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Nuclear hTERT Immunohistochemical Expression is Associated with Survival of Patients with Urothelial Bladder Cancer

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Abstract. *Background: The role of telomere in tumorigenesis is complex. While telomerase activation is suggested to be necessary for tumor growth, it may also help in diminishing genetic instability. The expressions of the telomerase reverse transcriptase/hTERT and the telomerase associated protein-1/hTEP-1 were investigated in relation to clinicopathological parameters and various proliferative and apoptotic biological markers. Materials and Methods: The immunohistochemical method ABC/HRP was performed on paraffin sections of 132 patients with urothelial bladder carcinomas to detect the proteins hTERT, hTEP-1, Ki-67, bcl-2, p53 and caspase-3. Results: The hTEP-1 protein was localized in the cytoplasm of cancerous cells (56.6%), while the hTERT protein was detected in the nuclei and the cytoplasm of cancerous cells (57.6% and 45.5%, respectively). hTEP-1 demonstrated an association with lower stage of the disease (p=0.036), as well as both nuclear and cytoplasmic hTERT (p=0.018 and p=0.0001, respectively). Cytoplasmic hTERT showed inverse correlation with the mutant p53 protein (p=0.047), while both cytoplasmic hTERT and hTEP-1 demonstrated parallel correlation with caspase-3 (p=0.004 and p=0.048, respectively). Nuclear hTERT associated with improved overall survival in multivariate analysis (p=0.007). Conclusion: The association of the hTERT protein with low stage urothelial carcinomas and improved patients' survival is in keeping with the idea that the early activation of telomerase may protect against genetic instability and the prevalence of aggressive malignant clones.*

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Telomeres are distinctive DNA-protein structures that cap the ends of linear chromosomes, enabling cells to distinguish chromosome ends from double-strand breaks in the genome (1-3). Uncapped chromosome ends are at great risk of degradation, recombination or fusion, leading to loss of genetic information, rearrangements and genetic instability (1-3). Human telomeres in somatic cells undergo progressive shortening with each cell division and critically short telomeres trigger either replicative senescence or apoptosis (4, 5). Telomerase is a large ribonucleoprotein complex that stabilizes and extends telomeres of eukaryotic chromosomes, and thereby regulates cell replicative potential and lifespan (6). Human telomerase is composed of an RNA subunit and several protein components (7-9). The RNA subunit (hTR) contains the template for telomeric DNA addition and is essential for telomerase activity (7). The protein components include the catalyst telomerase reverse transcriptase (hTERT) and telomerase-associated protein 1 (hTEP-1) (8, 9). The hTERT gene codes for a 127 kDa protein that is concomitantly expressed with the activation of telomerase during cellular immortalization (8). In contrast, hTR and hTEP-1 expressions do not correlate with telomerase activity in cells and tissues (10). Binding to telomerase RNA, hTEP-1 probably serves not only as a structural protein, but also as a regulatory subunit in mediating telomerase interaction with other molecules (9). Moreover, for hTEP-1, a "helper role" in telomerase activity regulation has been proposed, and recently hTEP-1 was found to be of phospho-proteinaceous nature, with protein kinase C alpha-mediated phosphorylation as a prerequisite for the activation of telomerase (11).

The activation of telomerase is associated with the proliferation of cells which are required to undergo repeated clonal expansion such as embryonic cells during development and bone marrow blood cell progenitors, whereas inhibition of telomerase is implicated in cellular senescence, apoptosis, premature aging and degenerative disease (2, 12). Furthermore, it is believed that telomerase reactivation is a critical step in allowing a tumor cell

population to avoid critical telomere shortening and apoptosis *in vivo (*4), which has been confirmed by surveys of human tumor samples showing that over 90% of cancers are telomerase-positive, whereas nearly all normal or benign tumor tissues are negative (2, 13).

