chromosomal "caps," preventing chromosome degradation and fusion with neighboring chromosomes during mitosis. Their three-dimensional structure (i.e. the telomeric T-loop) is stabilized by telomere-binding proteins (TBP), including telomeric repeat binding factors (TRF1 and TRF2), tankyrase (TANK) and poly (ADP-ribose) polymerase 1 (PARP-1). Each TBP binds to a specific nucleotide sequence and performs a specific role in mediating the function of the telomere.

In human somatic cells, telomeres are shortened by approximately five to 20 repetitions for each cell division. Successive telomere shortening is responsible for the onset of replicative senescence,[1] which is thought to counteract malignant transformation.[2,3] The passage of cells from replicative senescence to the stage of crisis is linked to a critical reduction in telomere length (TL). Crisis, characterized by chromosomal rearrangements and genome

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instability, results in the death of most cells. However, rare cells, with heightened telomere stability, can emerge immortalized from crisis.[4]

Telomerase activity (TA) is one of the main factors contributing to telomere stability. Telomerase is an enzyme consisting of two subunits, hTERC (the RNA component of telomerase) and hTERT (the catalytic portion of the enzyme). With its own RNA, telomerase functions as a reverse transcription and, through this action, is responsible for telomere maintenance and lengthening. Accordingly, cells with elevated TA are more likely to survive crisis and achieve immortalization. Even if other oncogenic events are required, cellular immortalization is considered a pre-requisite for malignant transformation, and it has been observed that over 80% of human cancers demonstrate persistent TA associated with critical dysfunction of TBP and the maintenance of stable telomeres.

The aim of our study was to assess whether similar mechanisms are associated with papillary urothelial bladder cancer (BC) and to determine if the molecular parameters (TL and expression of telomerase [hTERT], TRF1, TRF2, TANK and PARP-1) are related to tumor grade and risk of papillary urothelial BC recurrence.

MATERIALS AND METHODS

Patients

This study included 58 patients: An NMIBC group comprised of 35 patients and a control group of 23 patients, who visited our clinic between October 2009 and January 2011. Of the 35 patients in the NMIBC group, 12 were female (mean age 63.8 ± 7.7 years) and 23 were male (mean age 63.9 ± 12.0 years). The mean age of the NMIBC group was 63.9 ± 10.6 years. The control group included 23 patients (mean age 65.3 ± 10.1 years), seven females (mean age 64.0 ± 13.5 years) and 16 males (mean age 65.8 ± 8.8 years), selected according to the absence of risk factors for BC (positive familiar history or personal anamnesis for tobacco use).

Intervention and histology

The NMIBC patients underwent trans-urethral resection of tumors and cold-forceps biopsies of macroscopically unaffected bladder mucosa. The control group subjects underwent two to three endoscopic biopsies of the bladder mucosaduring endoscopic procedures performed for benign pathologies.

All the specimen from both groups were submitted for histological and molecular examination. Samples for the histological examination were formalin fixed, paraffin embedded and sliced into 4 μm sections stained with hematoxylin and eosin for evaluation using a light microscope. Microscopic examination was carried out by a pathologist who was unaware of the molecular findings, who diagnosed the presence of papillary urothelial BC and graded it according to the WHO/ISUP classification system[5] into low grade (LG) and high grade (HG). The depth of infiltration within the bladder wall, according to the TNM classification system,[6] was also assessed by histological examination. In detail, non-invasive papillary urothelial carcinomas were classified as pTa, while tumors invading the sub-epithelial connective tissue were considered as pT1. Samples collected for molecular analysis were frozen in the operating theater immediately following resection using liquid nitrogen and were then stored at -80°C until they were analyzed further.

In addition, patients in the NMIBC group received post-operative, intravesical immunotherapy or chemotherapy, depending on the results of the histological evaluation. Follow-up was conducted by cystoscopic examination every 3 months for 1 year.

All patients provided written informed consent to participate in the study; the study was granted ethical approval by an institutional review board.

