Tumori, 95: 744-752, 2009

Telomerase RNA expression and DNA ploidy as prognostic markers of prostate carcinomas

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ABSTRACT

Aims and background. The objective of this study was to determine whether there was a correlation between telomerase RNA expression and DNA ploidy status with clinicopathological parameters and biochemical recurrence after radical prostatectomy.

Study design. Telomerase RNA expression and DNA ploidy were evaluated in imprint smear samples obtained from 112 prostates after radical prostatectomy. The results were correlated with pathological stage, Gleason score and serum PSA.

Results. Positive telomerse RNA expression was detected in 67.8% of prostate carcinomas. The multiple linear regression model showed a statistically significance increase in telomerase RNA expression with increased Gleason score (P <0.0001) and preoperative serum PSA values (P = 0.0125). DNA ploidy status also varied significantly with Gleason score (P <0.0001) and preoperative serum PSA values (P = 0.0110). Five patients with diploid tumors and negative telomerase RNA expression developed a recurrence. However, recurrence was associated with DNA aneuploidy (P = 0.001) as well as with high telomerase RNA overexpression (P = 0.001).

Conclusions. We conclude that telomerase RNA expression and DNA ploidy could be additional markers in the field of prognosis of prostate carcinomas.

Introduction

Telomerase is a ribonucleoprotein complex, containing both a catalytic protein component and an RNA component, associated with cellular immortality[1-4]. It catalyzes the de novo addition of telomeric repeats on to telomeres. Recently, the structure of human telomerase has been determined, and its three major subunits – hTR, a ribonucleic acid subunit (human telomerase RNA), telomerase-associated protein 1, and a protein catalytic subunit hTERT (human telomerase reverse transcriptase) – have been identified[5-8]. Of these subunits, telomerase activity requires the presence of hTR, which is the RNA template for the telomeric repeat, and hTERT, which is a reverse transcriptase. For many years, hTERT has been regarded as the limiting component of telomerase. However, recent evidence suggests that the tumorigenic effects of TERT overexpression are reliant on hTR expression, and, in fact, TERT overexpression in hTR-deficient mice has anti-tumorigenic effects[9]. Overexpression of hTR and hTERT in two cancer cell line models and primary human lung fibroblasts markedly induced telomerase activity and telomere length elongation, thereby indicating that both components are required for functional telomerase activity and the existence of a co-regulatory mechanism[10]. Promoter studies have shown that hTR may be more tightly regulated *in vivo* than in cultured cells, as hTR promoter activity is silenced in normal cells compared to tumor cells[11].

Telomerase activity is strongly up-regulated in most cancer cells, whereas it is not detected in normal cells except for stem cells and germ cells[12-18]. In some tumor types,

Key words: DNA ploidy, prognostic factors, prostate adenocarcinoma, telomerase RNA.

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Received October 13, 2008;
accepted May 14, 2009.

levels of hTR expression are more closely related with tumor grade than telomerase activity or hTERT expression[9]. Human telomerase detected by in situ hybridization (ISH) has been recently demonstrated to be a useful tool for the diagnosis of malignancy including prostate carcinoma[18-20]. Telomerase activity has been detected in 47-100% of prostate cancers. Such results are in keeping with results demonstrating that prostate cancer cells have shorter than normal telomeres, thus telomerase activity is presumably required to allow continued tumor proliferation[2,13,19,21].

Image cytometry is a slow and tedious process but helpful to measure DNA content. DNA ploidy status determination has been suggested as an important predictor for evaluation of the prognosis of prostate cancer and can be used in planning therapy[4,22-26]. It has been stated that DNA aneuploidy correlates with a poor prognosis[1,27,28].

Conventional pathological variables may not provide optimal prediction of outcome after radical prostatectomy because of the heterogeneity of prostate carcinoma. This is one of the reasons for the pretreatment underestimation of tumor aggressiveness[29-31]. The value of different biomarkers remains to be applied in clinical practice[32-34].

