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Prostate Cancer Cell Telomere Length Variability and Stromal Cell Telomere Length as Prognostic Markers for Metastasis and Death

Christopher M. Heaphy[1,*], **Ghil Suk Yoon**[1,2,*], **Sarah B. Peskoe**[3], **Corinne E. Joshu**[3], **Thomas K. Lee**[1], **Edward Giovannucci**[4,5,6], **Lorelei A. Mucci**[5,6], **Stacey A. Kenfield**[5,6], **Meir J. Stampfer**[4,5,6], **Jessica L. Hicks**[1], **Angelo M. De Marzo**[1,7,8], **Elizabeth A. Platz**[3,7,8,**,†], and **Alan K. Meeker**[1,7,8,**,†]

[1]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD

[2]Department of Pathology, Kyungpook National University School of Medicine, Daegu, Korea

[3]Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

[4]Department of Nutrition, Harvard School of Public Health, Boston, MA

[5]Department of Epidemiology, Harvard School of Public Health, Boston, MA

[6]Department of Medicine, Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

[7]James Buchanan Brady Urological Institute, Johns Hopkins School of Medicine, Baltimore, MD

[8]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD

Abstract

Current prognostic indicators are imperfect predictors of outcome in men with clinicallylocalized prostate cancer. Thus, tissue-based markers are urgently needed to improve treatment and surveillance decision-making. Given that shortened telomeres enhance chromosomal instability and such instability is a hallmark of metastatic lesions, we hypothesized that alterations in telomere length in the primary cancer would predict risk of progression to metastasis and prostate cancer death. To test this hypothesis, we conducted a prospective cohort study of 596 surgically treated men who participated in the ongoing Health Professionals Follow-up Study. Men who had the combination of more variable telomere length among prostate cancer cells (cell-to-cell) and shorter telomere length in prostate cancer-associated stromal cells were substantially more likely to progress to metastasis or die of their prostate cancer. These findings point to the translational potential of this telomere biomarker for prognostication and risk stratification for individualized therapeutic and surveillance strategies.

[†]To whom correspondence should be addressed. Elizabeth A. Platz, ScD, MPH, Professor, Department of Epidemiology, John Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Rm E6132, Baltimore, MD 21205: eplatz@jhsph.edu, Alan K. Meeker, PhD, Assistant Professor, Department of Pathology, Johns Hopkins School of Medicine, 411 N. Caroline St, Bond Street B301, Baltimore, MD 21231 ameeker1@jhmi.edu.
[*]These authors contributed equally to this study
[**]These authors contributed equally to this study

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Keywords

Telomere; prostate cancer; prognostic marker

Introduction

Currently used prognostic indicators inadequately predict prostate cancer behavior, particularly in men with clinically-localized disease. To target men with appropriate, individualized treatment strategies and surveillance, new molecular markers that improve prognostic accuracy beyond the currently used indicators – stage, Gleason sum and PSA concentration at diagnosis – are urgently needed.

One such possible molecular marker is telomere length. Telomeres are specialized nucleoprotein structures that are essential for maintaining chromosomal integrity by protecting the ends of chromosomes from degradation and recombination (1-3). Critical telomere shortening is a common abnormality observed early in prostate tumorigenesis, where it likely helps drive malignant transformation and tumor progression via telomere destabilization and concomitant chromosomal instability (4).

Preliminary investigations have suggested that reduced telomere length in prostate cancer tissue may be associated with poor clinical outcome in prostate cancer (5-7). While intriguing, these studies used bulk measures of tissue telomere length, and without individual cell resolution, could not address associations with outcome for telomere length in specific cells types or for variability in telomere length from cell-to-cell. Some have suggested measures of genetic or phenotypic variability or diversity at the cellular level may prove to be useful prognostic biomarkers of cancer behavior (8).

Thus, we prospectively evaluated the association of telomere length and variability in telomere length among prostate cancer cells and other prostate cell types with risk of prostate cancer outcomes, including prostate cancer death, and also non-prostate cancer death in 596 men who were surgically treated for clinically-localized prostate cancer; the men were participants in the large, well-characterized Health Professionals Follow-up Study (HPFS) (https://www.hsph.harvard.edu/hpfs). Using a telomere-specific fluorescence *in situ* hybridization (FISH) assay that provides single cell resolution of telomere length while maintaining tissue architecture, we report below that men whose prostate cancer cells had more variable telomere length from cell to cell and whose prostate cancer-associated stromal (CAS) cells had shorter telomeres, when in combination, were substantially more likely to progress to metastasis and prostate cancer death than other men. Notably, these findings for the telomere biomarker were independent of currently used prognostic indicators, including in men with intermediate risk disease. If confirmed, the biomarker has the potential to aid in making better treatment and surveillance decisions.

Results

Characteristics of the men

The men were 65.3 years old on average at diagnosis, the majority were white, had a prostatectomy Gleason sum of 7 (3+4 or 4+3), had pathologically organ-confined disease, and of those for whom PSA concentration at diagnosis was available, had a PSA concentration <10 ng/mL (Table 1). The mean follow-up times were 10.7 years for biochemical recurrence, 13.1 years for lethal prostate cancer (either progression to distant metastasis or prostate cancer death), and 13.2 years for prostate cancer death and for non-prostate cancer death. The Kaplan-Meier estimates of the cumulative incidences over

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follow-up were: biochemical recurrence 33%, lethal prostate cancer 19%, prostate cancer death 17%, and non-prostate cancer death 56%, all over a maximum follow-up of 23 years. Given the men's characteristics and rates of recurrence, this cohort is relevant to men in the PSA era diagnosed with clinically-localized disease.

Telomere FISH staining provides single cell resolution, allowing high-resolution assessment of telomere length and variability in length from cell to cell

Telomere-specific FISH signals are linearly proportional to telomere length and thus, telomere length can be quantified via digital image analysis (9). As expected, signals were less intense (i.e., telomere length was shorter) in cancer cells, on average, than in adjacent cells in prostate tissue samples from the men in the HPFS. Figure 1 shows representative examples of telomere signals for individual cells. For some men, telomere signals were variable in intensity from cancer cell to cancer cell (Fig. 1A). For other men, telomere signals were less variable in intensity; Fig. 1B shows an example where telomere signals were uniformly diminished in cancer cells. We also observed that telomere signals were decreased in CAS cells in some of the men (Fig. 1C) compared with other men (Fig. 1D).

Shorter telomeres in prostate CAS cells, and more variable telomere length among prostate cancer cells are associated with increased risk of poor prostate cancer outcomes

We assessed telomere length, on a per cell basis, as the ratio of the total intensity of telomeric signals in each cell to the total intensity of the DAPI stained nuclear DNA signal in the same cell (see **Methods**). Then, we examined the association of median telomere length and the standard deviation of telomere length (as a measure of cell-to-cell variability), which we calculated for each man separately by cell type, with prostate cancer outcomes and with non-prostate cancer death after adjusting for commonly used prognostic indicators.