Analysis of telomere length

Terminal restriction fragment (TRF) length measurements in tumor and normal samples were quantitatively determined by Southern blot using a Telo-TTAGGG TL assay kit (Roche Diagnostics, Milan, Italy) according to the manufacturer's instructions. Briefly, 2 μg sample of DNA was digested with restriction enzymes *Hin*fI/*Rsa*I; fractionated DNA fragments were electrophoresed on 0.8% agarose gel and were transferred to nylon membranes (Hybond-N +, Amersham, UK) by an alkaline transfer technique using capillary blotting, followed by hybridization with a $(TTAGGG)_4$ probe labeled with [α-^{32}P] ATP (Amersham) at the 5'. TL was detected using chemiluminescence. The intensity of the hybridization was evaluated by densitometric analysis with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Analysis of TBP and telomerase expression

The expression of TBP (TRF1, TRF2, PARP-1, TANK) and telomerase (hTERT) was investigated by Western blot analysis. Forty milligrams of frozen tissues were harvested by homogenization with a Potter homogenizer in a 15-volume ice cold triple protein lysis buffer. Total protein extraction was carried out using standard methods and protein concentrations were determined by the BCA assay (Sigma).

Twenty-five milligrams of total protein were separated by electrophoresis on SDS polyacrylamide gel (SDS-PAGE). The separated proteins were transferred onto a nitrocellulose membrane and incubated with mouse monoclonal TRF1, TRF2, PARP-1, TANK antibodies and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Following incubation with primary antibody, blots were incubated

with a peroxidase-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark) at room temperature for 1 h. Enhanced chemiluminescence reagents were used to visualize immunolabeling on a Kodak Biomax ML chemiluminescent film (ECL, Amersham Biosciences, Little Chalfont, Buckingamshire, UK).

Detection was performed by enhanced chemiluminescence (ECL) using a Western blotting luminol reagent (Tiangen Biotechnology, China) according to the manufacturer's instructions. Film data were analyzed using AlphaImager 2200 soft, compute gray value (see ß-actin as a reference).

Statistical analysis

Molecular parameters were compared among groups using the Kruskal–Wallis non-parametric tests as the assumptions of normality and homogeneity of variance were not met. Pair-wise, *post hoc* comparisons were conducted using two-tailed Mann–Whitney U-tests with Bonferroni-alfa correction. Results were considered significant at a *P* value less than 0.05 and were verified with the Monte Carlo method (with 10,000 samples). All calculations were performed using the R statistical language package version 2.13.

RESULTS

Histology

Histological examination found HG papillary urothelial carcinomas of the bladder in 15 of 35 patients (42.9%; five women and 10 men) and LG papillary urothelial carcinomas in 20 of the 35 NMIBC subjects (57.1%; seven women and 13 men). Ta-LG and three patients with T1-LG (single lesion with diameter <2 cm) did not undergo any intravesical adjuvant chemotherapeutic treatment. The remaining three T1-LG patients underwent adjuvant chemotherapy with 4-epirubicin at a dose of 50 mg. All patients with HG carcinoma underwent immunotherapy with BCG (Immucyst®) at a dose of 81 mg. The histological examination of samples from unaffected mucosa of cancer patients and from control group patients confirmed the absence of BC in all cases.

Molecular parameters

The mean TL and relative expression levels of TBP (TRF1, TRF2, TANK, PARP-1) and telomerase (hTERT) are summarized in Table 1. Tumor patients differed significantly from control group patients ($P < 0.05$) in all parameters [Figures 1-3]. Significant differences were also shown between HG and LG, with the exception of TRF2 expression. Despite having similar TL, disease-free mucosa of cancer patients differed significantly ($P < 0.05$) in TBP in the HG and LG tumors. Samples of unaffected mucosa from NMIBC patients differed significantly for all parameters including TL from the control group [Figure 4].

Recurrence

Follow-up examinations found recurrence of BC in 11 patients, eight with an original diagnosis of HG and three with LG carcinomas. Significant differences ($P < 0.05$) in TL, hTERT and PARP-1 values were found among patients with different risks of recurrence. The data are summarized in Table 2.