With such findings in mind, the aim of the present study was to investigate the prognostic value of telomerase RNA expression and DNA ploidy in smears of prostate adenocarcinoma (PAC) in relation to conventional prognostic variables and clinical outcome.

Materials and methods

Samples were obtained immediately after prostate removal in the operating theater from 112 patients (mean age, 67.11 years) who underwent radical prostatectomy for PAC. Furthermore, 25 samples with benign prostate hyperplasia confirm histologically were examined as the control group. Imprint smears were taken from different areas of macroscopically estimated prostate cancer, immediately after prostate removal at the operation theater. From each specimen, a minimum of four smears was prepared and stained according to the Papanicolaou and Giemsa procedure to confirm the presence of cancer cells from the tumor site. After air drying, additional cytologic smears were fixed in ethanol-acetone 1:1 for 10 min and stored at -70 ºC until used for the ISH procedure. Other smears were fixed with 10% formalin for 30 min, washed in water and phosphate-buffered saline and then stained with the Feulgen technique.

All histopathological diagnoses were performed by two pathologists using sections from the samples that were used for the imprints.

The TNM system (of the American Joint Committee on Cancer)[35] was used for pathological staging (pT2a, tumor involves 50% of a lobe or less; pT2b, tumor involves more than 50% of a lobe; pT2c, tumor involves both lobes; pT3a, unilateral extracapsular extension of the tumor).

Grading was evaluated by the Gleason score system. Serum prostate-specific antigen (PSA) was determined by the tandem method (Hybritech, San Diego, CA, USA). The patients had not undergone any preoperative treatment for prostate cancer.

The mean postsurgery follow-up period was 60 months, and all patients were followed clinically at regular 6-month intervals. A PSA concentration greater than 0.2 ng/ml on two successive measurements was defined as biochemical disease progression. None of the patients received any kind of therapy up to the time of biochemical recurrence.

Telomerase RNA (hTR) expression by ISH

For the detection of the hTR component on smear cells, an immunodetection system for ISH was used (BioGenex, San Ramon, CA, USA). The BioGenex Telomerase hTR probe is ready to use with no dilution required and is biotin labeled. The staining protocol was applied according to the manufacturer's instructions. For highly reproducible results, we performed three experiments after standardizing the protocol and using control slides in every run.

The steps of the procedure were as follows. A prehybridization solution was applied to the smears, and the slides were then incubated for 60 min at 37 °C in a humidity chamber. After that, the slides were rinsed in two changes of 100% ethanol, 1 drop of the hybridization solution was applied to the smears, and they were heated in an oven at 95 °C for 10 min. The slides, with a coverslip, were placed in a humidity chamber and incubated at 37 °C overnight. The smears were soaked in a 2X saline sodium citrate solution until the coverslips fell off. The slides were washed in saline sodium citrate, and drops of mouse antibiotin antibody were then applied to the smears, which were incubated at room temperature for 40 min. Drops of biotinylated Fab2 fragments of antimouse immunoglobulins and then drops of alkaline phosphatase-labeled streptavidin were added for 30 min, respectively, at room temperature. Drops of a 5-bromo-4-chloro-3 indolyl phosphate/nitro blue tetrazolium solution as chromogen were added for 30 min.

As counterstain, Mayer's hematoxylin was used for 30 sec. Negative control included omission of the probe from the hybridization buffer and replacement by normal rabbit immunoglobulin in appropriate concentrations.

All immunostained smears were examined using an ×40 objective. At least 200 cells, in randomly selected fields, were counted in every case by two independent observers. Tumor cells were designated as telomerase RNA positive if ≥10% of nuclei were stained (Figure 1).