Compared with the longest tertile, the shortest and middle tertiles of median telomere length in CAS cells had a statistically significant increased risk of lethal prostate cancer (shortest: hazard ratio [HR]=2.42, 95% CI 1.16-5.07; middle: HR=2.44, 95% CI 1.17-5.11; *P*-trend=0.02) and prostate cancer death (shortest: HR=2.85, 95% CI 1.22-6.69; middle: HR=3.02, 95% CI 1.31-6.97; *P*-trend=0.02), but not of biochemical recurrence or non-prostate cancer death. Median telomere length in the other cell types assessed (cancer, cancer-associated luminal epithelial, cancer-associated basal epithelial, normal luminal epithelial, normal basal epithelial, and normal stromal) was not associated with the outcomes. Considering telomere length variability, compared with the least variable tertile, the most variable tertile among prostate cancer cells had a higher risk of biochemical recurrence (*P*-trend=0.01), and while not statistically significant, the HR for the most variable tertile compared with the least variable tertile was above 1.0 for both lethal prostate cancer (HR=1.39, 95% CI 0.73-2.67; *P*-trend=0.17) and prostate cancer death (HR=1.52, 95% CI 0.73-3.18; *P*-trend=0.12). This pattern was also evident for non-prostate cancer death (primarily death due to cardiovascular diseases and other cancers; HR=1.26, 95% CI 0.83-1.92; *P*-trend=0.23). Risk was similar when comparing the middle and least variable tertiles among cancer cells for all four outcomes. None of the other cell types assessed had a consistent pattern of association for telomere length variability. We also evaluated the association for telomere length variability from cancer cell to cancer cell using nonparametric measures and the inferences were comparable. The coefficient of variation (CV%; i.e., standard deviation/mean) for telomere length among the cancer cells was not associated with the outcomes.

Based on these findings, we focused on cancer cells and CAS cells for the subsequent analyses. We calculated telomere length and variability in telomere length among the cancer cells and in the CAS cells, and explored the relationship between length and variability in

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length within and between the two cell types. Cancer cells (median=12.9 [ratio of the total intensity of telomeric signals to the total intensity of the DAPI signal]) had substantially shorter telomere length than CAS cells (median=55.8). Cancer cells had a smaller standard deviation for telomere length, but when standardized for the mean length (i.e., the CV%), the relative variability was greater among cancer cells (standard deviation=8.4, CV %=58.1%) than in CAS cells (standard deviation=24.6, CV%=44.4%). Median telomere length in CAS cells increased across tertiles of variability in telomere length (least to most variable tertiles: 43.4, 56.3, 66.7). Variability in telomere length among the prostate cancer cells increased across tertiles of median telomere length in CAS cells (shortest to longest tertiles: 6.4, 8.1, 11.0). For cancer cells, the midpoint of the most variable tertile was 3.6 times larger than the midpoint of the least variable tertile. For the CAS cells, the midpoint of the longest tertile of median telomere length was 2.5 times larger than the midpoint of the shortest tertile of median telomere length, indicating a wide dynamic range in both telomere length and variability in length.

Because the HRs of lethal prostate cancer for the shortest and middle tertiles of median telomere length in CAS cells were similar, we combined those tertiles to form a single “shorter” length group. Likewise, because the HRs of lethal prostate cancer for the least and middle tertiles of variability in telomere length among prostate cancer cells were similar, we combined those tertiles to form a single “less variable” length group.

Prostate cancer cell-to-cell variability in telomere length and CAS cell telomere length are associated with prognostic indicators

We next determined how variability in telomere length among cancer cells and telomere length in CAS cells relate to currently used prognostic indicators. Median variability in telomere length from cancer cell to cancer cell increased with increasing prostatectomy Gleason sum (P-trend=0.0002) and was higher in T3b or worse disease (P=0.05), but did not notably increase with increasing PSA concentration at diagnosis (P-trend=0.45). Median telomere length in CAS cells did not differ across prostatectomy Gleason sum (P-trend=0.38), pathologic stage (P=0.60), or PSA concentration at diagnosis (P-trend=0.11).

More variable telomere length among prostate cancer cells and shorter telomeres in CAS cells are associated with an increased risk of poor prostate cancer outcomes independent of prognostic indicators

Shown in Table 2 are the associations for more versus less variable telomere length among cancer cells and shorter versus longer telomere length in CAS cells for each outcome after a) adjustment for age and year of diagnosis, b) further adjustment for prognostic indicators, and c) because the median telomere length in the CAS cells was related to the variability in telomere length among the cancer cells and vice versa, even further adjustment for shorter length in the variability analysis and for more variability in the shorter length analysis. For lethal prostate cancer and for prostate cancer death, both more variable telomere length among cancer cells and shorter telomeres in CAS cells were associated with increased risk. The association for variability in telomere length among cancer cells was attenuated after adjustment for the prognostic indicators, but was strengthened and was significant after further adjustment for shorter telomeres in CAS cells. The association for shorter telomeres in CAS cells was strengthened after adjustment for the prognostic indicators and even further strengthened after adjustment for variability in telomere length among cancer cells. For biochemical recurrence, more variable telomere length among cancer cells was associated with increased risk even after adjustment for the prognostic indicators, while shorter telomeres in CAS cells was not associated with risk. For non-prostate cancer death, variability in telomere length among cancer cells was not statistically significantly

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associated with risk, although shorter telomere length in CAS cells was possibly, but not statistically significantly, inversely associated.

Defining the "the telomere biomarker" and its association with prognostic indicators

Given that the associations of more variable telomere length from cancer cell to cancer cell and shorter telomeres in CAS cells with prostate cancer outcomes were independent of one another, we combined them to form four groups: less variable [among cancer cells]/longer [in CAS cells] (reference), more variable/longer, less variable/shorter, and more variable/shorter. We refer to the combined groups as "the telomere biomarker". Prostatectomy Gleason sum, pathologic stage, and PSA concentration at diagnosis differed among the telomere biomarker groups (Table 1). The less variable/longer combination tended to have the most favorable clinicopathologic prognostic indicators, the more variable/shorter and more variable/longer combinations tended to have similar and less favorable indicators, and the less variable/shorter combination had indicators that were intermediate (Table 1).

Men with the more variable/shorter combination of the telomere biomarker had poorer disease-free survival than other men

Figure 2 shows survival curves for the four telomere biomarker groups; these results are unadjusted. For biochemical recurrence (Fig. 2A), men with the more variable/shorter and more variable/longer combinations had similarly higher risk over time, men with the less variable/longer combination had the lowest risk, and men with the less variable/shorter combination had an intermediate risk (overall log-rank test comparing the 4 combinations: P=0.002). Men with the more variable/shorter combination were the most likely to experience lethal prostate cancer (Fig. 2B) and prostate cancer death (Fig. 2C) over time, whereas the men with the less variable/longer combination were the least likely to experience these outcomes, and men with the other two combinations had intermediate risk (for both outcomes - logrank test comparing the 4 combinations: P<0.0001; logrank test comparing the more variable/shorter to less variable/longer: P<0.0001). The telomere biomarker groups did not statistically significantly differ (P=0.20) on risk of non-prostate cancer death over time (Fig. 2D), supporting the specificity of the biomarker for prostate-cancer outcomes.

