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ORIGINAL ARTICLE

The prostate tissue-based telomere biomarker as a prognostic tool for metastasis and death from prostate cancer after prostatectomy

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Abstract

Current biomarkers are inadequate prognostic predictors in localized prostate cancer making treatment decision-making challenging. Previously, we observed that the combination of more variable telomere length among prostate cancer cells and shorter telomere length in prostate cancer-associated stromal cells – the telomere biomarker – is strongly associated with progression to metastasis and prostate cancer death after prostatectomy independent of currently used pathologic indicators. Here, we optimized our method allowing for semi-automated telomere length determination in single cells in fixed tissue, and tested the telomere biomarker in five cohort studies of men surgically treated for clinically localized disease ($N = 2{,}255$). We estimated the relative risk (RR) of progression to metastasis ($N = 311$) and prostate cancer death ($N = 85$) using models appropriate to each study's design adjusting for age, prostatectomy stage, and tumor grade, which then we meta-analyzed using inverse variance weights. Compared with men who had less variable telomere length among prostate cancer cells and longer telomere length in prostate cancer-associated stromal cells, men with the combination of more variable and shorter telomere length had 3.76 times the risk of prostate cancer death (95% confidence interval [CI] 1.37–10.3, $p = 0.01$) and had 2.23 times the risk of progression to metastasis (95% CI 0.99–5.02, $p = 0.05$). The telomere biomarker was associated with prostate cancer death in men with intermediate risk disease (grade groups 2/3: RR = 9.18, 95% CI 1.14–74.0, $p = 0.037$) and with PTEN protein intact tumors (RR = 6.74, 95% CI 1.46–37.6, $p = 0.015$). In summary, the telomere biomarker is robust and associated with poor outcome independent of current pathologic indicators in surgically treated men.

Keywords: biomarker; prognosis; prostate cancer; metastasis; telomeres

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Introduction

Currently used pathologic prognostic indicators do not adequately predict prostate cancer behavior in men with clinically localized disease [1]. To target men with appropriate, individualized treatment strategies or surveillance, new molecular markers that improve prognostic accuracy beyond the currently used pathologic stage, and

Gleason sum (or grade group) are urgently needed. One such molecular tissue-based marker is the measurement of telomeres – the repetitive DNA sequence at the ends of the chromosomes, which are pivotal for maintenance of genome integrity [2–4]. Telomere dysfunction is common in precancerous lesions (e.g. high-grade prostatic intraepithelial neoplasia) and continued critical telomere shortening and chromosomal breakage–fusion–bridge cycles lead to chromosomal instability, thereby driving malignant transformation and cancer progression [5,6].

In our prior cohort study, we discovered that men surgically treated for clinically localized disease who had more variable telomere length among cancer cells and shorter telomere length in prostate cancer-associated stromal cells (i.e. the 'telomere biomarker') had a substantially higher risk of progression to metastasis and prostate cancer death than men who had less variable telomere length in cancer cells and longer telomere length in cancer-associated stromal cells [7]. Importantly, these findings were independent of the pathologic prognostic indicators and added prognostic information to those indicators including in men with Gleason 7 disease, who tend to have a more variable course. Notably, men with the less variable/longer combination of the telomere biomarker rarely died of their cancer over 15 years. The telomere biomarker was not prognostic for death from other causes, supporting its specificity for aggressive disease. Moreover, we found that variability in telomere length among cancer cells, one component of the telomere biomarker, was associated with recurrence after surgery. Our prior work used the manual method that we developed to measure telomere length with single-cell resolution, assessing 30–50 user-selected cells per cell type, while maintaining tissue architecture in archival, formalin-fixed paraffin-embedded (FFPE) prostate tissues [5].

As our manual method was very labor intensive and possibly susceptible to user bias during the manual selection of the cells to be analyzed, we now have developed a robust, semi-automated method to quantitate cell type-specific telomere length at single-cell resolution. Our semi-automated method is based on performing telomere-specific fluorescence *in situ* hybridization (FISH) combined with multiplex immunofluorescence to detect a basal cell-specific cytokeratin, prostate epithelial-cell specific nuclear markers (NKX3.1 and FOXA1), and lymphocyte-specific markers (CD3 and CD20) using FFPE tissue samples. This staining process is then followed by semi-automated slide scanning and multi-channel acquisition of fluorescent microscopy images. Cell-type specific telomere and nuclear DNA content data are then obtained from these collected images via semi-automated image analysis allowing us to measure telomere length in all cells of the specified type in focal plane without selection by the operator.

While our original findings pointed to the potential prognostic utility of the telomere biomarker, we next sought to confirm, in a larger study of five cohorts, that the telomere biomarker indeed is independently associated with risk of poor outcome in men surgically treated for clinically localized prostate cancer using our optimized, semi-automated method. This confirmation is an important and necessary step toward clinical implementation. Thus, we expanded the number of men surgically treated for prostate cancer from 596 to 2,255, the number of metastatic or rapidly rising prostate-specific antigen (PSA) events from 54 to 311, and the number of prostate cancer deaths from 46 to 85. We again assessed the telomere biomarker in men with Gleason 7 disease (grade groups 2/3 [8]), a group for whom clinical management decisions are challenging. In addition, we assessed, for the first time, whether the telomere biomarker is associated with outcomes among men with PTEN protein intact tumors, as PTEN protein loss has been associated with poor prognosis [9–11].