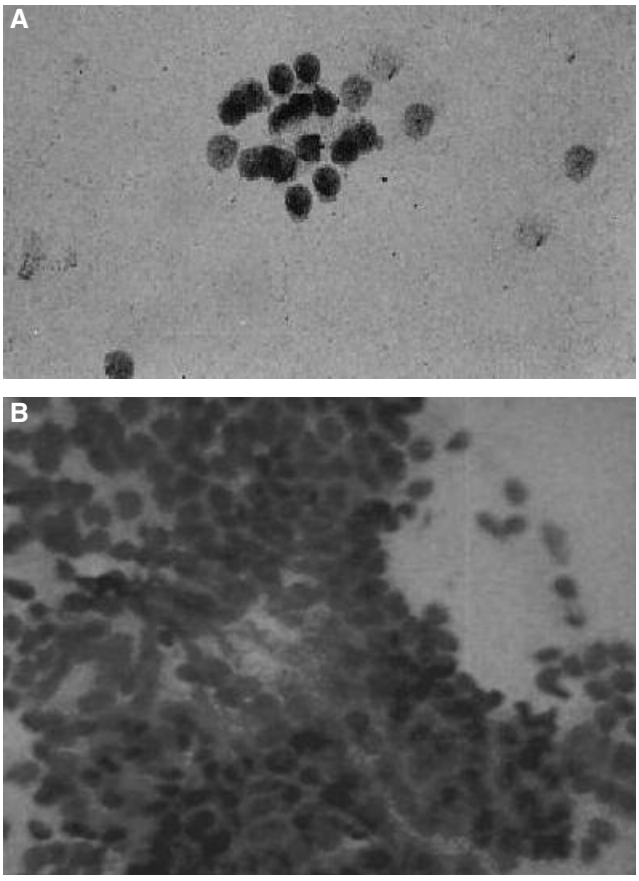

Figure 1 - A) Cluster of low-grade (Gleason score <5) prostate adenocarcinoma with positive nuclear expression for telomerase RNA (x500). B) Prostate hyperplasia with negative hTR expression.

This cutoff has been validated in several reports[20,36]. Furthermore, with the logrank test, it can distinguish patients in two prognostic groups with or without recurrence.

DNA analysis

DNA analysis was performed on imprints stained by the Feulgen technique using a SAMBA 2005 image analyzer according to the customer protocol, a Zeiss Axioplan microscope with an ×40 plan achromatic lens, a Sony three-color camera CCD and a Compaq Computer. From each smear slide, at least four optical fields were selected (hot spots) and captured as images. All images were implemented with the following automation: a) image calibration based on stain and optical density, b) image calibration based on microscope lens with a micrometric scale in microns, c) control cell detection and counting (at least 800 lymphocytes), d) tumor cell detection and counting (at least 300 cells), and e) measurement of area.

Data were automatically exported to Microsoft Excel tables, and DNA index and ploidy histograms were produced. Each image was a "true color" image, meaning that we had three channels that represented the three basic known colors, red, green and blue. Each channel recognizes and separates the light information in "gray levels". These gray levels range from 0 (black) to 255 (white). This range is proportional and expresses the transmitted intensity of the image, so each pixel (dot) in the image caries its light information in 3 gray level channels (0-255). Our material was divided into the already known groups: type I and II with a diploid DNA content (1.8c-2.2c), and type III and IV with an aneuploid DNA content (>3c) with one peak or more outside the diploid or tetraploid region[4,5] (Figure 2).

Statistical analysis

The relationship of telomerase RNA expression and DNA with all prognostic variables (stage, Gleason score and preoperative PSA serum levels) was assessed by one-way analysis of variance followed by tests of multiple comparisons, since telomerase RNA expression and DNA ploidy did not deviate from normality (Kolmogorov-Smirnof test: $P = 0.281$). The simultaneous effect of all variables on telomerase RNA expression and DNA ploidy was investigated by multiple linear regression. Survival rates were calculated using the Kaplan-Meier method. A multivariate survival analysis was performed using Cox's proportional hazard regression model. Statistical significance was accepted when $P < 0.05$.

Results

Positive telomerase RNA expression was detected in 67.8% of prostate carcinomas. Telomerase RNA staining was almost entirely confined to the nucleus of the tumor cells and showed a granular pattern. The clinicopathological features of the PAC cases are summarized in Table 1.