The more variable/shorter combination of the telomere biomarker is strongly associated with increased risk of poor prostate cancer outcomes independent of prognostic indicators

Table 3 shows the association between each telomere biomarker group and outcomes after a) adjustment for age and year of diagnosis, and b) further adjustment for the commonly used prognostic indicators. Men with the more variable/shorter combination were more likely to subsequently progress to lethal prostate cancer and prostate cancer death after adjustment for age and year of diagnosis, and even after further taking the prognostic indicators into account. When compared with men with the less variable/longer combination, men with the more variable/shorter combination had 8 times the risk of lethal prostate cancer (P=0.005) and 14 times the risk of prostate cancer death (P=0.01) after multivariable adjustment. Men with the other two combinations possibly had a higher risk of lethal prostate cancer and prostate cancer death, although the results were not statistically significant. The more variable/shorter combination was weakly associated with biochemical recurrence. The telomere biomarker was not associated with non-prostate cancer death. Taken together, these results support the potential utility of the telomere biomarker as a prognostic indicator specifically of poor prostate cancer outcome.

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Men with the less variable/longer combination of the telomere biomarker were much less likely to die of their prostate cancer than expected, and their time to poor outcome was much longer

Only 1 man (expected 5.8 men) in the less variable/longer combination died of his prostate cancer, whereas 20 men (expected 10.4 men) in the more variable/shorter combination died of their prostate cancers. The time from diagnosis to prostate cancer death was 16.5 years for the former man despite having Gleason 9 (stage T2aN0M0) disease, whereas the median time from diagnosis to prostate cancer death was 8.4 years for the latter men. Adjusting for the prognostic indicators, men with the less variable/longer combination had an HR of lethal prostate cancer of 0.23 (95% CI 0.06-0.93; P=0.04) and an HR of prostate cancer death of 0.13 (95% CI 0.02-0.96; P=0.05) when compared to men with all other telomere biomarker combinations. These findings support that the telomere biomarker may point to men who are unlikely to progress or who may progress more slowly after surgical treatment.

The telomere biomarker adds to the predictive capability of the currently used prognostic indicators

We considered the predictive capability of the telomere biomarker for prostate cancer outcomes relative to the currently used prognostic indicators. In the multivariable model that included the prognostic indicators plus the telomere biomarker, the HRs of lethal prostate cancer and prostate cancer death for the more variable/shorter combination were of the same order of magnitude and statistical significance as for prostatectomy Gleason sum and pathologic stage (Table 4). We also calculated the C-statistic (i.e., the area under the receiver operating characteristics curve) for the telomere biomarker. For lethal prostate cancer, the C-statistic improved from 0.63 to 0.74 when adding the telomere biomarker to the model with age and year of diagnosis. When adding the telomere biomarker to the model with the prognostic indicators stage, prostatectomy Gleason sum, and PSA concentration at diagnosis, the C-statistic improved from 0.85 to 0.87. For prostate cancer death, the C-statistic improved from 0.67 to 0.79 when adding the telomere biomarker to the model with age and year of diagnosis. When adding the telomere biomarker to the model with the prognostic indicators, the C-statistic improved from 0.91 to 0.93. Thus, we have documented that the telomere biomarker is an independent predictor of poor outcome, and it has the potential to add to the predictive capability of the currently used prognostic indicators.

Notably, the telomere biomarker predicts poor outcome even in men with intermediate risk disease

Treatment decision-making for clinically-localized Gleason 7 prostate cancer is difficult because of variable disease course. Thus, we determined whether the telomere biomarker improves prognostication for these men (N=351). When comparing the more variable/shorter combination to all other combinations, the HR of lethal prostate cancer was 3.67 (95% CI 1.60-8.40, P=0.002) in men with Gleason 7 disease (in all men: HR=2.83, 95% CI 1.59-5.03, P=0.0004). For prostate cancer death, the HR was 7.13 (95% CI 2.71-18.77; P<0.0001) in men with Gleason 7 disease (in all men: HR=3.12, 95% CI 1.68-5.77, P=0.0003). Further, among men with Gleason 7 disease, when adding the telomere biomarker to the currently used prognostic indicators, the C-statistic improved from 0.82 to 0.84 for lethal prostate cancer, and from 0.85 to 0.90 for prostate cancer death. Importantly, even in intermediate risk disease, the telomere biomarker may identify men who are more or less likely to experience poor outcome.

Discussion

In this prospective study, men with more variable telomere length from prostate cancer cell to prostate cancer cell and shorter telomeres in prostate cancer-associated stromal cells had 8

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times the risk of progressing to lethal prostate cancer, and 14 times the risk of dying of their prostate cancer when compared with men with less variable telomere length among prostate cancer cells and longer telomeres in prostate-cancer associated stromal cells. These associations were independent of the currently used prognostic indicators. In contrast, men who had less variable telomere length from cancer cell to cancer cell and had longer telomeres in CAS cells were 87% less likely to die of their prostate cancers. The telomere biomarker added to the capability of the currently used prognostic indicators for predicting poor outcome in men surgically treated for clinically-localized prostate cancer, even in men with intermediate risk disease. The excess of prostate cancer deaths in the more variable/shorter combination and the deficit of deaths in the less variable/longer combination suggest that the telomere biomarker may have utility in identifying men who may and may not require additional treatment and enhanced surveillance.

Telomeres are comprised of the repeating hexanucleotide DNA sequence, TTAGGG, bound by the six-member shelterin protein complex (1, 2). This telomere complex maintains chromosomal stability by inhibiting exonucleolytic degradation, inhibiting inappropriate homologous recombination, and preventing the chromosome ends from being recognized as double-strand breaks, thereby averting chromosomal fusions (3, 10). In normal somatic cells, critical telomere shortening leads to p53-dependent senescence or apoptosis (11, 12). In cancer cells, cell cycle checkpoints are typically abrogated. In this setting, critical telomere shortening and chromosomal breakage-fusion-bridge cycles may lead to genomic instability (13). Using high-resolution *in situ* methods, extensive telomere shortening has been observed in cancer cells compared with normal epithelial cells in the vast majority of prostate cancers and in high-grade prostatic intraepithelial neoplasia (4, 14).

Given that dysfunctional telomeres contribute to genomic instability and promotes tumorigenesis (15), we hypothesized that increased telomere shortening in prostate cancer cells would drive the evolution of cell clones capable of invasion, extravasation, and metastasis. Therefore, we expected that prostate cancers possessing the greatest degree of telomere loss would have a more aggressive disease phenotype and thus a worse outcome. While we verified that telomeres were shorter, on average, in cancer cells than in neighboring benign-appearing cells, we found that *variability* in telomere length among the cancer cells, rather than telomere length, was associated with risk of poor outcome. Shorter telomeres in cancer-associated stromal cells were even more strongly associated with risk of poor outcome. The combination of variability in telomere length among cancer cells and telomere length in cancer-associated stromal cells, which we call the “telomere biomarker”, was a stronger predictor of prostate cancer outcome than either alone. Notably, the telomere biomarker was also strongly associated and predictive of outcome in men with Gleason 7 disease.