We confirm here that the telomere biomarker is independently associated with progression to metastasis and prostate cancer death in men surgically treated for prostate cancer, including in men with intermediate disease (grade groups 2/3) and in men with PTEN protein intact tumors. We also confirm that variability in telomere length among cancer cells, but not the telomere biomarker, is associated with recurrence. Thus, the telomere biomarker has the promise to aid in better treatment and surveillance decision-making for these men.

Materials and methods

Study populations and designs

We used tissue and data from five cohorts: Health Professionals Follow-up Study (HPFS), the cohort in which we originally described the telomere biomarker [7], Physicians' Health Study (PHS), Johns Hopkins Recurrence Nested Case–Control Study, and two Johns Hopkins Intermediate-High-Risk Case–Cohort studies. This work was approved by the IRB at the Johns Hopkins University. The cohort study protocol was approved by the institutional review boards of the Brigham and Women's Hospital, Harvard T.H. Chan School of Public Health, and those of participating registries as required. The study populations were used as designed and are described in detail in the Supplementary materials and methods and supplementary material, Tables S1–S5.

Measurement of telomere length

For the original HPFS tissue microarrays (TMAs) ($N = 5$), we used the non-automated method described in Heaphy *et al* [7]. For the additional HPFS TMAs ($N = 2$), and the TMAs from the PHS, Johns Hopkins Recurrence Nested Case–Control Study, and the Johns Hopkins Intermediate-High Risk Case–Cohort Study I and II, we used our semi-automated, optimized method described in Heaphy *et al* [12] and summarized here.

Telomere-specific FISH and immunostaining

Deparaffinized TMA slides were stained for telomeres by telomere-specific FISH and co-labeled by multiplex immunofluorescence (see Supplementary materials and methods).

Microscopy and image analysis

The TissueFAXS Plus (Tissue Gnostics, Vienna, Austria) automated microscopy workstation, which contains an eight-slide ultra-precise motorized stage and utilizes a Zeiss Z2 Axioimager microscope (Zeiss, Oberkocken, Germany), was used for automated image acquisition. First, a DAPI preview image is captured with a ×10 objective to allow for appropriate orientation. Next, the TMA spots are identified and images are captured with a ×40 oil objective using the DAPI, GFP, Cy3, and Cy5 filters. An autofocus algorithm in the DAPI filter and the extended focus parameter by capturing three steps above and below (step size = 0.8 μm) was utilized. An entire TMA with 400 spots can be imaged in ∼14 h, which is faster than other current imaging modalities [13]. For image analysis, a separate high-performance workstation with the TissueQuest software module to analyze the fluorescent images with precise nuclear segmentation was used [12,14]. A region of interest is set (e.g. stroma or cancer) and processed for nuclear segmentation. If required, exclusion regions were set to exclude benign prostate glands.

Categorizing the telomere biomarker

After exporting the data to Excel spreadsheets, we converted them to an SAS dataset and merged the data with the TMA spot individual identifiers. For each man and for each cell, we calculated the ratio of Cy3 dot sum intensity and DAPI dot sum intensity and multiplied by 1,000; this ratio is the telomere ratio. The telomere ratio for each nucleus compensates for differences in nuclear cutting planes and ploidy. Separately, for each cohort we performed the following: for each man, we calculated median telomere ratio for cancer-associated stromal cells; this is the first of the two components of the telomere biomarker. For each man, we calculated the standard deviation of telomere ratio for cancer cells; this is second of the two components of the telomere biomarker. We determined whether the man's median telomere ratio for cancer-associated stromal cells was below the 66th percentile among the men in each TMA set; we categorized this group as having shorter telomeres in cancer-associated stromal cells. We determined whether a man's standard deviation of telomere ratio for cancer cells was above the 66th percentile of the distribution of the standard deviation of telomere ratio among men in each TMA set; we categorized this group as having more variable telomeres in cancer cells. Based on our prior study [7], individuals who have the combination of shorter telomeres in cancer-associated stromal cells and more variable telomeres in cancer cells have the poorest prognosis; individuals who either have shorter telomeres in cancer-associated stromal cells or more variable telomeres in cancer cells have an intermediate prognosis; and individuals who have neither shorter telomeres in cancer-associated stromal cells nor more variable telomeres in cancer cells have the best prognosis.