The median follow-up was 54 months (range, 2-60). Of the 112 patients with analyzed specimens, 15 (13.39%) patients died from their disease, 2 each at 5, 7, 14 and 36 months and 1 each at 18, 24, 25, 26, 42, 48 and 54 months. The remaining patients were alive and well at the last follow-up. In all control cases, diploid DNA content and negative telomerase RNA expression were observed. Table 2 summarizes the DNA index and telomerase activity of all 112 cases.

The multiple linear regression model showed that the most significant variables associated with DNA ploidy and telomerase RNA expression were Gleason score ($P < 0.0001$) and preoperative PSA serum levels ($P = 0.0110$). The mean hTR was higher for those with PSA score ≥10. Specifically, the average hTR score was 58.52 for patients with a PSA score ≥10 (SD = 20.24 and 95% CI, 49.31-67.73), 38.53 for patients with a PSA score between 5.1 and 9.9 (SD = 18.55 and 95% CI, 32.25-44.80),

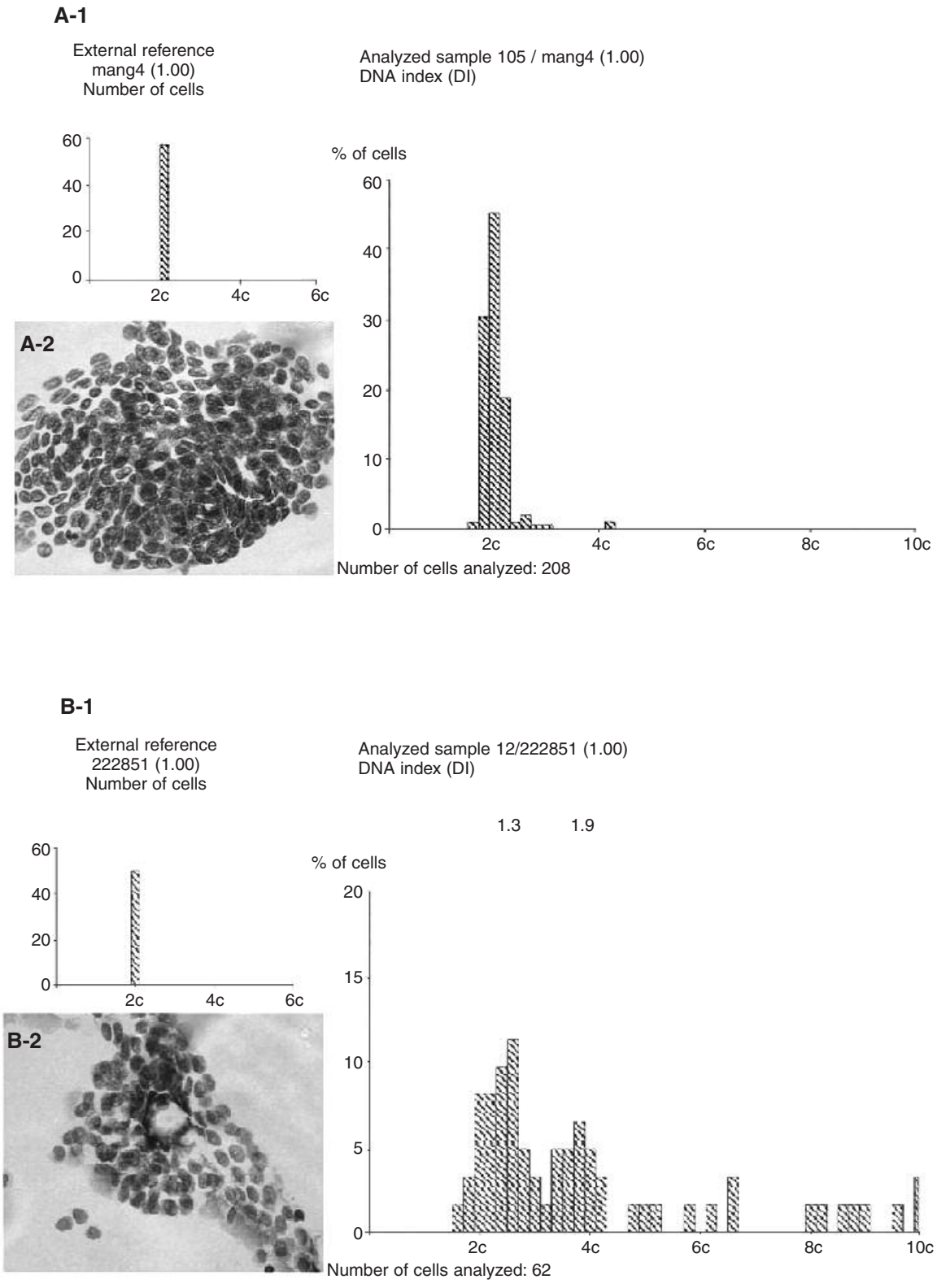

Figure 2 - Aneuploid (A) and diploid (D) DNA histograms. Nuclear DNA content of the cells, given on the horizontal axis (2c denotes diploid DNA content). A-1: Histogram (<3c) from a case of well-differentiated prostate adenocarcinoma with low Gleason score (2-4). A-2: Prostate adenocarcinoma cells from the same case: nuclear staining with Feulgen method (x500). B-1: Histogram (>3c) from a case of poorly differentiated prostate adenocarcinoma with high Gleason score (4 = 4). B-2: Cluster of prostate adenocarcinoma cells, from the same case, stained with Feulgen method (x500).

Table 1 - Clinical characteristics of 112 patients with prostate cancer

	No.	(%)
Age (yr)		
<65	28	25.0
65-69	49	43.7
>70	35	31.3
Pathological stage		
pT2a	53	47.0
pT2b	30	26.7
pT2c	16	14.2
pT3a	13	12.1
Gleason score		
2-4	33	29.4
5-6	44	39.2
