

TELOMERASE ACTIVITY IN LOCALIZED PROSTATE CANCER: CORRELATION WITH HISTOLOGICAL PARAMETERS

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ABSTRACT

Objective: Telomerase is an enzyme responsible for lengthening the telomere, active during the embryonic development and detected, in the adult life, only at low levels in germinative cells and some stem cells. Tumor cells need to be immortalized and the reactivation of telomerase activity, or alternative mechanisms for lengthening the telomeres is necessary for tumor progression. Some studies have correlated telomerase activity with bad prognostic parameters in prostate cancer. Our objective is to detect telomerase activity in invasive prostatic adenocarcinoma and to correlate its presence with the stage, histological differentiation, and volume of the cancer in radical prostatectomy specimens. As well as in PIN adjacent to the cancer.

Material and Methods: Tissue samples from 75 patients submitted to radical prostatectomy for localized prostate cancer were studied using the telomeric repeat amplification protocol (TRAP).

Results: Telomerase activity was detected in 29% of 21 high grade PIN and in 80% of 54 invasive cancer. There was no statistically significant difference between the telomerase-positive and telomerase-negative groups in Gleason grade, tumor volume or tumor stage.

Conclusion: We conclude that telomerase activity is common in prostate cancer and may appear early in the development of the disease. Its presence is unrelated to classical prognostic parameters.

Key words: prostate; prostatic neoplasia; prognosis; telomerase

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INTRODUCTION

The enzyme telomerase is important for the immortalization of cancer cells because it maintains the telomeres (1). Telomeres cap the ends of chromosomes and protect the DNA from recombination, fusion, and loss of terminal sequences during replication. They consist of tandem repeats of a G-rich sequence (TTAGGG in vertebrates) up to 15 kilobases long (2). With each round of DNA replication the telomeres become 100 base pairs (bp) shorter because the RNA primers that initiate polymerization of a new DNA at the 3' single strand tail are then degraded, leaving a gap that is never filled in (3). When the telomere reaches a critical length, a

cell becomes senescent and unable to divide. If the cell is forced into mitosis by an oncogene, the complete loss of the telomeres results in massive fusion of chromosomes, and the cell dies by apoptosis. This mechanism, which is called the mitotic clock, has been implicated in the control of cell proliferation, and is believed to work as a tumor suppressor (4).

Telomerase, an enzyme capable of elongating telomeres, was discovered in 1985 (5). It is a ribonucleoprotein composed of an RNA primer (3'-CAAUCCCAAUC-5'), that hybridizes with the end of the telomere, and an enzyme with reverse transcriptase activity (5). Telomerase is normally expressed only by germ cells and, at low levels, by some stem cells; it is repressed in somatic tissues (6).

The maintaining of telomere length by the re-expression of telomerase has been described in more than 85% of malignant human tumors (1), as well as other alternative mechanism for it (7), and it is considered an essential step in tumor progression, promoting cell immortalization.

The re-expression of telomerase is believed to be one step in the transformation of benign prostatic epithelium to prostatic intraepithelial neoplasia (PIN) and has been detected in 16% of the cases (8), as well as in benign prostatic hyperplasia (BPH) adjacent to invasive adenocarcinoma (9,10).

There is no definitive prognostic parameter for prostate cancer. Histological differentiation, tumor volume, tumor stage, and DNA ploidy are important for prognosis, but some cases behave unpredictably. This lack of predictability is inconvenient when different therapeutic approaches should be considered (11,12). If telomerase has a role in prostate cancer, perhaps its detection could be useful for prognosis.

We wanted to determine the frequency of telomerase expression in samples of PIN and invasive cancer from 75 prostate cancer patients submitted to radical prostatectomy, and to examine the relationship of telomerase expression to prognostically important tumor characteristics (stage, histological differentiation, and tumor volume). To this purpose, we used the telomeric repeat amplification protocol (TRAP) (13), a very sensitive method based on a polymerase chain reaction, to detect telomerase activity.