Only three studies have investigated telomere length and prostate cancer outcomes previously; these were small retrospective studies (5-7) that used DNA extracted from cancer-containing tissue sections. These studies observed statistically significant independent associations of reduced telomeric DNA content, reflecting shorter telomeres, in prostatectomy specimens (5, 7) and in biopsy specimens (6) with risk of prostate cancer recurrence or death. In contrast to those studies, our study was prospective, 5-times larger, and our method provided individual cell resolution, thus enabling us to evaluate the contributions of telomere length differences in specific malignant and benign-appearing cell types to clinical outcome.

Recent studies have observed telomere shortening in cancer-associated stromal cells (16, 17); such shortening may reflect a microenvironment that promotes tumor progression or may be a consequence of the tumor on surrounding cells (16). Regarding the former

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possibility, mounting evidence suggests that microenvironmental alterations may initiate and promote prostate carcinogenesis. During normal development, stromal cells profoundly influence epithelial differentiation. In prostate tumors, the cancer-associated stroma frequently displays an altered gene expression profile (18, 19) and an increase in myofibroblasts and fibroblasts mimicking wound repair, a phenotype known as “reactive stroma” (20). Cunha and colleagues have demonstrated that prostate cancer-associated fibroblasts can induce proliferation and malignant transformation of cultured benign prostate epithelial cells (21). The prostate cancer-associated stroma can help promote tumor progression via several mechanisms including the expression of pro-tumorigenic factors (22). Relevant to our finding of an increased risk of poor outcome in men with shorter stromal telomeres, telomere shortening in fibroblasts has been shown to lead to a senescent phenotype that includes an altered pattern of secreted factors, many of which are known to be tumor promoting, including pro-inflammatory cytokines and matrix-degrading proteases (23). How might the stromal cells develop telomere shortening? While speculative, these reactive stromal cells may be developing telomere shortening as a response to tissue injury caused by the tumor cells. How might the tumor develop increased telomere length variability from cell to cell? Perhaps this variability reflects or leads to more generalized genetic instability, which in multiple cancer types tends to be related to more aggressive features (24). Future studies in which whole genome sequencing is employed in cases with and without high variability in telomere length from cancer cell to cancer cell could help answer this question.

Several aspects of our study merit discussion. With respect to generalizability, the men we studied are highly relevant to men who are being diagnosed with clinicallylocalized disease today. While the majority of men in the study were white, reflecting the demographics of the men who entered the health professions during a prior era, we do not have any evidence that our findings would not also apply to men of other racial/ethnic backgrounds. We selected the largest and usually the highest Gleason sum tumor focus then sampled multiple regions of that tumor focus selected to capture within-tumor heterogeneity. Given our tumor sampling strategy, we could not determine whether the telomere biomarker had different predictive capability by tumor focus in men with multiple foci. We used a method of telomere length determination that we previously documented to be both valid and reliable (9). For the assessment of telomere length we evaluated each cell type, where available. For some men, the tissue microarray (TMA) spots, which were sampled from areas of adenocarcinoma, did not contain sufficient normal-appearing luminal epithelial, basal epithelial, or stromal cells for analysis. Thus, the number of men in those analyses was smaller than for the cancer cell analysis. Cells of each type that were in sharp focus in the digital image of the TMA spots were selected for telomere length determination, but otherwise were not sampled with respect to the appearance of the cells. Nevertheless, the evaluated cells were not a random sample and it was not feasible to evaluate all potentially evaluable cells because the assay in its current implementation is extremely labor intensive. We used the ratio of the total intensity of telomeric signals to the total intensity of the DAPI signal to correct the telomere signals for the amount of DNA that was in the evaluable tissue plane of the stained tissue sections. We confirmed that variability in DAPI signals, which, in theory would be higher in aneuploid cancer cells, did not explain our findings (data not shown).

The number of men who experienced progression to metastasis and prostate cancer death was relatively small especially when we divided the men into the four telomere biomarker groups. In the less variable/longer combination, only 1 man died of prostate cancer; this group had a reduced risk of the outcome and thus a deficit of events is expected. Indeed, if the four telomere biomarker groups each had had the same risk of poor outcome, then the number of prostate cancer deaths expected in each group each exceeded 5. While variability

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in telomere length among the cancer cells captured some of the same risk prediction as pathologic stage and grade, the telomere biomarker associations with outcome were independent of the currently used prognostic indicators and telomere biomarker added to the predictive capability of the prognostic indicators. The residual prediction suggests that the telomere biomarker may capture other features of disease aggressiveness that stage and grade do not capture.

In summary, we have identified that the combination of more variable telomere length among prostate cancer cells and shorter telomeres in prostate-cancer associated stromal cells is potentially a new and independent tissue-based marker of prognosis in men surgically treated for clinically-localized prostate cancer, including in men with intermediate risk disease. Individually both telomere measurements are associated with an increased risk of lethal prostate cancer and prostate cancer death, but in combination (i.e. the telomere biomarker), these measurements are even more strongly positively associated with these outcomes. Future steps toward verifying the prognostic utility of the telomere biomarker include automating the assay for increased throughput and application to other cohorts of men. Also, future studies should address the utility of the telomere biomarker as a prognostic tool at the time of biopsy and in risk stratification for individualizing treatment and surveillance strategies.

Materials and Methods

Study Population

The study population was drawn from men participating in the Health Professionals Follow-up Study (HPFS), an ongoing prospective cohort study on risk factors for cancer and other chronic diseases (https://www.hsph.harvard.edu/hpfs). In 1986, 51,529 men aged 40-75 years old enrolled. We asked them to complete a mailed questionnaire on their medical history and lifestyle factors at baseline and then again every two years. The conduct of the HPFS was approved by the Human Subjects Committee of the Harvard School of Public Health. The study on telomere length in prostate tissue and risk of aggressive prostate cancer was additionally approved by the Institutional Review Board at the Johns Hopkins Bloomberg School of Public Health.

Ascertainment of Prostate Cancer Cases and Their Follow-up

On each follow-up questionnaire, we asked the men to report a diagnosis of prostate cancer. We were able to obtain medical records and pathology reports pertaining to their diagnosis for 94.5% of the men who reported a prostate cancer diagnosis or for whom prostate cancer was mentioned on the death certificate. We abstracted TNM stage and PSA concentration at diagnosis from these records. We followed these men from the date of their diagnosis through January 2010 for the development of biochemical recurrence, distant metastasis, prostate cancer death, and non-prostate cancer death. The diagnosis of biochemical recurrence and distant metastasis (to bone or other organs) was collected by mailed questionnaire and then confirmed by the treating doctor. We learned of a participant's death from family members, the postal system, or by searches of the National Death Index, which is estimated to have a sensitivity of more than 98% (25). Men were classified as having died from their prostate cancer if they had documented extensive metastatic disease. Follow-up for death is more than 98% complete for the HPFS cohort.