A significant negative correlation was found between risk of recurrence and TL, while a significant positive correlation was observed between recurrence and hTERT and PARP-1 expression [Figure 5]. However, a statistically significant difference between the risk of recurrence and the other analyzed factors (TRF1, TRF2, TANK) ($P > 0.05$) was not

Table 1: Histological results and mean values of the analyzed parameters in both groups (tumor group and control group)

	Tumor group		Control group
	HG	LG	
Ta	*n*=8 (22.9%)	*n*=14 (40%)	
	3 ♀	5 ♀	
	5 ♂	9 ♂	
T1	*n*=7 (20%)	*n*=6 (17.1%)	
	2 ♀	2 ♀	
	5 ♂	4 ♂	
TL (KB)	6.23±0.26	7.28±0.35	9.21±0.24
TRF1 (A.U.)	0.19±0.09	0.31±0.07	8.20±0.87
TRF2 (A.U.)	0.17±0.04	0.39±0.06	2.24±0.43
hTERT (A.U.)	14.82±0.99	8.58±0.50	0.25±0.16
TANK (A.U.)	10.79±1.5	4.46±0.66	1.26±0.33
PARP-1 (A.U.)	17.37±0.74	12.05±0.70	0.97±0.30

KB = Kilo-bases, A.U. = Arbitrary units, TL = Telomere length, TRF1 = Telomeric repeat binding factors, TA = Telomerase activity, hTERT = Human telomerase reverse transcriptase, TANK = Tankyrase, PARP = poly (ADP-ribose) polymerase 1

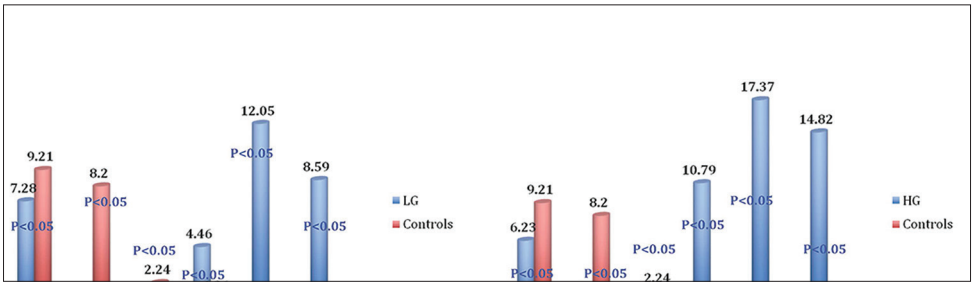

Figure 1: Comparison of molecular parameters between the cancer and the control groups

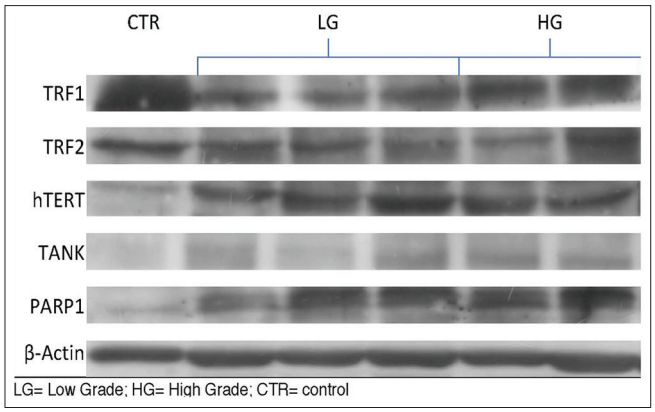

Figure 2: Analysis of telomere-binding proteins and telomerase expression investigated by Western blotting