≥7	35	31.4
Preoperative PSA (ng/ml)		
0-5	27	24.1
5.1-9.9	50	44.6
≥10	35	31.3

T2a, tumor involves 50% of a lobe or less; T2b, tumor involves more than 50% of a lobe; T2c, tumor involves both lobes; T3a, unilateral extracapsular extension of the tumor.

Table 2 - Combination of hTR expression with DNA ploidy in patients with prostate cancer

hTR/DNA ploidy	Patients	
	No.	%
<9.9/Diploid	36	32
<9.9/Aneuploid	13	12
≥10/Diploid	52	46
≥10/Aneuploid	11	10
Total	112	100

and 44.08 for those with a PSA score less than 5 (SD = 22.49 and 95% CI, 30.48-57.67). The mean hTR increased with increasing Gleason score. Those with a Gleason score between 2 and 4 had a mean hTR of 26.05 (SD = 11.09 and 95% CI, 20.71-31.40), those with a score between 5 and 6 had a mean hTR of 45.30 (SD = 17.63 and 95% CI, 38.72-51.88), and those with a Gleason score of more than 7 had a mean hTR of 63.57 (SD = 17.84 and 95% CI, 55.45-71.69). Increased stage was associated with increased mean hTR. Stages T2c and T3a had a higher mean hTR than stages T2a and T2b. Patients with stage T3a had a mean hTR score of 73.04 (SD = 41.63 and 95% CI, 48.75-100.00), patients with stage T2c had a mean hTR score of 62.57 (SD = 29.23 and 95% CI, 35.53-89.61), whereas patients with stages T2a and T2b had significantly lower mean values for hTR - 43.93 (SD = 20.70 and 95% CI, 37.64-50.23) and 40.24 (SD = 16.83 and 95% CI, 31.58-48.89), respectively.

A higher incidence of recurrence was noted in those patients whose tumor had aneuploid DNA content (type III, IV) than in those whose tumor had diploid DNA content. Cox's model demonstrated that DNA ploidy and telomerase RNA expression had significant prognostic value for disease-free survival ($P = 0.007$ [HR, 1.142; CI, 1.027-1.270] and $P = 0.009$ [HR, 1.159; CI, 1.083-1.294], respectively).