In human cancers, telomerase has mainly been investigated through the TRAP assay, while a very few immunohistochemical studies have been performed (14-16). hTERT protein expression is, however, considered as the immunohistochemical equivalent of the TRAP assay for the determination of telomerase activity, offering in addition the ability to detect the cellular source of telomerase activity and the exact localization of telomerase expression (14, 17). In view of the above, we investigated immunohistochemically, hTERT and hTEP-1 protein expressions in 132 urothelial bladder carcinomas, in relation to clinicopathological parameters (age, gender, histological grade, stage) and patients' survival. Moreover, we investigated hTERT and hTEP-1 expressions in relation to markers of cellular proliferation (Ki-67) and apoptosis (bcl-2, p53, caspase-3), since telomerase is considered to be the critical enzyme in overcoming growth limitations due to telomere dysfunction and in achieving cell immortalization.

Materials and Methods

Patients and tumor specimens. Specimens were obtained from 132 patients with urothelial bladder carcinomas. The age ranged from 31 to 89 years (mean age: 70.08 years) and the male/female ratio was 113/19. Transurethral biopsy was performed in all the patients. None of the patients had received prior chemotherapy, intravesical instillation therapy or radiation. Tα and T1 tumors were treated with transurethral resection and intravesical instillations of either bacillus Calmette-Guerin or epirubicin, while for muscle invasive carcinomas, cystectomy, radiation or systemic chemotherapy was used according to our clinical protocols.

Transitional cell carcinomas were graded histologically according to the criteria of the Ancona 2001 Refinement of the WHO 1973 classification (18): 25 carcinomas were classified as grade I, 43 as grade II and 64 as grade III (Table I). Tumor staging was performed based on the results of excreting pyelography, transurethral resection, cystectomy, bimanual palpation under anesthesia, computed tomography and ultrasonography and was assessed according to the criteria of the International Union Against Cancer Staging System (19): Twenty-six tumors were non invasive papillary carcinomas (Ta), 50 carcinomas invaded the subepithelial connective tissue (T_1), while the remaining 56 tumors were muscle invasive (27 T_2, 22 T_3, 7 T_4) urothelial carcinomas (Table I).

Survival analysis was based on data available from 131 patients. The follow-up of patients ranged from 6 to 145 months, with a mean follow-up of 48.13 months (interquartile range: 16 months - 77 months). Forty-nine out of 131 patients died from the disease (cause-specific deaths) during the observation period.

Immunohistochemistry. For the detection of hTEP-1, hTERT proteins, as well as for the coestimated proteins Ki-67, p53, bcl-2 and caspase-3, the avidin-biotin immunoperoxidase method (ABC-HRP) was performed on routinely processed formalin-fixed, paraffin-embedded 4-μm-thick sections, as previously described (20). A polyclonal antibody against hTERT [goat polyclonal IgG, SC-7215 (Santa Cruz Biotechnology Inc., CA, USA)] was used at a dilution of 1:60. This antibody is specific for hTERT and reacts with an epitope mapping at the carboxy terminus of telomerase reverse transcriptase of human origin. For the detection of hTEP-1, a polyclonal antibody [goat polyclonal IgG, SC-6370 (Santa Cruz Biotechnology Inc.)], which reacts with an epitope mapping at the carboxy terminus of hTEP-1 of human origin, was used at a dilution of 1:60. For better hTERT antigen retrieval, sections were pretreated in an autoclave for 20 min at 126˚C.

The other immunomarkers assessed in this study in combination with hTERT and TEP-1 (Ki-67, p53, bcl-2 and caspase-3) have also been detected immunohistochemically with the following antibodies: i) rabbit anti-human Ki-67 (Dako, Glostrup, Denmark) at a dilution of 1:100; ii) anti-p53 clone BP53.12.1 (Oncogene, Cambridge, USA) at a dilution of 1:50; iii) anti-bcl-2 clone 124 (Dako) at a dilution of 1:100; iv) a goat polyclonal antibody for caspase-3 (Santa Cruz Biotechnology Inc.), raised against a peptide mapping at the amino acid terminus of the p20 subunit of caspase-3 and its precursor of human origin, at a dilution of 1:80. Positive controls included bladder cancer tissue with known immunoreactivity for the above markers, while negative controls had the primary antibodies omitted and replaced by PBS.