Confirmation of Pathologic Tumor Characteristics and Construction of Tissue Microarrays (TMAs)

After receiving participant permission, we requested tissue blocks of the prostatectomy specimens for the men who underwent surgical treatment for their prostate cancer from

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hospitals around the US. Study pathologists re-reviewed H&E-stained tissue sections containing prostate cancer and assigned a standardized Gleason sum as previously described (26); we used this Gleason sum in the analyses. We used five TMAs that were constructed for 631 prostate cancer cases as previously described (27). Briefly, a study pathologist selected the tumor focus that was the largest and/or had the highest Gleason sum, selected at least three areas of that focus, and then sampled them using 0.6 mm biopsy needles. For this analysis on telomere length, we excluded one man whose date of diagnosis and death were the same, men who had a prior history of a different primary cancer (N=7), and men who were diagnosed with prostate cancer incidentally after having undergone a transurethral resection of the prostate for the treatment of symptomatic benign prostatic hyperplasia (N=27). After these exclusions, 596 men were available for this analysis.

Measurement of Telomere Length Using FISH

FISH staining—Telomere length was assessed by telomere-specific fluorescence *in situ* hybridization (FISH) staining for telomeric DNA as previously described (9). Deparaffinized TMA slides were hydrated through a graded ethanol series, placed in deionized water, followed by deionized water plus 0.1% Tween-20. The TMA slides were steamed for 14 minutes (Black and Decker Handy Steamer Plus; Black and Decker) in citrate buffer (catalog No. H-3300; Vector Laboratories), removed and allowed to cool at room temperature for 5 minutes. The TMA slides were placed in PBS with Tween (PBST; catalog No. P-3563; Sigma) for 5 minutes, thoroughly rinsed with deionized water, followed by 95% ethanol for 5 minutes, and then air-dried. Twenty-five μL of a Cy3-labeled telomere-specific peptide nucleic acid hybridization probe (0.3 μg/mL peptide nucleic acid in 70% formamide, 10 mmol/L Tris, pH 7.5, 0.5% B/M Blocking reagent (catalog No. 1814-320; Boehringer-Mannheim) was applied, coverslipped, and denaturated by incubation for 4 minutes at 83°C. The TMA slides were then hybridized at room temperature for 2 hours in the dark. Following hybridization, the coverslips were then carefully removed and the slides were washed twice in peptide nucleic acid wash solution (70% formamide, 10 mmol/L Tris, pH 7.5, 0.1% albumin (from 30% albumin solution, catalog No. A-7284; Sigma) for 15 minutes each. The slides were rinsed in PBST followed by application of primary antibody (anticytokeratin antibody 34βE12, catalog no. 30904; Enzo Diagnostics, Farmingdale, NY) and incubated overnight at 4°C. After the incubation, the TMA slides were rinsed in PBST followed by application of fluorescent secondary antibody labeled with Alexa Fluor 488 (Molecular Probes) diluted 1:100 in Dulbecco's PBS, and incubated at room temperature for 30 minutes. The TMA slides were then rinsed in PBST, thoroughly washed in deionized water, drained and counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (500 ng/mL in deionized water, Sigma Chemical Co. Cat #D-8417) for 5 minutes at room temperature. The TMA slides were then mounted with Prolong anti-fade mounting medium (catalog No. P-7481; Molecular Probes) and imaged. The peptide nucleic acid probe complementary to the mammalian telomere repeat sequence was obtained from Applied Biosystems. The probe has the sequence (N-terminus to C-terminus) CCCTAACCCTAACCCTAA with an N-terminal covalently linked Cy3 fluorescent dye. As a positive control for hybridization efficiency, a FITC-labeled peptide nucleic acid probe having the sequence ATTCGTTGGAAACGGGA with specificity for human centromeric DNA repeats (CENP-B binding sequence) was also included in the hybridization solution.

Microscopy—The TMA slides were imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, CA) using a 40X/0.95 NA PlanApo lens with correction collar. Fluorescence excitation/emission filters are as follows: Cy3 excitation, 546 nm/10 nm BP; emission, 578 nm LP (Carl Zeiss Inc.); DAPI excitation, 330 nm; emission, 400 nm via an XF02 fluorescence set (Omega Optical, Brattleboro, VT); Alexa Fluor 488 excitation, 475 nm; emission, 535 via a B-2E/C

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filter set. For each color channel, separate grayscale images were captured using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital cooled CCD camera, and saved as 12-bit uncompressed TIFF files for use in downstream image analysis. Exposure times were set such that fluorescence signal saturation was avoided. Integration times typically ranged from 400 to 800 milliseconds for Cy3 (telomere) and FITC (centromere) signal capture, 50 to 100 milliseconds for the DAPI nuclear counter-stain, and 100 ms to 400 ms for Alexa Fluor 488-conjugated antibodies. For cases with differing exposure times, arithmetic adjustment was made based on the known linear response characteristics of the imaging system. In all cases, telomeric signals were within the linear response range of the charge-coupled device camera, which was confirmed by use of fluorescent microbead intensity standards (InSpeck microscope image intensity calibration fluorescent microspheres; Molecular Probes).

Telomere length assessment—The digitized fluorescent telomere FISH signals were quantified using the open source, JAVA-based image analysis software package ImageJ (http://rsb.info.nih.gov/ij/) and a custom designed plugin ("Telometer"; http://demarzolab.pathology.jhmi.edu/telometer/). Matched telomeric and nuclear DNA grayscale TIFF image files were normalized by simple background subtraction, and the resulting telomere image was then run through a sharpening filter, followed by enhancement using a rolling ball algorithm for contouring of telomeric spots. A binarized mask of the telomere signals was then created and applied to the original unfiltered Cy3 telomere fluorescence image for data extraction. Data were recorded on an individual cell basis. For each cell, a region of interest was manually defined on the DAPI image by use of the freeform drawing tool in ImageJ. Guidance for cell type selection was provided by comparison to a separate 3-color merged image showing the combined DAPI, the telomere stain as well as the immunofluorescence stain; in this case delineating benign prostatic basal cells. Telomeric signals identified by the binary segment mask, which were contained within the area inscribed by each circled nuclear DNA (DAPI) signal area, were then measured, and the data for each telomeric spot was tabulated. The total DAPI (DNA) fluorescence signal for each selected nucleus was likewise quantified. For each selected cell, the individual telomere intensities were summed ("telomere sum"), and this total was divided by the total DAPI fluorescence signal ("DAPI sum") for that same nucleus. This normalization to the nuclear DAPI signal corrects for differences in nuclear cutting planes and ploidy.

In TMA spots containing cancer, we evaluated prostate adenocarcinoma cells and the following cancer-associated cell types: benign-appearing prostate luminal epithelial, basal epithelial, and stromal (fibroblasts and smooth muscle). A small number of TMA spots did not contain cancer because of purposive sampling or because the cancer focus was exhausted during prior serial sectioning, leaving only benign-appearing tissue (N=133 men). In these TMA spots, we were able to evaluate benign-appearing prostate luminal epithelial, basal epithelial, and stromal cells. For each of the above cell types, we selected and analyzed 30 to 50 individual cells per man; not all cell types were available for evaluation for each man. For all TMA spots, other cell types, such as infiltrating lymphocytes, were excluded from the image analysis based on morphologic features. Tabulated data were stored in a MySQL (http://www.mysql.com) database and viewed through Microsoft Access (Microsoft Corp.).