Table 3 shows the correlation of risk factors for progression to recurrence status of the tumors, including ploidy status (diploid or aneuploid), telomerase RNA expression (<9.9 and ≥10), postoperative Gleason score (<5, ≥6) and preoperative serum PSA value (<5.9, ≥6). The Kaplan-Meier plots of progression to recurrence, stratified according to these four variables, are shown in Figure 3. Sixty months after the diagnosis, 5 patients (9 to 13.8%) with a Gleason score of less than 5, PSA <5, telomerase RNA expression <9.9, and diploid DNA status developed a recurrence. In contrast, 10 patients (13.1-17.5%) with a Gleason score of more than 6, PSA ≥6, telomerase RNA expression ≥10, and aneuploid DNA status developed a recurrence. Of the 112 men, 97 had not developed a recurrence in the 60-month follow-up. Fifty of them (91%) had a postoperative Gleason score of less than 5, 47 (90.4%) initial serum PSA value <5.9 ng/ml, 31 (86.2%) with telomerase RNA expression <9.9,

Table 3 - Correlation of risk factors with progression to recurrence status of the tumors (logrank test)

	No. (%)	NR No. (%)	R No. (%)	Mean time to recurrence (mo)	*P*
Gleason score					
<5	55 (49.1)	50 (91)	5 (9.0)	28	0.0128
≥6	57 (50.9)	47 (82.5)	10 (17.5)	26	
Preoperative PSA, ng/ml					
<5.9	52 (46.4)	47 (90.4)	5 (9.6)	27	0.216
≥6	60 (53.6)	50 (83.4)	10 (16.6)	27	
Telomerase RNA (%)					
<9.9	36 (32.2)	31 (86.2)	5 (13.8)	27	0.001
≥10	76 (67.8)	66 (86.9)	10 (13.1)	26	
DNA ploidy					
Diploid	48 (42.8)	43 (89.6)	5 (10.4)	30	0.001
Aneuploid	64 (57.2)	54 (84.4)	10 (15.6)	29	

NR, no recurrence; R, recurrence.

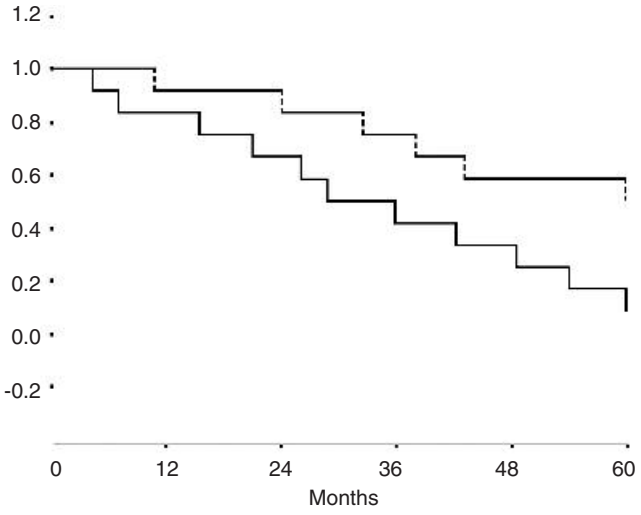

Figure 3 - Kaplan-Meier plot of progression to recurrence (R) 60 months after diagnosis stratified according to preoperative serum PSA, Gleason score, telomerase RNA expression and DNA ploidy status. The dotted line is the (R) group of 5 patients with DNA diploidy, PSA <5.9 ng/ml, Gleason score <5, telomerase RNA expression <9.9%. The solid line is the (R) group of 10 patients with DNA aneuploidy, PSA ≥6 ng/ml, Gleason score ≥6 and telomerase RNA expression ≥10%.

and 43 (89.6%) with diploid DNA status. Mean time to recurrence for Gleason score <5, serum PSA <5.9, telomerase RNA expression <9.9, and diploid tumors was 28 months. For Gleason score ≥6, initial serum PSA ≥6, telomerase RNA expression ≥10 and aneuploid tumors, mean time to recurrence was 27.2 months. For recurrence, the denominator for each risk factor differed: for postoperative Gleason score, $P = 0.0128$; initial serum PSA value, $P = 0.216$; and $P < 0.001$ for telomerase RNA expression and DNA ploidy status (Table 3).

