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Original Article

Comparison of telomerase activity in prostate cancer, prostatic intraepithelial neoplasia and benign prostatic hyperplasia

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Abstract

BACKGROUND: Telomerase is a reverse transcriptase enzyme that synthesizes telomeric DNA on chromosome ends. The enzyme is important for the immortalization of cancer cells because it maintains the telomeres.

METHODS: Telomerase activity (TA) was measured by fluorescence-based telomeric repeat amplification protocol (F-TRAP) assay in prostate carcinoma and benign prostatic hyperplasia (BPH).

RESULTS: TA was present in 91.4% of 70 prostate cancers, 68.8% of 16 prostatic intraepithelial neoplasia (PIN), 43.3% of 30 BPH*, 21.4% of 14 atrophy and 20% of 15 normal samples adjacent to tumor. There was not any significant correlation between TA, histopathological tumor stage or gleason score. In contrast to high TA in the BPH* tissue from the cancer-bearing gland, only 6.3% of 32 BPH specimens from patients only diagnosed with BPH were telomerase activity-positive.

CONCLUSIONS: These results indicate that TA is present in most prostate cancers. The high rate of TA in tissue adjacent to tumor may be attributed either to early molecular alteration of cancer that was histologically unapparent, or to the presence of occult cancer cells. Our findings suggest that the re-expression of telomerase activity could be one step in the transformation of BPH to PIN.

KEY WORDS: Telomerase activity, prostate cancer, prostatic intraepithelial neoplasia, benign prostatic hyperplasia.

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Telomerase is a complex ribonucleoprotein enzyme with reverse transcriptase activity that adds hexanucleotide repeats to the end of chromosomes and forms telomeres [1,2]. Telomere shortening is correlated with cell senescence in vitro and cell aging in vivo. The telomere hypothesis suggests that telomere length serves as a mitotic clock for timing cellular replicative lifespan. Expression of telomerase stabilizes telomere length and allows for continual replication or cell immortality. The enzyme contains protein components that include reverse transcriptase activity and a RNA molecule that contains the C-rich strand of telomere repeats in the template region [3,4]. Transcriptase activity is directly involved in telomere maintenance, and its activation may play a role in cell immortality. It has been postulated that the immortalization of tumor cells relies on the aberrant reactivation of telomerase enzyme. The maintaining of telomere length by the re-expression of telomerase has been described in more than 85% of malignant human tumors and it is considered an essential step in tumor progression, promoting cell immortalization [5,6].

Prostate cancer is the most common solid cancer among men and is the second leading cause of cancer deaths. Prostate carcinoma is usually curable if caught in its early stages. The risk of prostate cancer rises with age and continues to increase by 3-4% each year as fewer

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men die from cardiovascular disease [7,8]. We urgently need biochemical and molecular markers to diagnose malignancy and pathological states of human prostate and detect prostate carcinoma in early stages [9].

There is no definitive prognostic parameter for prostate cancer. Histological differentiation, tumor stage, tumor volume, DNA ploidy and PSA are important prognostic indicators, but some cases behave unpredictably. This lack of predictability is inconvenient when different therapeutic approaches should be considered [10-12]. The purpose of this study was to determine and compare telomerase activity in prostate cancer and prostatic intraepithelial neoplasia (PIN), benign prostatic hyperplasia (BPH*), atrophy and normal samples adjacent to tumor. In addition, we evaluated telomerase activity in BPH specimens from patients only diagnosed with BPH.

Methods

Tissue sampling

Tissue samples were obtained from 70 patients who underwent radical prostatectomy for prostatic adenocarcinoma. Specimens had been stored at -80°C in the tissue bank. The slides were prepared from the frozen material and stained with H & E. All samples were histologically characterized as tumor, PIN, BPH*, atrophy or normal. In addition, 31 BPH samples obtained by cold-cup technique from non-cancer patients at the time of transurethral prostatectomy were analyzed for telomerase activity. The TNM system was used for tumor staging [13]. The malignancy grade was evaluated according to the Gleason score.