Statistical Analysis

For each man and each of his cell types, we calculated (i) the median ratio of telomere sum to DAPI sum as the measure of central tendency; (ii) the standard deviation and the 25^{th} to 75^{th}, 10^{th} to 90^{th}, and 5^{th} to 95^{th} percentile ranges as measures of variability from cell to cell; and (iii) the coefficient of variation (the standard deviation divided by the mean) as a

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standardized measure of variability. We divided the distribution of these measures into tertiles. We combined over adjacent tertiles that had similar associations. After viewing these results, we combined over telomere length in the CAS cells (shorter/longer) and variability in telomere length among the prostate cancer cells (more/less) to create four groups. We characterized the men by their demographic and prognostic indicators overall and by the combination of telomere length in CAS cells and variability in telomere length among the prostate cancer cells and tested for differences across the combinations using the chi-square test for proportions and one-way ANOVA for means. We determined whether length or variability in length differed across prognostic indicators – pathologic stage, prostatectomy Gleason sum, and PSA concentration at diagnosis.

To evaluate the association of telomere length, variability in length, and the telomere biomarker with prostate cancer outcomes and non-prostate cancer death, we generated two analytic cohorts. For progression to biochemical recurrence and lethal prostate cancer (defined as the subsequent development of distant metastasis or prostate cancer death), the analytic cohort consisted of men with clinically-localized disease without pathologic stage N1 or M1 (excluded N=7, total N=589 of which 560 were in the telomere biomarker analysis) at the time of prostatectomy. For prostate cancer death and non-prostate cancer death, the analytic cohort consisted of men with clinically-localized disease irrespective of pathologic stage (N=596). For each outcome, we generated Kaplan-Meier curves for the four telomere biomarker groups and tested differences in the curves using the log-rank test. Separately by cell type, we estimated the hazard ratio (HR) and 95% confidence interval (CI) of each outcome using Cox proportional hazards regression. For telomere length in CAS cells and for telomere length variability among prostate cancer cells, we ran three models that were (i) adjusted for age (continuous) and calendar year (continuous) at diagnosis; (ii) further adjusted for prostatectomy Gleason sum (indicator variables: 3+4, 4+3, ≥8, versus ≤6 (reference)), pathologic TNM stage (≥T3b versus < T3b (reference)) and PSA at diagnosis (indicator variables: 10-20, >20, unknown, versus <10 ng/mL (reference)); and (iii) additionally mutually adjusted for telomere length and variability in length. For the combination of telomere length and variability in telomere length, we also ran models (i) and (ii). We calculated the C-statistic (28) for the model that included age and date of diagnosis, and the model the further included the prognostic indicators. Then, we added the telomere biomarker to these models. All analyses were performed using SAS v 9.2 (SAS Institute, Cary, NC). All statistical tests were two-sided, with $P<0.05$ considered to be statistically significant.

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Statement of Significance

In this prospective study, the combination of more variable telomere length among cancer cells and shorter telomere length in cancer-associated stromal cells was strongly associated with progression to metastasis and prostate cancer death, pointing to the translational potential for prognostication and risk stratification for individualized therapeutic and surveillance strategies.

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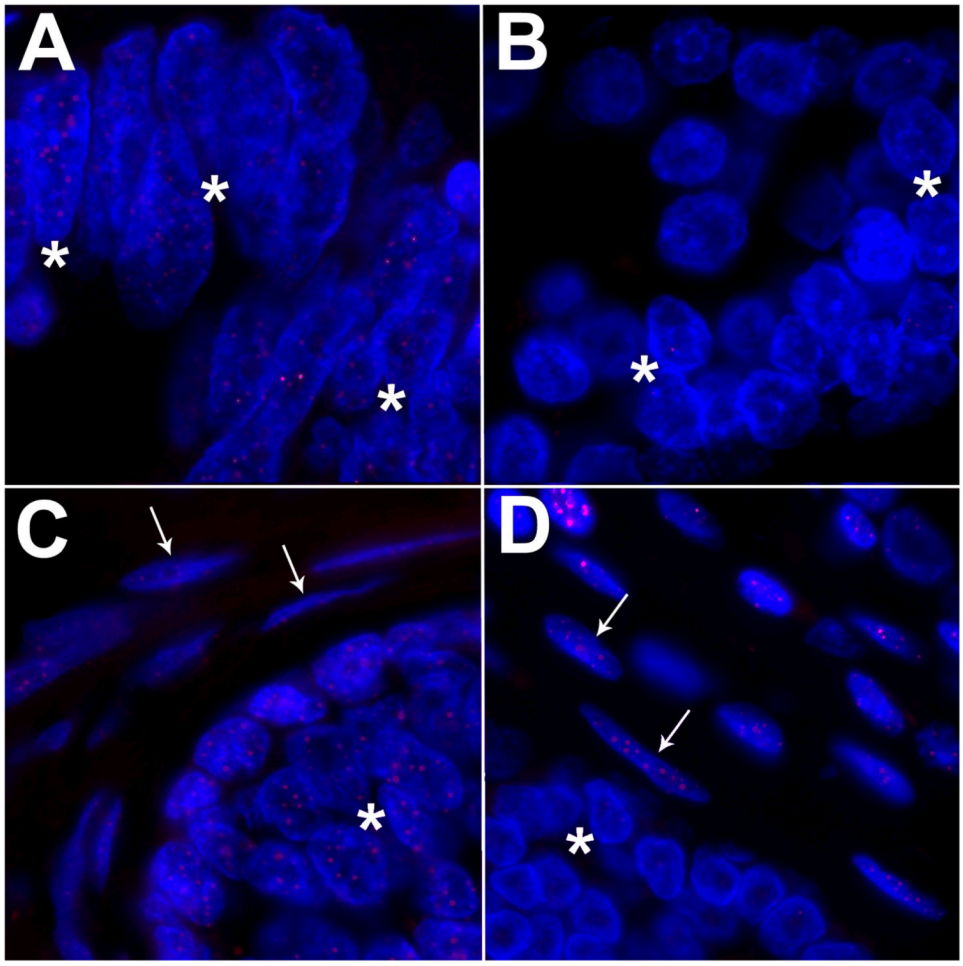

Figure 1. Telomere-specific FISH in prostate adenocarcinomas

Panels A–D show examples of telomere length and cell-to-cell variability in telomere length in malignant and benign prostate tissue from men in the Health Professionals Follow-up Study who were surgically treated for clinically-localized prostate cancer. **(A)** This case has strikingly variable telomere signals among the cancer cells. **(B)** This case has extremely short telomere signals and low variability in telomere length from cancer cell to cancer cell. **(C)** This case has weak telomere signals in the cancer-associated stromal cells. **(D)** This case has strong telomere signals in cancer-associated stromal cells. In all the images, the DNA is stained with DAPI (blue) and telomere DNA is stained with the Cy3-labeled telomere-specific peptide nucleic acid probe (red). Of note, the centromere DNA, stained with the FITC-labeled centromere-specific peptide nucleic acid probe, has been omitted from the image to emphasize the differences in the telomere lengths. In all panels, the asterisks highlight the cancer cells and the arrows point to the cancer-associated stromal cells. Original magnification × 400.