Evaluation of immunohistochemistry. A semi-quantitative evaluation based on the percentage of immunoreactive cells and staining intensity was carried out by three independent observers and was scored using a scale of 0 to 1, as follows: score 0: <10% positive cells per 10 high-power fields (HPF) (X400) with weak immunoreactivity, score 1: ≥10% positive cells per 10 HPF (X400) with moderate or intense immunoreactivity. Cell immunoreactivity in cases with <10% positive cells was too weak and, thus, these cases were considered as negative. For hTERT, a separate evaluation was performed for nuclear and cytoplasmic expression.

The extent of Ki-67 expression was evaluated using a score of 0 to 3, as follows: score 0: <10% positive cells per 10 HPF (X400); score 1: 11-30% positive cells; score 2: 31-50% positive cells; and score 3: >50% positive cells, as previously reported (21).

Bcl-2 expression was scored as negative (score 0) if less than 10% of tumor cells were positive, slightly positive (score 1) if 10-50% of tumor cells were positive and as strongly positive (score 2) if more than 50% of neoplastic cells showed cytoplasmic staining, as previously reported (21).

The fraction of p53-positive-stained nuclei was scored on a scale of 0-3, as follows: 0 (negative): <10% of positive tumor nuclei; 1: 10-25% positive tumor nuclei; 2: 26-50%; and 3: >50% of positive tumor nuclei, as previously described (22).

Finally, the caspase-3 expression was scored as negative if less than 20% of the tumor cells were found positive per 10 HPF (X400) and as positive if ≥20% of the tumor cells per 10 HPF (X400) were positive, as previously described (23).

Statistical analysis. Pearson's Chi-square test with continuity correction and *t*-test were employed in order to assess correlations of nuclear or cytoplasmic hTERT as well as of hTEP-1 with the clinicopathological parameters of bladder carcinomas and the immunohistochemical expressions of Ki-67, p53, bcl-2 and caspase-3. The interrelations between cytoplasmic and nuclear hTERT expressions were investigated through the McNeamar's test. The

Table I. *hTERT (nuclear or cytoplasmic) and hTEP-1 protein expressions in relation to clinicopathological parameters and immunohistochemical expression of p53, Ki-67, bcl-2 and caspase-3 (CPP32) proteins.*

			Nuclear hTERT ≥10%			Cytoplasmic hTERT ≥10%			hTEP-1 ≥10%			
		Total	N	%	*p*	N	%	*p*	Total	N	%	*p*
Gender	Male	113	66	58.4	NS	54	47.8	NS	108	59	54.6	NS
	Female	19	10	52.6		6	31.6		19	11	57.9	
Grade	1	25	16	64.0	NS	13	52.0	NS	25	15	60.0	NS
	2	43	24	55.8		21	48.8		40	25	62.5	
	3	64	36	56.3		26	40.6		62	30	48.4	
Stage	Tα	26	22	84.6	**0.018**	5	19.2	**<0.0001**	28	14	50.0	**0.033**
	T1	50	25	50.0		32	64.0		49	32	72.7	
	T2	27	13	48.1		15	55.6		27	12	44.4	
	T3-T4	29	16	55.2		8	27.6		28	70	42.9	
p53	Neg	60	32	53.3	0.709	33	55.0	**0.047**	54	35	64.9	NS
	Pos	67	39	58.2		24	35.8		66	32	48.5	
Ki-67	Neg	75	44	58.7	0.568	30	40.0	0.251	75	43	57.3	NS
	Pos	52	27	51.9		27	51.9		45	24	53.3	
bcl-2	Neg	74	44	59.5	0.283	31	41.9	0.255	66	33	50.0	NS
	Pos	50	24	48.0		26	52.0		49	30	61.2	
CPP32	<20%	42	14	33.3	0.106	14	33.3	**0.004**	46	20	43.5	**0.048**
	≥20%	35	19	54.3		24	68.6		38	27	71.1	

Neg=Negative, Pos=Positive

effect of nuclear or cytoplasmic hTERT and hTEP-1 expressions on postoperative survival rates was assessed by univariate analysis (log-rank test) and multivariate analysis using Cox's proportional hazards regression model. A p value of <0.05 was considered as statistically significant.