TRAP assay

Telomerase activity was assessed by using a modified version of the fluorescence-based telomeric repeat amplification protocol (F-TRAP) as described [5,14,15]. Briefly, tumor samples were lysed in 100 µl of freshly prepared CHAPS buffer (10 mmol/L) Tris-HCl (pH 7.5), 1 mmol/L MgCl2, 1 mmol/L EGTA, 10% glycerol, 0.5% 3-[(3-cholaunidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 1 mmol/L 4-(2-aminoethylbenzenesulfonyl fluoride (AEBSF) (Sigma, Hamburg, Germany)), 1 mmol/L dithiothreitol (DTT), and 1 µl (100 U) RNase inhibitor (MBI Fermentas, St. Leon-Rot, Germany) for 30 minutes on ice. All other reagents were purchased from Merck (Darmstadt, Germany) unless stated otherwise. After centrifugation (20,000 × g) for 30 minutes at 4°C, the supernatants were divided into 20-µl aliquots for further use and storage. Protein concentrations were measured using Bradford assay (BioRad, Munich, Germany). TRAP assay was performed in two steps [16,17]. In the elongation step, 5 µg protein was incubated in a final volume of 50 µl of assay buffer containing 20 mmol/L Tris-HCl (pH 8.3), 63 mmol/L KCl, 1.5 mmol/L MgCl2, 0.005% Tween 20, 50 µmol/L of each deoxynucleoside triphosphate (MBI Fermentas), and 10 pmol N,N,N',N'-tetramethyl-6-carboxyfluorescein (TAMRA)-labeled forward primer TS (Eurogentec, Seraing, Belgium) for 30 minutes at 30°C. The samples were extracted once with phenol-chloroform and once with chloroform alone, followed by ethanol precipitation. The pellets were then re-dissolved in 50 µl of assay buffer (as above) supplemented with 10 pmol fluorescein-labeled reverse primer CX-ext (Eurogentec), 2 U Taq-polymerase (MBI Fermentas), and 0.01 attomole of an internal amplification standard (ITAS) prepared as described [18]. This internal standard is necessary for reliable quantification of telomerase activity in different tissue extracts because the polymerase chain reaction (PCR) amplification of telomerase products may be influenced by variable protein concentrations and the presence of inhibitors. Samples were transferred to a thermocycler for 30 cycles of PCR amplification (30 seconds at 95°C, 30 seconds at 50°C, and 30 seconds at 72°C) and subsequently analyzed by capillary electrophoresis (ABI prism 310; Perkin-Elmer, Foster City, CA). The number of cycles was optimized using the cell line L428 [17]. Integrated values were added up for all telomerase products containing five (one repeat beyond the primer dimer size) to 9 telomeric hexamer repeats and calibrated by dividing by

the value obtained for ITAS. Different concentrations (500, 250, 100, and 50 cells/50 µl assay, corresponding to 200, 100, 40, and 20 ng protein/assay, respectively) of the highly telomerase-active cell line L428 served as positive controls. Negative control was predigestion of extracts from a positive control with RNase A (0.5 µg for 10 µl extract, 15 minutes at 37 °C). These were analyzed analogously and used to generate a calibration curve. In this way, telomerase activity of the samples under investigation can be expressed in terms of cell equivalents (CE) corresponding to multiples of the activity of one L428 cell. All telomerase assays were done at least in duplicate. When the ITAS product became undetectable in the presence of exceedingly high relative telomerase levels, dilution steps were performed until quantitation became possible.

The significance of the differences among proportions was evaluated using the chi-square test. The correlation between telomerase activity and other clinicopathological characteristics was determined using t-test. P values less than 0.05 were considered as statistically significant.