MATERIAL AND METHODS

Seventy-five patients with prostate cancer, clinically staged T1c-T2 were submitted to radical prostatectomy at the Sirio Libanes Hospital from March 1998 to March 1999. Immediately after the gland was removed it was sent to the laboratory, and a thin, 1-cm² square fragment of glandular tissue was frozen at -80°C in liquid nitrogen. After this, the prostate was fixed in formalin for 4 hours, surgical margins were stained with India ink, and the whole gland, including the bladder neck and prostatic apex,

was submitted for pathological evaluation of surgical margins and extraprostatic infiltration. Serial 3-mm cuts were taken from each prostatic lobe. The seminal vesicles and pelvic lymph nodes were also submitted, and cuts were examined for tumor involvement. Extraprostatic infiltration was defined as tumor infiltrating the adipose tissue or neurovascular plexus. The Gleason score was used for histological classification. The tumor volume was evaluated as described by Humphrey et al. (1). Briefly, a grid was placed below the slides, on which the area involved by the tumor had been previously sketched out. The percentage of tumor on a slide was determined by dividing the number of squares involved by tumor by the number of squares occupied by the whole section on the slide. Tumor volume was defined as the mean percentage of tumor in the prostate gland (the percentage of tumor on each slide divided by the number of slides from the prostate gland). Tumors were staged according to the TNM classification (1992) (15).

Before telomerase activity was assessed a section of the frozen prostate fragment was stained with hematoxylin and eosin and examined microscopically by the pathologist in order to confirm the presence of invasive cancer or PIN. Ten 10- μ m sections of the frozen prostate fragment were cut in a cryostat and used for the telomerase assay.

The TRAPeze Telomerase Detection Kit (Intergene, Purchase NY, USA) and the methods described in the kit were used for detection of telomerase activity. To extract RNA and protein, the samples were incubated for 30 minutes on ice in 200 μ L of CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanane sulfonate) lysis buffer (10 mM Tris-HCl, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β -mercaptoethanol, 0.5% CHAPS, and 10% glycerol) containing RNase inhibitor (RNAGuard[®], Pharmacia Biotech, Uppsala, Sweden) at 200 units/mL. The samples were then spun in a microcentrifuge at 12,000g for 20 minutes at 4°C, and the supernatant was retained as the extract.

To detect telomerase activity, the TRAPeze kit follows the telomeric repeat amplification protocol (13), except that it does not require a wax

barrier hot start. For each sample, 2 μ L of the RNA-protein extract was added to a 48- μ L reaction mixture containing 10X TRAP Buffer (200 mM Tris-HCl, 15 mM MgCl₂, 630 mM KCl, 0.5% Tween 20, and 10 mM EGTA), 50X dNTP mix, TS primer (5'-AATCCGTCGAGCAGAGTT-3'), TRAP primer mix (RP primer, K1 primer, and TSK1 template), Taq polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden), and distilled water. The reaction mixture also contained a 36-bp positive control to allow recognition of false negatives, which could result if an inhibitor of Taq-polymerase were present. All measures to prevent RNA degradation were taken. In a thermocycler block (Perkin Elmer GeneAmp Thermal Cycler 9600, Foster City, California, USA), a 30-minute incubation at 30°C was provided in which telomerase (if present) could hybridize with and extend the TS primer. Then a polymerase chain reaction was run for 30 cycles of 30 seconds at 94°C, 30 seconds at 59°C, and 30 seconds at 72°C to amplify the first extension, adding 6 base pairs per cycle.

To identify a positive or negative assay, 20 μ L of the amplification product was submitted to electrophoresis for 2.5 hours at 200 V on a 10% nondenaturing polyacrylamide gel in 0.5X Tris-borate-EDTA (TBE) buffer was used. The gel was then silver-stained according to the method of Budowle et al. (16). Briefly, the gel was placed in 10% ethanol for 5 minutes, oxidized in 1% nitric acid for 3 minutes, placed in 0.012 M silver nitrate for 30 minutes, and reduced in a solution containing 0.28 M sodium carbonate and 0.019%

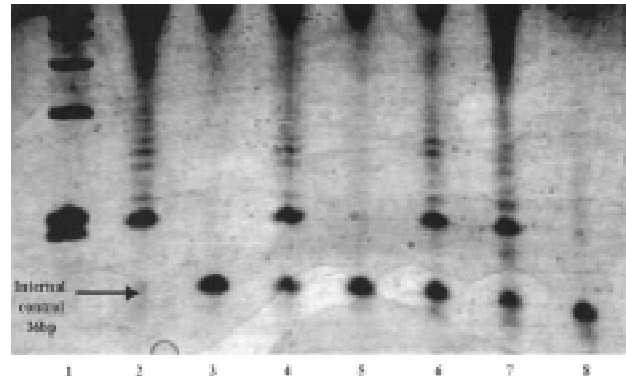


Figure - Detection of telomerase activity in extracts of invasive prostate cancer. Lane 1, ladder 50bp. Lanes 2, 4 and 6, show telomerase activity characterized by a ladder pattern with 6bp increment in bands. Lanes 3 and 5 show 2 negative cases. Lanes 7 and 8, have the positive and negative control, respectively.

formalin until bands developed. Placing the gel in 10% glacial acetic acid for 2 minutes stopped reduction, and the gel was then placed in distilled water. Samples that exhibited a ladder beginning at 50 bp with 6-bp increments were considered positive (Figure). Since the telomerase is a heat sensitive enzyme, the negative control is one tumor sample submitted to a heat treatment (85°C) for 10 minutes.