Results

hTEP-1 protein expression was observed in the cytoplasm of cancerous cells with a positive reaction in 56/99 cases (56.6%) (Figure 1). hTERT protein was localized in the nuclei of cancerous cells with a positive reaction in 76/132 cases (57.6%) (Figure 2). A specific granular cytoplasmic localization was also observed for hTERT in 60/132 cases (45.5%) (Figures 3, 4).

As far as interrelations among the different components of human telomerase are concerned, a statistically significant correlation was observed between cytoplasmic hTERT and hTEP-1 protein expressions ($p=0.008$, $\chi^2=7.082$, df=1), as well as between cytoplasmic and nuclear hTERT expressions (McNeamar Test: $p=0.093$).

No associations were found between the different components of human telomerase and the gender or age of patients. A statistically significant association was observed between hTEP-1 protein expression and the stage of urothelial carcinomas, in that loss of hTEP-1 expression was seen in high stage carcinomas ($p=0.033$) (Table I, Figure 5). Neither hTERT (nuclear or cytoplasmic), nor hTEP-1 was associated with the histological grade of the carcinomas (Table I). On the other hand, both nuclear and cytoplasmic hTERT expressions were significantly associated with the stage of the urothelial carcinomas ($p=0.018$, $\chi^2=10.008$, df=3 and $p=0.0001$, $\chi^2=18.993$, df=3, respectively), in that loss of nuclear or cytoplasmic hTERT was seen in advanced stages (Table I, Figure 5). Moreover, statistically significant inverse association was observed between cytoplasmic, but not nuclear, hTERT and p53 protein expressions ($p=0.047$, $\chi^2=3.963$, df=1) (Table I, Figure 5), whereas no relationship was found between hTEP-1 and p53 (Table I). Significant parallel associations were observed between hTEP-1 and caspase-3, as well as between cytoplasmic hTERT and caspase-3 expressions ($p=0.048$, $\chi^2=3.023$, df=1 and $p=0.004$, $\chi^2=8.126$, df=1, respectively) (Table I, Figure 5). No significant correlations were found between hTEP-1 or hTERT and the Ki-67 proliferation index or the anti-apoptotic bcl-2 protein (Table I). The multivariate survival analysis (Cox's regression model) revealed an independent favorable impact of nuclear hTERT on patients' survival ($p=0.007$), along with histological grade ($p=0.043$) and stage of the disease ($p=0.0001$) (Table II).

Discussion

In the present study, the telomerase catalytic subunit/hTERT and the telomerase-associated protein-1/hTEP-1, were investigated for the first time by means of immuno-

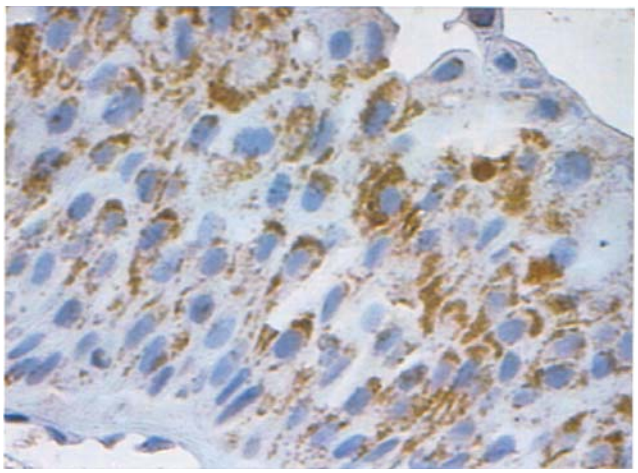

Figure 1. *Cytoplasmic granular hTEP-1 protein expression in a well-differentiated urothelial carcinoma (ABC-HRP X400).*