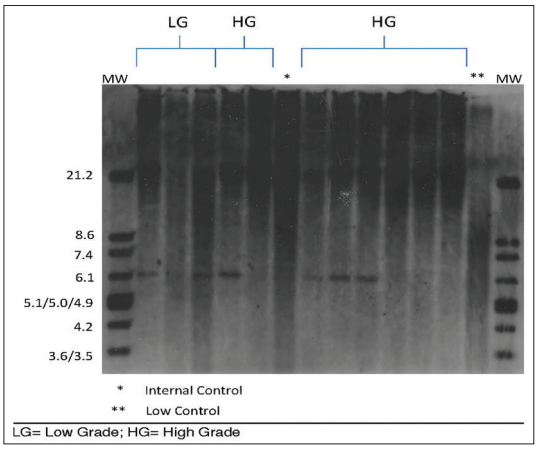

Figure 3: Analysis of telomere length determined by Southern blot

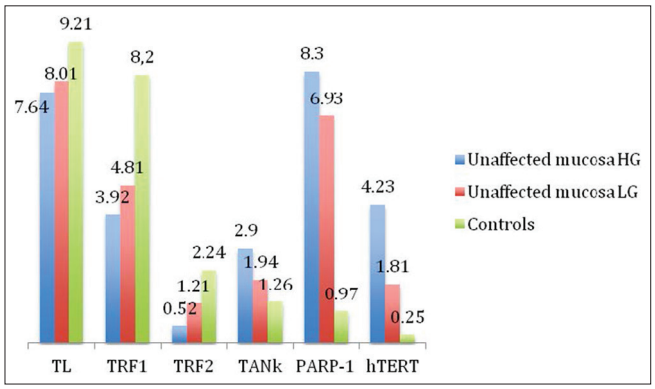

Figure 4: Comparison unaffected mucosa high grade/unaffected mucosa low grade/controls

observed [Table 2]. Finally, we assessed the risk of recurrence at 3, 6 and 12 months in relation to the molecular characteristics of the normal mucosa samples from NMIBC patients. In this case, significant differences were observed only for TRF1 and TRF2 ($P < 0.05$) [Table 3]. In contrast, biopsies from the unaffected mucosa of cancer patients differed from control subjects for all parameters analyzed ($P < 0.05$).

DISCUSSION

Almost 25% of the newly diagnosed bladder cancer patients present with muscle-invasive cancers.[7] More than 70% of

Table 2: Correlation between recurrence and analyzed parameters in the tumor groups

Months	N° pts	Mean value	*P*
TL			
3	2	5.85±0.07 KB	<0.002
6	5	6.26±0.37 KB	
12	4	6.67±0.37 KB	
TRF1			
3	2	0.14±0.49 A.U.	>0.560
6	5	0.25±0.10 A.U.	
12	4	0.17±0.07 A.U.	
TRF2			
3	2	0.14±0.00 A.U.	>0.088
6	5	0.21±0.11 A.U.	
12	4	0.33±0.17 A.U.	
hTERT			
3	2	15.35±0.35 A.U.	<0.05
6	5	13.63±3.33 A.U.	
12	4	11.18±3.58 A.U.	
TANK			
3	2	9.67±0.31 A.U.	>0.403
6	5	8.68±2.66 A.U.	
12	4	7.72±4.09 A.U.	
PARP-1			
3	2	17.50±0.14 A.U.	<0.012
6	5	16.94±2.40 A.U.	
12	4	15.05±3.46 A.U.	

N° pts = Number of patients, KB = Kilo-bases, A.U. = Arbitrary units.
>0.05 = Not statistical significant

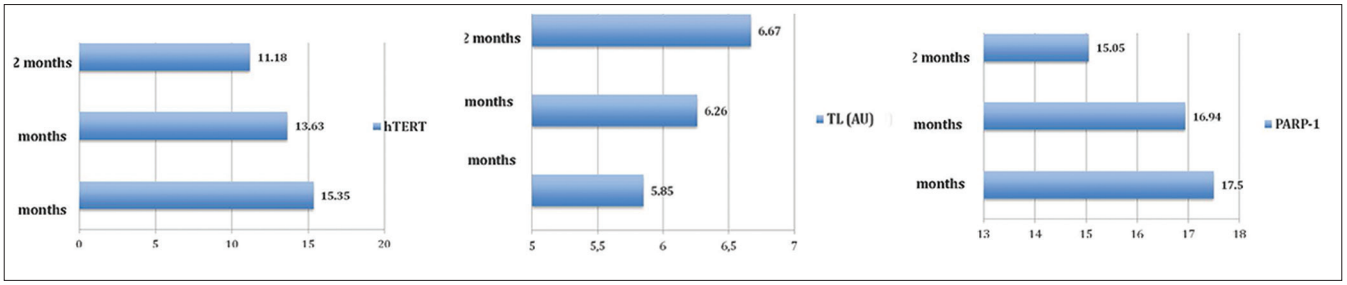

Figure 5: Mean telomere length, hTERT and polymerase 1 values and risk of recurrence