Discussion

Prognosis after radical prostatectomy is usually based on clinical and conventional pathological variables such as Gleason score, preoperative PSA serum values and pathological stage. In recent years, other variables that may predict the biological behavior of prostate cancer including DNA ploidy status and telomerase activity have been found[24,26,32,37].

The reaction of telomerase activity has been considered to be one of the key mechanisms in cell immortalization, and telomerase may be considered a new target in cancer therapy. In previous studies, telomerase activity has been shown to be positive in about 80% of prostate carcinomas, but the relationship between such activity and prognosis has not been clearly demonstrated[3,38,39]. Most of the studies used the telomeric repeat amplification protocol assay, which detects the biochemical activity of telomerase[40]. Human telomerase detected by ISH has been demonstrated to be a useful tool for the diagnosis of malignancy[3].

Recent studies of the RNA component (hTR) of telomerase by ISH have shown a good correlation with the expression of telomerase activity in some malignancies[8,12,23,41]. In spite of the intratumor heterogeneity of telomerase activity, its expression can be detected in surgical specimens and needle biopsy material, as well as in the urine and prostate fluids of patients with prostate cancer[37,42].

On the whole, the potential diagnostic use of telomerase hTR expression in prostate cancer appears limited, primarily because of problems having to do with specificity for the detection of hTR by ISH. As regards telomerase activity, most studies examined radical prostatectomy samples and detected activity in most prostate carcinomas, but the rates of activity in their detection varied widely in cancer-associated normal and benign prostate hyperplasia tissues. However, normal prostate epithelial cells typically lack detectable telomerase activity[9].

In this study, we used an ISH method for the detection of the RNA component of telomerase (hTR) in imprints of surgical specimens of PAC. Telomerase RNA expression was positive in 67.8% of PAC smears and negative in 32.2%. In most previous reports on various malignant tumors, telomerase activity was not necessarily positive in all cases[43,44].

The negative results in 32.2% of our carcinoma specimens, although each one undeniably contained histopathologically confirmed carcinoma, are of considerable interest in view of the extensive evidence from several other reports of a strong association between telomerase activity and malignancy. A possible explanation for such an absence of telomerase activity in some of the tumors is the notion that most cancer cells in these negative cases may still be mortal according to the telomerase hypothesis. The latter proposes that cells can proliferate without telomeres until the second stage of mortality, at which point telomerase may be activated in response to the drastic reduction in telomere length[19].

Furthermore, the presence of some tumor factors is capable of inhibiting telomerase, which becomes ineffective upon dilution[37,40-42,45-48]. Approximately 10-20% of prostate cancer cases appear to be telomerase negative. These tumors may have limited growth potential and might therefore be expected to have a more favorable clinical course than their telomerase-positive, immortal counterparts[19].

A recent report has clearly shown that several tumor cell lines keep their telomere length without telomerase activity. Consequently, some unidentified mechanisms for restoring the telomere length must exist[49]. A substantial number of human tumors utilize a telomerase-independent length maintenance mechanism referred

to as alternative lengthening of telomeres (ALT). ALT involves lengthening of telomeres by homologous recombination-mediated replication of telomeric DNA[50]. Thus, it seems that a small number of PAC actually do not posses telomerase activity. However, because telomerase is a ribonucleoprotein, it is possible that the lack of telomerase activity is related to degradation of essential telomerase-templating RNA before sampling or during storage[38].

Reports on the association between telomerase activity and established prognostic indicators have been mixed. A positive correlation between telomerase activity and tumor grade was reported in 2 of 6 studies, whereas 4 other studies found no correlation with grade, stage or preoperative PSA levels[38,42-45,49,51,52]. Iczkowski *et al.*[7] showed a direct relationship between telomerase activity and tumor grade. In their study, in spite of a stronger signal intensity in Gleason score 8 and 9, no significant difference with lower scores could be demonstrated.