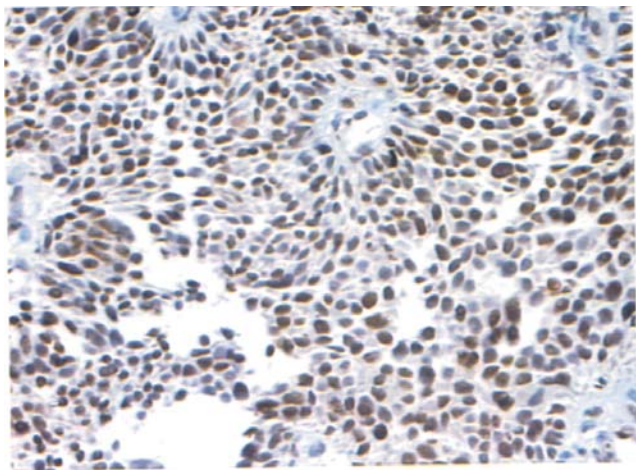

Figure 2. *Nuclear hTERT protein expression in many nuclei of a well-differentiated urothelial carcinoma (ABC-HRP X250).*

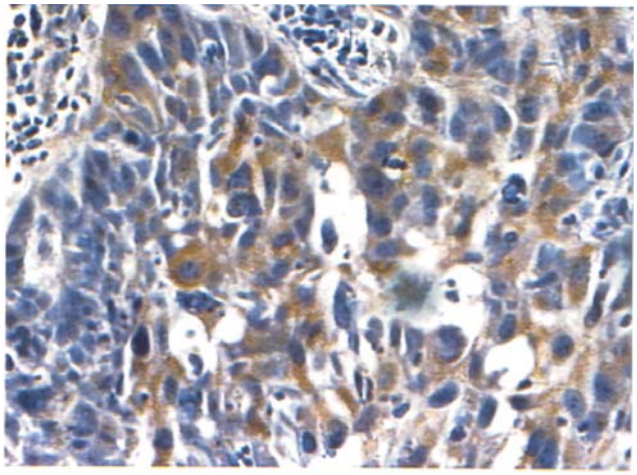

Figure 3. *Cytoplasmic granular hTERT protein expression in many cells of a high-grade urothelial carcinoma (ABC-HRP X400).*

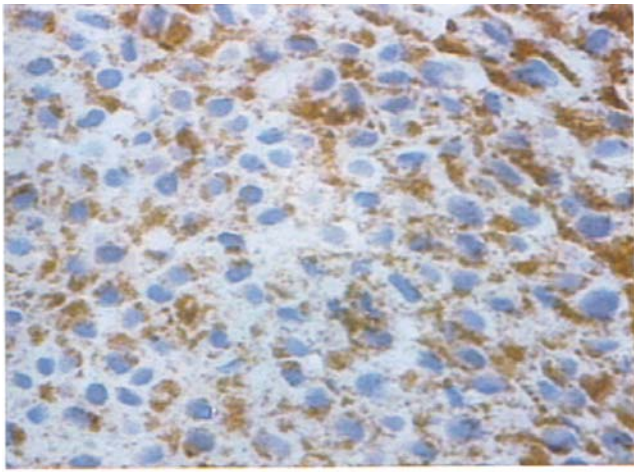

Figure 4. *Cytoplasmic granular hTERT protein expression in many cells of a well-differentiated urothelial carcinoma (ABC-HRP X400).*

histochemistry in urothelial bladder carcinomas. hTEP-1 protein expression was observed in the cytoplasm of cancerous cells. For hTERT, a nuclear localization was found as well as a specific cytoplasmic granular staining. Although very few immunohistochemical studies have been performed for the detection of hTERT, a granular cytoplasmic staining has also been reported previously, in addition to its nuclear localization, in vulvar intraepithelial neoplasia and lung cancer (15, 16). This cytoplasmic localization of hTERT is of unclear significance. However, a potentially interesting mechanism of telomerase regulation through hTERT phosphorylation linked to its nuclear localization has recently been reported in human T-lymphocytes (24). During $CD4^+$ T cell activation, hTERT is phosphorylated and translocated from the cytoplasm to the nucleus (24). Thus, the nuclear translocation of telomerase, from a presumably non-functional cytosolic location to a physiologically relevant nuclear compartment, may be one regulatory mechanism of telomerase function in cells (5).