The relationship between stage and telomerase activity was analyzed with the Pearson Chi-square test. The Gleason score and tumor volume of patients with or without telomerase activity were compared using the Mann-Whitney test because of the non-normal distribution of the numbers. P-values less than 0.05 were considered statistically significant.

Table - Gleason score, tumor volume and tumor stage for carcinomas telomerase-positive and telomerase-negative.

	Telomerase-positive		Telomerase-negative		
Gleason score (median-range)	6.3	(4 - 10)	6.2	(4 - 9)	p = 0.733
Tumor volume % (median-range)	19	(0.3 - 56)	12	(0.7 - 53)	p = 0.193
Stage					
pT2	25	(58%)	7	(64%)	p = 0.990
pT3	18	(42%)	4	(36%)	

RESULTS

The pathological stage was pT2 for 49 (65%) patients and pT3 for 26 (35%) patients. Only one patient presented lymph node metastasis, and was staged T3cN1. The mean Gleason score was 6, and the median 5. Twenty-seven (36%) patients had high-grade tumors (Gleason \geq 7), and 48 (64%) had low-grade tumors (Gleason \leq 6).

For 21 patients the specimen submitted to the TRAP assay contained only high grade PIN. For the remaining 54 patients the assayed specimen contained only invasive adenocarcinoma.

Telomerase activity was found in 43 (80%) of the invasive cancer specimens. There was no correlation between Gleason score and Telomerase activity. The enzyme activity was positive in 81% (26/32) of well differentiated tumors (Gleason 4-6), in 80% (4/5) of Gleason grade 7 tumors and in 76% (13/17) of poor differentiated tumors. The results of telomerase activity detected in the invasive carcinoma and tumor stage, tumor volume and Gleason score are shown in Table. Telomerase activity was found in 6 (29%) of the PIN specimens. The characteristics of the invasive carcinoma adjacent to those PIN areas were: The median of Gleason score was 5 for either group, telomerase-positive and telomerase-negative. The median of tumor volume was 11% for telomerase-negative PIN and 3.5% for telomerase-positive PIN. Seventy three percent and 100% of telomerase-negative and telomerase-positive cases, respectively, were staged pT2. The presence or absence of telomerase activity in PIN did not correlate to the tumor stage ($p = 0.429$), tumor volume ($p = 0.310$) or Gleason score ($p = 0.619$) of the invasive carcinoma adjacent to the PIN lesion.

DISCUSSION

Our results show that the re-expression of telomerase occurs early in the development of prostate cancer, since we found telomerase activity in one third of the samples of PIN. Koeneman et al. (8) found telomerase activity in 16% of 25 cases of PIN, a frequency much lower than ours, that can be partially

explained by technical problems, since the authors, different from all previous studies, have found much lower telomerase activity in carcinoma, also (69%). They claim the presence of non-neoplastic cells, telomerase negative, interfering in the results, and suggest that the microdissection of the specimen could enrich the sample with tumor cells, telomerase positive, increasing the frequency of telomerase positive lesions. Currently there is no way to identify patients with PIN who will progress to invasive cancer or who already have invasive cancer that was not reached by the biopsy. We cannot assess from our study whether telomerase activity in a PIN specimen predicts prostate cancer, because all of our cases had invasive cancer. We can, however, point out that the absence of telomerase activity in a PIN specimen certainly does not rule out cancer elsewhere in the prostate gland.

Other cancer precursors, such as oral leukoplakias, express some telomerase activity, as does some normal lung tissue from lung cancer patients and its detection has been useful for surveillance of these patients (17,18). It has also been suggested that telomerase detection by the TRAP assay could be used for early detection of cancer in brushings, fine needle aspiration biopsy specimens, or voided urine (19-21).

Telomerase activity has been detected in 11% to 38% of biopsies containing only benign tissue (22,23). The presence of telomerase activity in BPH adjacent to cancer suggests that this information could be used to indicate patients who are more likely to have cancer elsewhere in the prostate or to develop it later. Since the TRAP assay is extremely sensitive, and can detect very few cells telomerase-active, we assume that the recovering of telomerase activity occurs previously to morphological alterations, and should be a marker for patients potentially vulnerable to develop prostate cancer.