In the present study, 82.5% of the advanced grade tumors (Gleason score ≥6) showed telomerase RNA activity, whereas this activation was undetectable in 91% of low-grade (Gleason score <5) PAC (P = 0.0128). Such findings suggest that telomerase-positive PAC has a more malignant potential than does PAC without telomerase activity.

Tumors with high telomerase RNA activity (62-82%) were generally large and at an advanced pathological stage (pT2c-pT3a). Such results suggest that telomerase is not always activated in PAC in early stage tumors, so the activity may occur as a late event of cancer progression. However, because 28.93% of PAC stage pT2a had telomerase RNA activity, this activation is not always a late event in PAC cell progression. According to a study of Wullich *et al.*[53], a strong association was also found between telomerase RNA activity pattern and histopathological stage, although the association did not achieve statistical significance.

Sommerfeld *et al.*[39] reported their findings in 25 prostate carcinomas, emphasizing that all four telomerase-negative tumors were strictly organ confined and did not exhibit either capsular infiltration or capsular penetration. Similar observations were reported by Lin *et al.*[46]

The serum concentration of PSA has become generally recognized as an important tumor marker for carcinoma of the prostate[54,55]. The serum concentration of PSA before radical prostatectomy was measured in all studied patients and compared to the results of telomerase RNA expression. We determined that preoperative PSA levels generally were higher when the telomerase RNA expression was increased using the high minimum value of >6 ng/ml (P = 0.0125).

In our study, 15 patients died from their disease after persistent recurrence. The smears of all these cases showed a strong expression of telomerase RNA. Thus, telomerase-positive cells might predict early disease recurrence, but a longer follow-up is needed to test this possibility.

There have been reports showing a direct correlation among increasing Gleason score, PSA level and DNA content[56]. Some investigators found that the use of DNA ploidy increases the prognostic value. Others have reported that DNA ploidy association with tumor stage and grade is questionable[4,57]. Many of these studies were retrospective image analyses of disaggregated paraffin-embedded and formalin-fixed specimens[15,26,56,58-61].

In comparison to the fresh imprint smears we used in our study, tissue sections present a lot of difficulties with regard to the estimation of DNA ploidy. Depending on the thickness of the section, there will always be a number of nuclei that are either sliced or overlapped, the first leading to false-low and the latter to false-high ploidy values. Consequently, researchers who study DNA ploidy in archive tissue sections should use internal control analysis such as that recommended by Green *et al.*[62]

DNA studies have shown that patients with diploid cancers have a longer disease-free interval and survival time than those with non-diploid tumors[28].

Using static cytometry, we found in our material a correlation between DNA ploidy and Gleason score (P <0.001), preoperative PSA value (P = 0.0110) and telomerase RNA expression (P <0.001). These results are in agreement with those described in previous reports[24-26]. As shown in Figure 3, recurring cases were found in the group of diploid tumors with telomerase RNA expression lower than 9.9% (low potential of malignant PAC). In contrast, a very high percentage of aneuploid tumors with telomerase RNA expression ≥10% recurred (high potential of malignant PAC).

In a study of Blute *et al.*[23], disease progression showed a significant relationship with ploidy only. Among patients with disease progression, 37% had diploid tumors and 63% had nondiploid tumors (tetraploid, aneuploid). In contrast, of patients without progression, 92% had diploid tumors and 8% nondiploid tumors (tetraploid). In the Mayo Clinic cancer prostatectomy series, ploidy was one of the significant predictive factors found in multivariate analysis of tumor characteristics[16]. In a study of stage C prostate cancer, Lee *et al.*[63] found that there was a greater likelihood for recurrent disease after surgery if tumors were nondiploid. The probability of a disease-free interval of 60 months was 85% for those with diploid tumors compared to only 9% for those with nondiploid tumors.

Thus, aneuploidy patterns and DNA histogram characteristics seem to be useful to differentiate between the stable and progressive characteristics of cancer. Since telomerase RNA expression and ploidy status could provide useful information about the biological behavior of PAC, further studies are required to estab-

lish the clinical utility of these parameters, especially the possibility that telomerase may be a therapeutic target for prostate cancer treatment.

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