In this study, apart from urothelial carcinoma cells, cells from dysplastic lesions, as well as apparently normal urothelial cells adjacent to carcinomas, displayed occasional hTERT positivity, which is in keeping with previous immunohistochemical observations in normal colonic and vulvar mucosa and VIN lesions (14, 15).

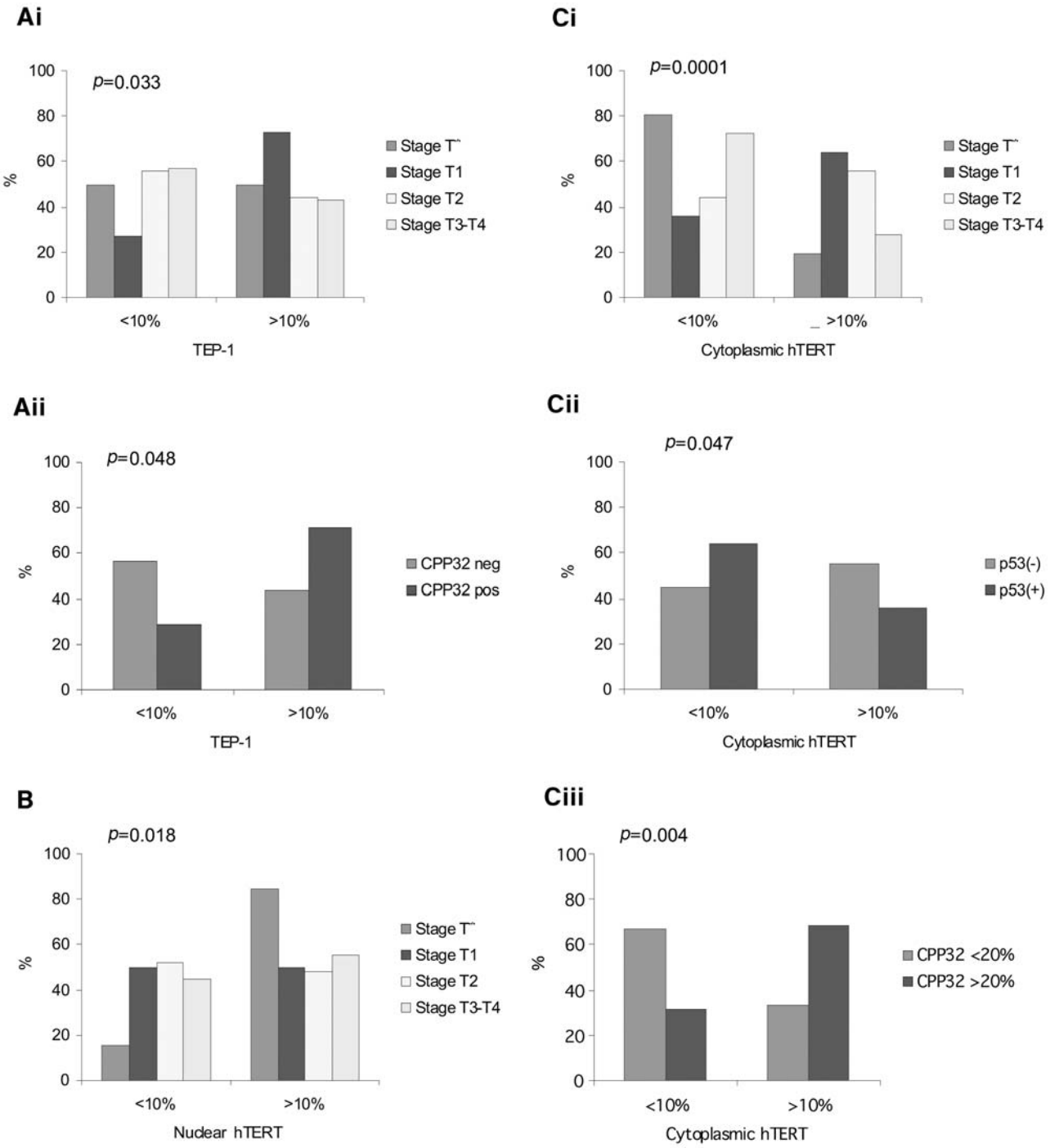

Figure 5. *Schematic representation of (A) hTEP-1 protein expression in relation with stage (Ai) and caspase-3 (CPP-32) expression (Aii), (B) nuclear hTERT expression in relation with stage and (C) cytoplasmic hTERT expression in relation with stage (Ci), p53 expression (Cii) and caspase-3 (CPP-32) expression (Ciii).*

In our study, positive hTERT nuclear and cytoplasmic expressions associated significantly with carcinomas of lower stages ($p=0.018$ and $p=0.0001$, respectively), suggesting that telomerase activation is a rather early event during urothelial cancer progression. Positive hTEP-1 expression was also significantly associated with carcinomas of less advanced stages ($p=0.036$). In the literature, there is confusion regarding the points at which telomerase is activated during

Table II. *Multivariate Cox's proportional hazards regression model on overall survival.*

		Variables in the equation							
								95% CI for Exp(B)	
		B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 3	Stage	1.351	0.265	26.002	1	0.000	3.862	2.298	6.493
	Grade	0.804	0.397	4.099	1	0.043	2.234	1.026	4.864
	Nuclear hTERT	-1.001	0.374	7.159	1	0.007	0.367	0.176	0.765

		Variables not in the equation[a]		
		Score	df	Sig.
Step 3	p53	0.066	1	0.797
	Ki-67	1.869	1	0.172
	bcl-2	0.825	1	0.364
	Gender	0.263	1	0.608
	Age	1.505	1	0.220
	hTEP1	0.024	1	0.876
	Cytoplasmic hTERT	0.056	1	0.813

[a] Residual Chi-square=4.698 with 7 df Sig.=0.697