Table 3: Risk of recurrence in relation to the molecular characteristics of the samples of normal mucosa of patients affected by bladder cancer

Month	N° pts	Mean value	*P*
TL			
3	2	7.35±0.77 KB	>0.678
6	5	7.92±0.61 KB	
12	4	6.37±2.91 KB	
TRF1			
3	2	3.85±0.55 A.U.	<0.011
6	5	3.48±0.89 A.U.	
12	4	4.68±0.76 A.U.	
TRF2			
3	2	0.50±0.16 A.U.	<0.027
6	5	0.61±0.27 A.U.	
12	4	0.86±0.33 A.U.	
hTERT			
3	2	4.23±0.28 A.U.	>0.137
6	5	3.62±1.06 A.U.	
12	4	2.80±1.16 A.U.	
TANK			
3	2	2.95±1.01 A.U.	>0.303
6	5	2.70±0.44 A.U.	
12	4	2.32±0.54 A.U.	
PARP-1			
3	2	8.95±1.76 A.U.	>0.158
6	5	7.72±0.90 A.U.	
12	4	8.32±0.87 A.U.	

N° pts = Number of patients, KB = Kilo-bases, A.U. = Arbitrary units. >0.05 = Not statistical significant. <0.05 = Statistical significant

urothelial BC recur after resection, and about 15% of them progress to invasive stages. The causes of BC recurrence are not clearly known, but the multiple foci of recurrence, common in BC cases, suggest widespread instability of the urothelium. The identification of prognostic markers, to serve as predictors of progression risk and/or recurrence in patients with superficial BC, is desirable.

Telomeric abnormalities are frequently observed during carcinogenesis and, in particular, their shortening seems to be a common early event in several human cancers.[8,9] After cancer initiation, most human tumors stabilize their telomeres through either activation of telomerase or, rarely, through a different mechanism - Alternative Lengthening of Telomeres (ALT).[9,10] In this study, we investigated the relationships between potential molecular markers of BC (TL, TA and TBP expression) and BC grade and recurrence. Our results indicate that several molecular parameters may serve as predictors of BC recurrence and also indicate widespread instability of the urothelium.

We found significant reductions in TL in samples from both HG and LG cancer patients, relative to the normal urothelium of patients without BC and without cancer-related risk factors. However, telomeres in HG tumors samples were shorter that those in LG carcinomas. We also observed significantly higher TA (hTERT expression) among BC patients and between HG and LG carcinomas. These findings confirm that telomeres play a role in carcinogenesis and indicate that TA likely stabilizes telomere length and promotes the neoplastic immortalization of bladder carcinoma cells.

We also observed differences in TBP expression between cancer and control groups. Expression of TRF1 and TRF2 was significantly lower, and the expression of TANK and PARP-1 was significantly higher in NMIBC patients. Statistical analysis also confirmed significant differences in TBP expression between tumor grades, with a lower expression of TRF1 and higher expression of PARP-1 and TANK in HG tumor samples as compared with LG carcinomas. TRF2 expression was also lower in HG than in LG patients, but this difference was not statistically significant. These results suggest the involvement of TBP in bladder carcinoma. Low levels of TRF1 and TRF2 would support the activity of telomerase by destabilizing the telomeric T-loop and allowing telomerase access to the telomere. Meanwhile, high levels of TANK and PARP-1 likely facilitate TA indirectly through inhibition of TRF1. The lower expression of TRF1 and elevated expression of TANK and PARP-1 indicate that this mechanism is responsible for elevated TA observed in our bladder carcinoma patients.