Telomerase activity was detected in 80% of our invasive adenocarcinoma samples, and we were unable to correlate the re-expression of telomerase with the Gleason grade, tumor volume or tumor stage.

Telomerase activity has been reported in 84 to 90% of prostate cancer (9,10). Sommerfeld et al.

(9) who were the first to detect telomerase activity in prostate cancer described the presence of it in all lymph node metastasis and the absence of it in 4 cases with organ-confined disease, suggesting that there could be a relationship between telomerase activity and unfavorable prognosis. Lin et al. (10) unlike us were able to correlate telomerase activity with histological differentiation: they found telomerase activity in 38% of well-differentiated prostate cancers, and in 91% of poorly differentiated prostate cancers. Also, they found strong telomerase activity in metastatic prostate cancer in lymph nodes and bones. Our study is the larger and strictly standardized. We included only surgical specimen obtained by radical prostatectomy, performed by the same team (MS), examined by the same pathologists (KRML, LHCL), and the whole gland was submitted for the evaluation of the prognostic parameters. The lack of correlation between telomerase activity and prognostic parameters could be related to the high prevalence and early occurrence of this phenomenon, and the follow-up of the patients could bring more information about the relevance of this event.

In other solid tumors telomerase activity has been associated with unfavorable characteristics or outcomes. Telomerase activity has been found in 92% of malignant ovarian tumors but only 17% of borderline tumors of the ovary (24). A lower survival rate and advanced stages have been described for telomerase-expressing gastric carcinomas (25). In meningiomas telomerase activity has been reported to predict recurrence (26). Some cases of stage 4S neuroblastoma with undetectable or low levels of telomerase have regressed spontaneously, suggesting that the lack of telomerase contributes to a favorable outcome (27).

Because telomerase is normally expressed only by germ cells and, at low levels, by some stem cells, it has been proposed that telomerase inhibitors could be used to treat cancer without affecting normal somatic cells (28). Antisense strategies directed toward the RNA template component of telomerase (29) and inhibitors of the reverse transcriptase (30) have been described. A recent, novel approach has been to use cationic porphyrins that interact with the

telomeric G-quadruplex inhibiting telomerase and preventing elongation of the telomere (31). However, there are tumors without telomerase activity in which telomeres are maintained by some alternative mechanism (7). Therefore, it will be important to identify those cancers that depend on telomerase for immortalization, as it is only these cancers that may be vulnerable to telomerase inhibitors.

In conclusion, our results demonstrate that the re-expression of telomerase is an early and frequent event in prostatic carcinoma, with telomerase activity present in 29% of our PIN specimens and 80% of our invasive cancer specimens. We could not find any correlation between telomerase activity and histological differentiation or tumor stage, parameters that reflect cancer behavior. The prognostic value of telomerase activity remains open for further investigation.

REFERENCES

1. Kim N, Piatyszek AM, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL, Shay JW: Specific association of human telomerase activity with immortal cells and cancer. *Science*, 266: 2011-2015 1994.
2. Lange T: Activation of telomerase in a human tumor. *Proc Natl Acad Sci USA*, 91: 2882-2885, 1994.
3. Lindsey J, MacGill NI, Lindsey LA, Green DK, Cooke HJ: In vivo loss of telomeric repeats with age in humans. *Mutat Res*, 256: 45-48, 1991.
4. Harley CB: Telomere loss: mitotic clock or genetic time bomb? *Mutat Res*, 256: 271-282, 1991.
5. Greider CW, Blackburn EH: Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell*, 43: 405-413, 1985.
6. Blackburn EH: Structure and fusion of telomeres. *Nature*, 350: 569-573, 1991.
7. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR: Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nature Med*, 3: 1271-1274, 1997.