Results

Of the 70 prostate cancer tissue specimens evaluated, 64 tumors (91.4%) were positive for telomerase activity. The pathological stage was pT1-2 for 46 (65.7%) patients and pT3-4 for 22 (34.3%) patients. Twenty-five (35.7%) patients had high-grade tumors (Gleason>7), and 45 (64.3%) had low-grade tumors (Gleason≤7). The enzyme activity was positive in 96% (24/25) of high-grade tumors (Gleason>7) and in 88.9% (40/45) of low-grade tumors (Gleason≤7). There was no correlation between telomerase activity, histopathological tumor stage, or Gleason score (P>0.05).

Telomerase activity was present in 11 of 16 (68.8%) PIN cases, 13 of 30 (43.3%) BPH* cases, 3 of 14 (21.4%) atrophy cases, and 3 of 15 (20%) normal samples adjacent to tumor.

In contrast to the BPH* tissue from cancer-bearing gland, only 2 of 32 (6.3%) BPH specimens from patients diagnosed with BPH were telomerase activity-positive (P<0.05).

Since regular amplification of the ITAS was seen with all specimens, lack of telomerase activity was not attributable to the failure of assay (e.g. because of the presence of inhibitors) (figure 1).

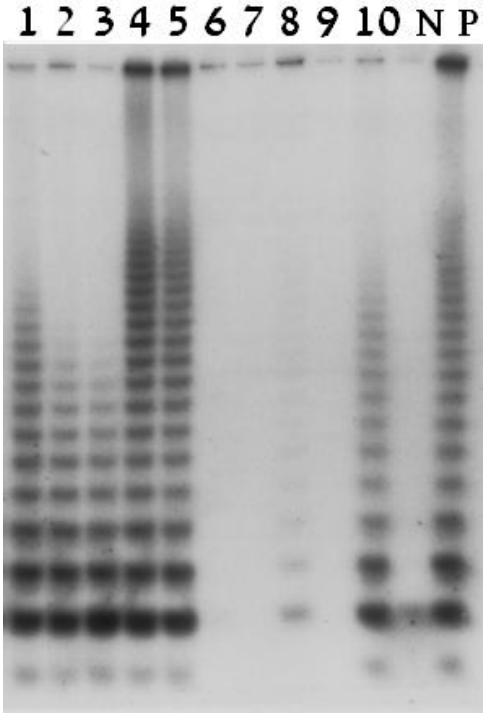

Figure 1. Telomerase activity in prostate carcinoma and other associated tissue histologies. Lines 1 and 2: Prostatic Intraepithelial Neoplasia (PIN); lane 3: Atrophy; lane 4 and 5: Prostate Cancer; lines 6 and 7: Normal; lines 8 and 9: Benign Prostatic Hyperplasia (BPH); line 10: BPH adjacent to tumor (BPH*); line N corresponds to RNase A pretreated sample and line P corresponds to a positive control.

Discussion

Using the telomeric repeat amplification protocol (TRAP) assay, a number of studies have demonstrated telomerase activity in a variety of malignant tumors, irrespective of tumor type. Thus, telomerase activation is likely an essential component of sustained malignant growth. The present study demonstrated detectable telomerase activity in 64 (91.4%) of 70 prostatic carcinomas, which confirms the

* **BPH:** BPH adjacent to tumor

high frequency of telomerase-positive prostatic tumors in earlier reports [7,9,19-22]. These findings suggest that telomerase activation may be crucial in the pathogenesis of human prostatic carcinoma.

We found no correlation between telomerase activity, histopathological tumor stage, or Gleason score. This result is in accordance with the study on prostatic tumors in which no significant correlation was found between telomerase activity and Gleason grade, tumor volume or tumor stage in 75 prostate cancer patients [12]. Sommerfeld et al reported their findings in 25 prostatic carcinomas, emphasizing that all four telomerase-negative tumors were strictly organ-confined and did not exhibit either capsular infiltration or capsular penetration [9]. This result contrasts with the report of Lin et al, who found that the level of telomerase activity in prostate cancer correlated with the primary Gleason pattern (although not with the total Gleason score) using a semiquantitative dilution technique [19].