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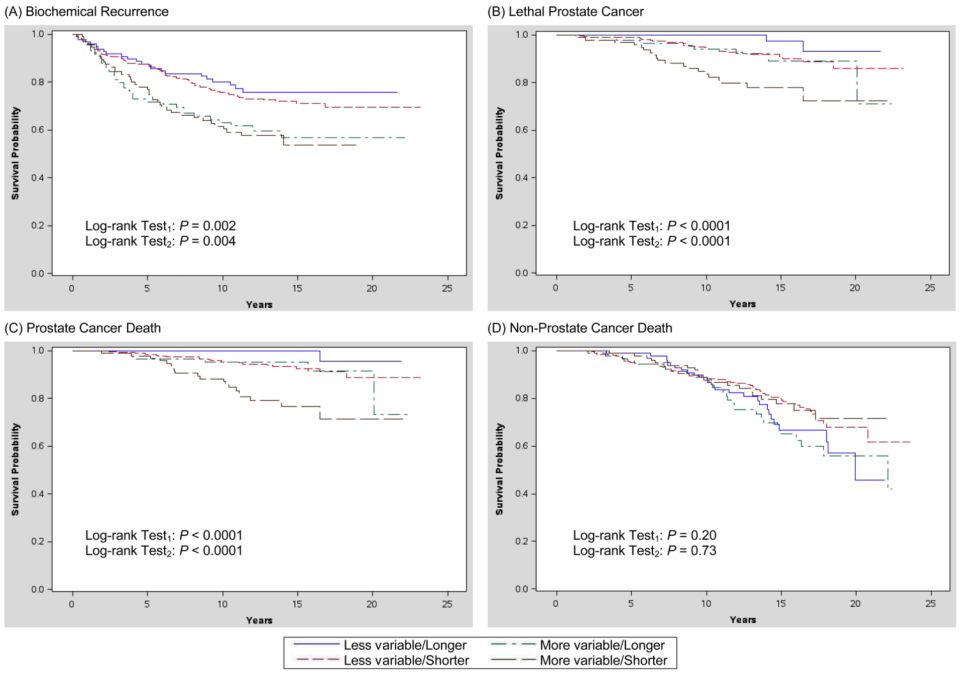

Figure 2. Prostate cancer outcome-specific survival and non-prostate cancer survival by the telomere biomarker combination of more variability in telomere length among prostate cancer cells and shorter telomeres in prostate cancer-associated stromal cells, Health Professionals Follow-up Study

(A) With respect to biochemical recurrence, men with the more variable/shorter and more variable/longer combinations had similarly higher risk over time, men with the less variable/longer combination had the lowest risk, and men with the less variable/shorter combination had an intermediate risk. **(B and C)** With respect to lethal prostate cancer and death from prostate cancer, men with the more variable/shorter combination were the most likely to experience these outcomes over time, whereas the men with the less variable/longer combination were the least likely to experience these outcomes, and men with the other two combinations had intermediate risk. **(D)** With respect to non-prostate cancer death, the telomere biomarker was not associated with risk, supporting the specificity of the biomarker for prostate cancer outcomes.

Log-rank $Test_1$: compares the survival distributions across all 4 telomere biomarker categories

Log-rank $Test_2$: compares the survival distributions of men with the less variable/longer combination to men with more variable/shorter combination of the telomere biomarker

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Table 1

Characteristics of prostate cancer cases at the time of prostatectomy, Health Professionals Follow-up Study

	All Men†	Telomere length among prostate cancer cells and telomere length in prostate cancer-associated strom cells ‡				
		Less variable/longer	More variable/longer	Less variable/longer	More variable/longer	*p1*
Number of men	596	98	91	280	98	
Mean ± standard deviation age at diagnosis (years)	65.3 ±6.1	65.7 ± 6.4	65.6 ± 6.2	65.0 ± 6.0	65.9 ± 6.2	0.51
White (%)	91.1	87.8	89.0	93.9	87.8	0.12
Mean ± standard deviation year of diagnosis (years)	1994.2 ± 3.2	1994.7 ± 3.3	1994.6 ± 3.4	1993.8 ± 3.1	1994.5 ± 3.1	0.04
Prostatectomy Gleason sum (%)						
≤6	21.3	21.4	11.0	23.9	13.3	
3+4	35.8	42.9	35.1	35.7	30.6	
4+3	24.8	23.5	29.7	25.4	26.5	0.006
≥8	18.1	12.2	24.2	15.0	29.6	
Pathologic stage ≥T3b (%)	12.6	5.1	18.7	12.1	17.3	0.02
Serum PSA concentration at diagnosis in ng/mL (%)						
<10	59.2	73.5	54.9	57.5	54.1	
10-20	17.6	14.3	17.6	17.5	20.4	
>20	10.1	4.1	14.3	10.7	11.2	0.17
Unknown	13.1	8.1	13.2	14.3	14.3	

*1*From a chi-square test for proportions and a one-way ANOVA for means

†Among all men

‡Among men for whom telomere length could be determined for both prostate cancer cells and prostate cancer-associated stromal cells (N=567)

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Table 2

Association of more variable telomere length among prostate cancer cells and shorter telomere length in prostate cancer-associated stromal cells with risk of biochemical recurrence, lethal prostate cancer, prostate cancer death, and non-prostate cancer death, Health Professionals Follow-up Study*

	Biochemical recurrence †		Lethal prostate cancer †		Prostate cancer death		Non-prostate cancer death	
	More variable length among cancer cells	Shorter length in associated stromal cells	More variable length among cancer cells	Shorter length in associated stromal cells	More variable length among cancer cells	Shorter length in associated stromal cells	More variable length among cancer cells	Shorter length in associated stromal cells
Age and year of diagnosis adjusted								
HR	1.88	0.92	2.56	1.95	3.07	2.37	1.29	0.73
95% CI	(1.39-2.55)	(0.67-1.26)	(1.50-4.37)	(1.01-3.78)	(1.71-5.51)	(1.11-5.08)	(0.91-1.82)	(0.52-1.02)
Multivariable adjusted‡								
HR	1.51	0.96	1.64	2.43	1.78	2.94	1.25	0.73
95% CI	(1.11-2.07)	(0.70-1.31)	(0.94-2.86)	(1.24-4.76)	(0.96-3.30)	(1.35-6.39)	(0.88-1.78)	(0.52-1.02)
Additionally mutually adjusted§								
HR	1.55	1.10	2.21	3.39	2.39	4.18	1.17	0.76
95% CI	(1.12-2.15)	(0.79-1.54)	(1.24-3.95)	(1.65-6.98)	(1.26-4.51)	(1.8-9.67)	(0.81-1.68)	(0.54-1.08)

*Associations are reported as hazard ratios (HR) and 95% confidence intervals (95% CI) for the following comparisons: more variable (top tertile of variability) in telomere length among prostate cancer cells versus less variable (bottom and middle tertiles), and shorter (shortest and middle tertiles) median telomere length in CAS cells versus longer (longest tertile).