In the literature, there are rather discordant results about the correlation between TA and grade or stage of BC. Lin *et al*.[11] observed a clear association between telomerase expression and grade and stage of disease. Similarly, De Kok *et al*.[12] showed that levels of hTERT expression are positively correlated with stage and degree of disease in urothelial cancer cells. In contrast, another study reported positive TA in 91.6% of LG/stage and 88.6% of HG/stage urothelial tumors.[13] Okumura *et al*.[14] also showed a significant reduction in TA in HG/stage BC. The authors observed TA in 83.3% of superficial tumors and 42.1% of invasive forms, with 83.3%, 66.7% and 40% observed among grades G1, G2 and G3, respectively. One possible explanation for these discrepancies could be the fact that immortalization of some cancers, especially the undifferentiated forms, is independent of TA. For example, Bryan *et al*.[15] found that of the 35 immortalized cell lines, 20 were positive and 15 were negative for telomerase, yet telomerase-negative cell lines had long and heterogeneous telomeres, indicating that TL maintenance was dependent on additional mechanisms.

Although discrepancy exists regarding the correlation between TA and tumor grade, studies focused on the TA in urine of patients with BC demonstrate its diagnostic potential.[16] Recently, Sanchini *et al.* evaluated TA in the urine of 134 patients affected by BC and in 84 healthy

individuals. Test sensitivity and specificity were 90% and 88%, respectively. The test therefore demonstrated the same predictive ability as was observed in patients affected by LG tumors.[17] Similar results were found in other studies, which stressed the potential of the test, even in superficial and LG carcinomas.[18-20]

Many studies in the literature have focused more on the activity of telomerase than on TL, but studies of the relationship between TL and tumor grade have produced mixed results. In 1996, Kamata *et al.*[21] studied tumor samples from 21 patients using the adjacent, apparently healthy urothelium as control. The authors reported a decrease in TL in cancer samples as compared with unaffected urothelium, but did not find any correlation with TA; superficial cancers (Ta/T1) had significantly shorter telomeres compared with invasive carcinomas. Fernandez-Gomez *et al.*,[22] using cell samples obtained from wash-out in patients affected by BC observed that more aggressive tumors displayed increased TL. TL was found to be higher in aneuploid than diploid tumors, but the authors also recognized the presence of some bias in their study, such as the lack of assessment of T1G3 tumors. In 2007, McGrath *et al.* demonstrated a significant difference in relative TL between men and women; women had greater relative TL than men.[23] At birth, this difference was undetectable, but it manifested in adulthood,[24] probably as a result of exposure to oxidative stress as well as cigarette smoking.[25]

Our results revealed a significant correlation between tumor grade and the risk of tumor recurrence. Moreover, the precocity of recurrence was higher in patients who had shorter telomeres and higher values of hTERT and PARP-1. Significant correlations between time to recurrence and the other TBP (TRF1, TRF2, TANK) were not observed.

All of the molecular parameters in samples of unaffected mucosa from NMIBC patients differed significantly from the control samples. This supports the notion that BC is not exclusively a disease of the tumor site but can be considered a disease of the entire urothelium, and confirms the potential for multifocal recurrence, common in cases of bladder cancer. Similarly, Leuenroyh *et al.*[26] observed a clear association between hTERT expression, in mucosal biopsies of tissue adjacent to the primary tumor, and the risk of recurrence; the non-statistical significance that was shown may be attributed to the paucity of the analyzed samples.

CONCLUSIONS

The results of this study affirm that telomere length likely plays a crucial role in the multi-step process of urothelial cell carcinogenesis. Telomeric instability appears to result from TBP dysfunction, evidenced by an up-regulation of TANK and PARP-1 expression and decrease in TRF-1. These changes likely destabilize the telomeric T-loop facilitating TA and telomere lengthening. Our results - significant differences in TA and TBP levels in unaffected mucosa of NMIBC patients relative to controls - also demonstrate widespread instability of the urothelium and offer an explanation for the high incidence of multifocal recurrence associated with bladder carcinoma. Changes in TA and TBP expression appear to be early events in urothelial carcinogenesis and should be considered diagnostic markers of bladder cancer. Further studies with larger sample size are still necessary before solid conclusions can be drawn.

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