PIN is a pre-malignant lesion. The prevalence of PIN is significantly higher in prostates harboring cancer (33-100%) than it is in prostates that do not contain invasive cancer (4-18%) [23]. PIN can precede carcinoma by 10 years or more [24]. The molecular pathogenesis of this precancerous lesion is still obscure.

Kim et al [5] reported telomerase activity in three of five cases of PIN and Leite et al reported TA in 6 of 21 cases (29%) of high-grade PIN [12].

We detected telomerase activity in 11 of 16 cases (68.8%) of PIN. The presence of telomerase in a high proportion of PIN cases supports the hypothesis that this lesion is a precursor of prostate cancer and harbors early molecular alterations leading to malignancy. Telomerase activity was detected in 43.3% BPH cases, 21.4% of atrophy cases, and 20% of normal tissue samples adjacent to tumor. All of the telomerase-positive non-cancerous tissue samples were derived from glands bearing cancers that were also telomerase-positive. The high proportion of telomerase-positive non-cancerous tissue is in accordance with data supplied by Wullich et al, who described TA in 45.9% of BPH* cases and 3 (33.3%) of 9 normal epithelium specimens, both from cancer-bearing glands [21]. It is also in agreement with data from other reports [7]. However, it contrasts with studies of Sommerfeld et al [9] and Lin et al [19] identifying TA in only 10-12% of BPH* samples.

Most of (93.7%) BPH specimens from patients who were only diagnosed with BPH were telomerase activity-negative. This contrasts with TA-positive BPH* from tissues adjacent to tumor. It is possible that occult cancer cells deep to the histologically characterized surface of the benign-appearing cores account for the high frequency of telomerase activity in this tissue. Pathological characterization merely evaluates the surface of the core. The TRAP assay is sensitive enough to detect telomerase activity of 1-10 telomerase-expressing cells [25]. This data could also be explained by the provocative hypothesis that early in the development of prostate cancer, telomerase activation may occur before histological alteration. Activation of telomerase in benign-appearing tissue may correlate with continued proliferation beyond the limits of the normal finite prostatic epithelial lifespan. This would represent an important step in the pre-malignant progression toward prostate cancers. This observation also raised the possibility that telomerase detection could provide a more sensitive means than conventional histology for the detection of infiltrating cancer cells in benign-appearing tissue. Conversely, the absence of telomerase activity in histological prostate cancer may portend a very favorable biological phenotype and provide a rationale for conservative management. Our results suggest that the re-expression of telomerase activation may be one step in the transformation of BPH to PIN.