carcinogenesis. Some studies have shown increased frequency or levels of telomerase activity in cancers of advanced stages or metastatic phenotypes (25-28), while others have failed to observe such correlations (29, 30). Studies of clinical samples have revealed telomerase activation not only in invasive cancers, but also in some types of premalignant lesions, such as cervical intraepithelial neoplasia (31, 32), prostate intraepithelial neoplasia (33) and even in preneoplastic bladder lesions (34), suggesting that telomerase activation is an early event in cancer progression. As far as urothelial carcinomas are concerned, in the study of Yoshida *et al. (*35), although no statistically significant correlation was observed between telomerase activity and tumor stage – probably due to the small sample – telomerase activity was detected in nearly all stage I carcinomas as well as in the dysplastic bladder lesions. Moreover, in the same study (35), most of the neoplasms that showed negative telomerase activity were carcinomas of advanced stages. Lee *et al. (*36) also reported positive telomerase activity in 80% of bladder washes obtained from patients with low-stage urothelial carcinomas, while cytology was abnormal in only 40% of them. It has been proposed that inappropriate activation of telomerase in a cell may lead to the evolution of a clonal line with the potential to develop into a tumor (37).

Interestingly, in our study, neither the nuclear or cytoplasmic immunohistochemical expression of hTERT associated with either the histological grade of urothelial carcinomas or with the proliferation index Ki-67, a finding that seems contradictory, since telomerase is the critical enzyme in overcoming growth limitations due to telomere dysfunction and in achieving cell immortalization. However, it has been pointed out that cell immortalization and growth deregulation are conceptually and physiologically separate processes. According to Harley (38), telomerase does not cause growth deregulation and, hence, it can not be considered as an oncogene. There are, in fact, dozens of normal cell types that have been immortalized with telomerase without signs of cancerous changes, without altering differentiation capacity, and without altering pre-existing genetic abnormalities (2, 38). Conversely, there are severely growth deregulated, malignant, metastatic tumor cells, which are telomerase-negative (25, 39).