†Restricted to men without metastatic prostate cancer at the time of diagnosis.

‡Adjusted for age (continuous) and year (continuous) of diagnosis, prostatectomy Gleason sum (categorical: ≤6, 3+4, 4+3, ≥8), pathologic TNM stage (categorical ≥T3b) and serum PSA concentration at diagnosis (categorical: <10, 10-20, >20 ng/mL, unknown).

§Association with more variable telomere length among prostate cancer cells further adjusted for shorter telomere length in prostate cancer-associated stromal cells; association with shorter telomere length in prostate cancer-associated stromal cells further adjusted for more variable telomere length among prostate cancer cells.

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Table 3

Association of the telomere biomarker* with risk of biochemical recurrence, lethal prostate cancer, prostate cancer death, and non-prostate cancer death, Health Professionals Follow-up Study

Outcome	Events/Person-years	Age and year of diagnosis adjusted			Multivariable adjusted‡		
		HR	(95% CI)	*P*	HR	(95% CI)	*P*
Biochemical recurrence†							
Less variable/Longer	22/1,083	1.00	(reference)	--	1.00	(reference)	--
More variable/Longer	35/841	1.96	(1.15-3.34)	0.014	1.42	(0.82-2.45)	0.21
Less variable/Shorter	74/3,149	1.12	(0.69-1.80)	0.66	1.02	(0.63-1.66)	0.93
More variable/Shorter	40/880	2.12	(1.26-3.57)	0.005	1.67	(0.98-2.83)	0.06
Lethal prostate cancer†,§							
Less variable/Longer	2/1,304	1.00	(reference)	--	1.00	(reference)	--
More variable/Longer	8/1,137	4.49	(0.95-21.16)	0.06	2.48	(0.52-11.93)	0.26
Less variable/Shorter	24/3,733	3.93	(0.93-16.66)	0.06	3.74	(0.88-15.96)	0.07
More variable/Shorter	20/1,148	12.31	(2.87-52.75)	0.0007	8.12	(1.88-34.97)	0.005
Prostate cancer death§							
Less variable/Longer	1/1,312	1.00	(reference)	--	1.00	(reference)	--
More variable/Longer	6/1,163	6.68	(0.80-55.55)	0.08	3.76	(0.44-31.79)	0.22
Less variable/Shorter	19/3,822	6.21	(0.83-46.46)	0.08	6.23	(0.82-47.06)	0.08
More variable/Shorter	20/1,194	24.59	(3.29-183.62)	0.002	14.10	(1.87-106.49)	0.01
Non-prostate cancer death							
Less variable/Longer	28/1,312	1.00	(reference)	--	1.00	(reference)	--
More variable/Longer	30/1,163	1.29	(0.77-2.17)	0.33	1.21	(0.71-2.07)	0.48
Less variable/Shorter	65/3,822	0.81	(0.52-1.26)	0.35	0.78	(0.50-1.23)	0.28
More variable/Shorter	21/1,194	0.92	(0.52-1.62)	0.76	0.88	(0.49-1.57)	0.66

*The combination of variability in telomere length among prostate cancer cells and telomere length in prostate cancer-associated stromal cells.

†Restricted to men without metastatic prostate cancer at the time of diagnosis.

‡Adjusted for age (continuous) and year (continuous) of diagnosis, prostatectomy Gleason sum (categorical: ≤6, 3+4, 4+3, ≥8), pathologic TNM stage (categorical ≥T3b) and serum PSA concentration at diagnosis (categorical: <10, 10-20, >20 ng/mL, unknown).

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[§] Using the group with the largest sample size – less variable/shorter – as the reference, the HRs are as follows: lethal prostate cancer – less variable/longer 0.27 (P=0.07), less variable/shorter 0.66 (P=0.33), more variable/shorter 2.17 (P=0.02); death from prostate cancer - less variable/longer 0.16 (P=0.08), less variable/shorter 0.61 (P=0.30), more variable/shorter 2.26 (P=0.02).

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Table 4

Hazard ratios (HR) of lethal prostate cancer and prostate cancer death for the telomere biomarker* and the currently used prognostic characteristics, Health Professionals Follow-up Study

Outcome	HR‡	(95% CI)	*P*
Lethal Prostate Cancer†			
Telomere biomarker			
Less variable/Longer	1.00	(ref)	--
More variable/Longer	2.48	(0.52-11.93)	0.26
Less variable/Shorter	3.74	(0.88-15.96)	0.07
More variable/Shorter	8.12	(1.88-34.97)	0.005
Prostatectomy Gleason sum			
≤6	0.27	(0.03-2.17)	0.22
3+4	1.00	(ref)	--
4+3	3.77	(1.59-8.96)	0.003
≥8	3.86	(1.57-9.49)	0.003
Pathologic stage ≥T3b	4.20	(2.27-7.75)	<0.0001
Serum PSA concentration at diagnosis (ng/mL)			
<10	1.00	(ref)	--
10-20	1.13	(0.52-2.45)	0.76
>20	1.12	(0.48-2.58)	0.80
Unknown	1.55	(0.68-3.55)	0.30
Prostate Cancer Death			
Telomere biomarker			
Less variable/Longer	1.00	(ref)	--
More variable/Longer	3.76	(0.44-31.79)	0.22
Less variable/Shorter	6.23	(0.82-47.06)	0.08
More variable/Shorter	14.10	(1.87-106.49)	0.01
Prostatectomy Gleason sum			
≤6§	0.00	--	0.99
3+4	1.00	(ref)	--
4+3	2.93	(1.04-8.25)	0.04
≥8	4.45	(1.6-12.44)	0.004
Pathologic stage ≥T3b	5.08	(2.63-9.84)	<0.0001
Serum PSA concentration at diagnosis (ng/mL)			
<10	1.00	(ref)	--
10-20	0.71	(0.26-1.94)	0.50
>20	1.19	(0.47-3)	0.71
Unknown	2.83	(1.21-6.63)	0.02

*The combination of variability in telomere length among cancer cells and telomere length in cancer-associated stromal cells.

†Restricted to men without metastatic prostate cancer at the time of diagnosis.

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[‡] Mutually adjusted and adjusted for age (continuous) and year (continuous) of diagnosis.

[§] No prostate cancer deaths occurred in these men. Combining ≤6 and 3+4 as the reference, the HRs of prostate cancer death were 4.54 (95% CI 1.60-12.91; *P*=0.004) for 4+3, and 6.84 (95% CI 2.42-19.34; *P*=0.0003) for ≥8. The HRs of prostate cancer death were unchanged for the telomere biomarker (Less variable/Longer: HR=1.00 (ref); More variable/Longer: HR=3.80 (95% CI 0.45-32.13; *P*=0.22); Less variable/Shorter: HR=6.19 (95% CI 0.82-46.74; *P*=0.08); More variable/Shorter: HR=14.21 (95% CI 1.88-107.28; *P*=0.01)).