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References

1. Royle NJ. **Telomeres and disease**. *J Pathol* 1996; 180(3):233-235.
2. Chiu CP, Harley CB. **Replicative senescence and cell immortality: the role of telomeres and telomerase**. *Proc Soc Exp Biol Med* 1997; 214(2):99-106.
3. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J et al. **Telomerase catalytic subunit homologs from fission yeast and human**. *Science* 1997; 277(5328):955-959.
4. Rhyu MS. **Telomeres, telomerase, and immortality**. *J Natl Cancer Inst* 1995; 87(12):884-894.
5. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL et al. **Specific association of human telomerase activity with immortal cells and cancer**. *Science* 1994; 266(5193):2011-2015.
6. Watanabe N. **[Telomerase, cell immortality and cancer]**. *Hokkaido Igaku Zasshi* 2001; 76(3):127-132.
7. Botchkina GI, Kim RH, Botchkina IL, Kirshenbaum A, Frischer Z, Adler HL. **Noninvasive detection of prostate cancer by quantitative analysis of telomerase activity**. *Clin Cancer Res* 2005; 11(9):3243-3249.
8. Biroccio A, Leonetti C. **Telomerase as a new target for the treatment of hormone-refractory prostate cancer**. *Endocr Relat Cancer* 2004; 11(3):407-421.
9. Sommerfeld HJ, Meeker AK, Piatyszek MA, Bova GS, Shay JW, Coffey DS. **Telomerase activity: a prevalent marker of malignant human prostate tissue**. *Cancer Res* 1996; 56(1):218-222.
10. Foster CS, McLoughlin J, Bashir I, Abel PD. **Markers of the metastatic phenotype in prostate cancer**. *Hum Pathol* 1992; 23(4):381-394.
11. Steinberg GD, Bales GT, Brendler CB. **An analysis of watchful waiting for clinically localized prostate cancer**. *J Urol* 1998; 159(5):1431-1436.
12. Leite KM, Srougi M, Darini E, Carvalho CM, Camara-Lopes LH. **Telomerase activity in localized prostate cancer: correlation with histological parameters**. *Brazilian Journal of Urology* 2001; 27:341-347.
13. Schroder FH, Hermanek P, Denis L, Fair WR, Gospodarowicz MK, Pavone-Macaluso M. **The TNM classification of prostate cancer**. *Prostate Suppl* 1992; 4:129-138.
14. Krams M, Claviez A, Heidorn K, Krupp G, Parwaresch R, Harms D et al. **Regulation of telomerase activity by alternate splicing of human telomerase reverse transcriptase mRNA in a subset of neuroblastomas**. *Am J Pathol* 2001; 159(5):1925-1932.
15. Rudolph P, Schubert C, Tamm S, Heidorn K, Hauschild A, Michalska I et al. **Telomerase activity in melanocytic lesions: A potential marker of tumor biology**. *Am J Pathol* 2000; 156(4):1425-1432.
16. Krupp G, Kuhne K, Tamm S, Klapper W, Heidorn K, Rott A et al. **Molecular basis of artifacts in the detection of telomerase activity and a modified primer for a more robust 'TRAP' assay**. *Nucleic Acids Res* 1997; 25(4):919-921.
17. Klapper W, Singh KK, Heidorn K, Parwaresch R, Krupp G. **Regulation of telomerase activity in quiescent immortalized human cells**. *Biochim Biophys Acta* 1998; 1442(2-3):120-126.
18. Wright WE, Shay JW, Piatyszek MA. **Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity**. *Nucleic Acids Res* 1995; 23(18):3794-3795.
19. Lin Y, Uemura H, Fujinami K, Hosaka M, Harada M, Kubota Y. **Telomerase activity in primary prostate cancer**. *J Urol* 1997; 157(3):1161-1165.
20. Scates DK, Muir GH, Venitt S, Carmichael PL. **Detection of telomerase activity in human prostate: a diagnostic marker for prostatic cancer?** *Br J Urol* 1997; 80(2):263-268.
21. Wullich B, Rohde V, Oehlenschlager B, Bonkhoff H, Ketter R, Zwergel T et al. **Focal intratumoral heterogeneity for telomerase activity in human prostate cancer**. *J Urol* 1999; 161(6):1997-2001.
22. Athanassiadou P, Bantis A, Gonidi M, Liossi A, Aggelonidou E, Petrakakou E et al. **Telomerase expression as a marker in prostate cancer: correlation to clinicopathologic predictors**. *J Exp Clin Cancer Res* 2003; 22(4):613-618.
23. Troncoso P, Babaian RJ, Ro JY, Grignon DJ, von Eschenbach AC, Ayala AG. **Prostatic intraepithelial neoplasia and invasive prostatic adenocarcinoma in cystoprostatectomy specimens**. *Urology* 1989; 34(6 Suppl):52-56.
24. Sakr WA, Haas GP, Cassin BF, Pontes JE, Crissman JD. **The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients**. *J Urol* 1993; 150(2 Pt 1):379-385.
25. Kim NW. **Clinical implications of telomerase in cancer**. *Eur J Cancer* 1997; 33(5):781-786.