It is well known that some cell cycle regulators, such as p53, are involved in telomerase regulation. In this study, we observed a statistically significant inverse correlation between cytoplasmic hTERT and p53 mutant protein expression ($p=0.047$). It has been shown that overexpression of wild-type p53 effectively represses telomerase activity through transcriptional down-regulation of hTERT in a variety of cancer cell lines (40, 41). This effect is independent of p53-induced apoptosis or induction of the p53 target gene p21 (40). On this basis, and if cytoplasmic hTERT is considered as a type of down-regulated hTERT, as we previously described, its association with low mutant p53 protein expression may suggest the failure of this mutant p53 protein to properly regulate telomerase activity, *i.e.* either to down-regulate nuclear hTERT expression or to up-regulate its cytoplasmic expression.

As far as apoptosis is concerned, in breast cancer cells, it has been shown that down-regulation of hTERT induces apoptosis (6). According to this observation, one should expect that cytoplasmic hTERT, a down-regulated form of hTERT, might be involved in the succeptibility of the cells to apoptosis. Indeed, in the present study, a positive correlation was observed between cytoplasmic hTERT and

caspase-3 protein expression ($p=0.004$), a critical component of the cell death machinery, being regarded as the most downstream enzyme in the apoptotic process because of its location in the protease cascade pathway (23, 42).

On the other hand, in this study neither nuclear nor cytoplasmic hTERT, nor hTEP-1 was associated with the expression of the anti-apoptotic protein bcl-2. This finding is in contrast to a previous study reporting that the anti-apoptotic factor bcl-2 activates telomerase (43). However, although the expression of bcl-2 is known to be regulated by p53 in several cell types, in a recent study, Kanaya *et al.* (40) demonstrated that deregulation of bcl-2 is not involved in telomerase repression by p53.

In the present study, multivariate survival analysis revealed an independent favorable impact of nuclear hTERT on patients' survival ($p=0.007$), along with histological grade ($p=0.043$) of neoplasms and stage of the disease ($p=0.0001$). This finding is striking, given the generally held view that activation of telomerase can facilitate tumor progression. Moreover, there are previous studies in other tumor types demonstrating an adverse prognostic value of human telomerase activation, which was determined using the TRAP assay, thus not permitting direct comparison (for review see 44). However, telomere dysfunction and telomerase activation are now considered to play paradoxical roles in tumor progression (45). Genetic instability, caused by telomere dysfunction resulting from a lack of telomerase, is now clearly implicated in tumor initiation in mice (38). Some evidence also points to a role for this mechanism in humans (46, 47). Thus, while telomerase inhibition may decrease the growth of late stage tumors, it may also select for more malignant subclones from the tumors by enhancing genomic instability (45). The associations observed in our study between hTERT and hTEP-1 expressions with low stage urothelial carcinomas are in keeping with the concept that telomerase activation occurs early during urothelial cancer progression, protecting against genomic instability and preventing the development of more aggressive clones, which could lead to the development of carcinomas with more aggressive behavior and impaired patients' outcome.

In conclusion, according to the results of this study, telomerase activation seems to be associated with low stage urothelial bladder cancer and with improved patients' survival, suggesting that this early activation might protect against the prevalence of more aggressive malignant clones. However, more studies are needed to clarify the complicated issue of telomerase activation and its precise role in cancer progression. Furthermore, as this study is one of the very few immunohistochemical studies performed for the detection of hTEP-1 and hTERT, suggesting different roles of nuclear and cytoplasmic hTERT with regard to apoptosis, further immunohistochemical studies are needed to establish the possible different roles of hTERT depending on its localization.

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