8. Koeneman KS, Pan C-X, Jin J-K, Pyle III JM, Flanigan RC, Shankey V, Diaz MO: Telomerase activity, telomere length, and DNA ploidy in prostatic intraepithelial neoplasia (PIN). *J Urol*, 160: 1533-1539, 1998.
9. Sommerfeld H-J, Meeker AK, Piatyszek MA, Bova GS, Shay JW, Coffey DS: Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res*, 56: 218-222, 1996.
10. Lin Y, Uemura H, Fujinami K, Hosaka M, Harada M, Kubota Y: Telomerase activity in primary prostate cancer. *J Urol*, 157: 1161-1165, 1997.
11. Foster CS, McLoughlin J, Bashir I, Abel PD: Markers of the metastatic phenotype in prostate cancer. *Hum Pathol*, 23: 381-394, 1992.
12. Steinberg GD, Bales GT, Brendler CB: An analysis of watchful waiting for clinically localized prostate cancer. *J Urol*, 159: 1431-1436, 1998.
13. Piatyszek MA, Kim NW, Weinrich SL, Hiyama K, Hiyama E, Wright WE, Shay JW: Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods in Cell Science*, 17: 1-15, 1995.
14. Humphrey PA, Vollmer RT: Intraglandular tumor extent and prognosis in prostatic carcinoma: application of a grid method to prostatectomy specimens. *Hum Pathol*, 21: 799-804, 1990.
15. Schroder FH, Hermanek P, Denis L, Fair WR, Gospodarowicz MK, Pavone-Macaluso M: The TNM classification of prostate carcinoma. *Prostate, Suppl*, 4: 129-138, 1992.
16. Budowle B, Chajraborty R, Giusti AM, Eisenberg AJ, Allen RC: Analysis of the VNTR locus D1S80 by the PCR followed by high-resolution PAGE. *Am J Hum Genet*, 48: 137-144, 1991.
17. Mutirangura A, Supiyaphun P, Trirekapan S, Sriuranpong V, Sakuntabhai A, Yenrudi S, Voravud N: Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma. *Cancer Res*, 56: 3530-3533, 1996.
18. Hiyama K, Hiyama E, Ishioka S, Yamakido M, Inai K, Gazdar AF, Piatyszek MA, Shay JW: Telomerase activity in small-cell and non-small-cell lung cancers. *J Natl Cancer Inst*, 87: 895-902, 1995.
19. Shay JW, and Gazdar AF: Telomerase in the early detection of cancer. *J Clin Pathol*, 50: 106-109, 1997.
20. Wymenga LF, Wisman GB, Veenstra R, Ruiters MH, Mensink HJ: Telomerase activity in needle biopsies from prostate cancer and benign prostates. *Eur J Clin Invest*, 30: 330-335, 2000.
21. Sugino T, Yoshida K, Bolodeoku J, Tahara H, Buley I, Manek S, Wells C, Goodison S, Ide T, Suzuki T, Tahara E, Tarin D: Telomerase activity in human breast cancer and benign breast lesions: diagnostic applications in clinical specimens, including fine needle aspirates. *Int J Cancer*, 69: 301-306, 1996.
22. Takahashi C, Miyagawa I, Kummano S, Oshimura M: Detection of telomerase activity in prostate cancer by needle biopsy. *Eur Urol*, 32: 494-498, 1997.
23. Scates DK, Muir GH, Venitt S, Carmichael PL: Detection of telomerase activity in human prostate: a diagnostic marker for prostatic cancer? *Br J Urol*, 80: 263-268, 1997.
24. Murakami J, Nagai N, Ohama K, Tahara H, Ide T: Telomerase activity in ovarian tumors. *Cancer*, 80: 1085-1092, 1997.
25. Hiyama E, Yokoyama T, Tatsumoto N, Hiyama K, Imamura Y, Murakami Y, Kodama T, Piatyszek MA, Shay JW, Matsuura Y: Telomerase activity in gastric cancer. *Cancer Res*, 55: 3258-3262, 1995.
26. Langford LA, Piatyszek MA, Xu R, Schold SC, Shay JW: Telomerase activity: a prognostic indicator in ordinary meningiomas. *Hum Pathol*, 28: 416-420, 1997.
27. Hiyama E, Hiyama K, Yokoyama T, Matsuura T, Piatyszek MA, Shay JW: Correlating telomerase activity with human neuroblastoma outcome. *Nat Med*, 3: 249-255, 1995.
28. Schalken J: Molecular diagnostics and therapy of prostate cancer: new avenues. *Eur Urol*, 34 (suppl 3): 3-6, 1998.
29. Norton JC, Piatyszek MA, Wright W, Shay JW, Corey DR: Inhibition of human telomerase

- activity by peptide nucleic acids. *Nat Biotechnol*, 14: 615-619, 1996.
30. Strahl K, and Blackburn E: Effects of reverse transcriptase inhibitor on telomere length and telomerase activity in two immortalized human tumor cell lines. *Mol Cell Biol*, 16: 53-65, 1996.
31. Izbicka E, Wheelhouse RT, Raymond E, Davidson KK, Lawrence A, Sun D, Windle BE,

Hurley LH, Von Hoff DD: Effects of cationic porphyrins as G-quadruplex interactive agents in human tumor cells. *Cancer Res*, 59: 639-644, 1999.

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