Statistical analysis

For all analyses, we used SAS v. 9.4 (Cary, NC, USA). For the HPFS and PHS studies, we used Cox proportional hazards regression to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) and adjusted for age at diagnosis, year of surgery, prostatectomy Gleason sum, pathologic stage, and preoperative PSA. For the Johns Hopkins Intermediate-High Risk Study I and II, we used Cox proportional hazards regression with robust variance correction to estimate HRs and 95% CIs and adjusted for age, race, pathologic stage, prostatectomy Gleason sum, year of surgery, preoperative PSA (if missing, a separate indicator variable was used), and surgical margins. For the Johns Hopkins Recurrence Nested Case–Control Study, we used conditional logistic regression to estimate odds ratios (as unbiased estimates of the HR) and 95% CIs and adjusted for year of surgery, primary and secondary Gleason pattern, preoperative PSA, and surgical margins (cases and controls were matched on age, race, categories of prostatectomy Gleason sum, and categories pathologic stage).

For each cohort, we estimated the association of more (versus less) variability in telomere length among cancer cells and shorter (versus longer) telomere length in cancer-associated stromal cells with recurrence, progression to metastasis, and prostate

cancer death. Next, for each study, we estimated the association between the telomere biomarker and these same outcomes using the less variable/longer combination as the reference group. We repeated this analysis stratified by Gleason sum (<7, 7, >7) and by PTEN protein status (intact, null).

Because of the differences in study design, disease severity at diagnosis, timing relative to the PSA era, the telomere length determination method used (original five HPFS TMAs non-automatic, all others optimized semi-automated), and the fact that the TMAs were run in different batches with slight modifications to the scanning parameters, we could not pool the data from the five cohorts. Instead, we used a meta-analytic approach to obtain summary estimates. To do so, we used inverse variance weights.

Results

Characteristics of the men in the five cohorts

We included men from five studies who were treated by radical prostatectomy for clinically localized prostate cancer. Table 1 provides the numbers of men and events included from each study. In total, we included 1,659 newly studied men along with 596 men we previously studied for whom we extended their follow-up. Across these studies, 654 men experienced recurrence, 311 men progressed to distant metastasis or had rapidly rising PSA indicative of likely metastasis (PSA doubling time <10 months), and 85 men died of their prostate cancer. The median follow-up times ranged from 3.0 to 14.6 years.

Measurement of telomere length in individual cells of specified type using the newly developed and optimized combined telomere FISH and IF staining method

As we previously demonstrated, telomere-specific FISH signal intensities are linearly proportional to telomere length and can be quantified via digital image analysis [15]. In our new method, we have combined telomere-specific FISH with multiplex immunofluorescence (IF) staining to better identify and more easily restrict analysis to specific cell types of interest. As shown in Figure 1A, basal-specific cytokeratin positivity (magenta) delineates benign prostate glands, the prostate epithelial-cell specific nuclear markers (NKX3.1 and FOXA1; green) highlight prostatic epithelial cells, and lymphocyte-specific markers (CD3 and CD20; magenta) identify lymphocytes in the surrounding microenvironment. Shown in Figure 1B are the telomere FISH signals that are robust in the benign gland, while diminished in the cancer cells. Using nuclear segmentation, the cancer cells (green^{+}/magenta^{-}) and cancer-associated stromal cells (green^{-}/magenta^{+}) can be identified, whereas the benign prostatic glands and lymphocytes are excluded from the analysis.

Table 1. Number of men who experienced prostate cancer outcomes, number of men in the cohort or comparison group, median time to event, and total follow-up time, five cohorts of men surgically treated for clinically localized disease

	HPFS	PHS	Johns Hopkins Recurrence	Johns Hopkins Intermediate-High Risk – I	Johns Hopkins Intermediate-High Risk – II
Study design	Cohort	Cohort	Nested case–control	Case–cohort	Case–cohort
Prostate cancer outcome					
Recurrence*	227	51	376	–	–
Metastasis	30	5	–	115	161†
Prostate cancer death	68‡	17	–	–	–
Cohort/controls/subcohort	755‡	151	376	253	192
Total number of men evaluated	755	151	752	306§	291¶
Median event and follow-up times (years)					
Case time to recurrence	3.8	4.2	2.0	–	1.0
Case time to metastasis	9.7	8.8		4.0	3.0
Case time to prostate cancer death	10.0	5.5	–	–	–
Cohort/control follow-up	14.6	13.8	6.0	10.0	3.0

*Includes biochemical recurrence.
†Of these, 125 had rapidly rising PSA (doubling time <10 months) indicative of a high risk of metastasis.
‡Of these, 50 prostate cancer deaths and 596 men were included in the original study in which we described the telomere biomarker.
§Sixty-two men in the subcohort progressed and 53 men outside of the subcohort progressed (253 subcohort + 53 progressed outside of the subcohort = 306).
¶Sixty-two men in the subcohort progressed and 99 men outside of the subcohort progressed (192 subcohort + 99 progressed outside of the subcohort = 291).

Components of the telomere biomarker and prostate cancer outcomes

Table 2 shows the summary associations between the two components of the telomere biomarker – variability in telomere length among cancer cells and telomere length in stromal cells – and recurrence, progression to metastasis, and prostate cancer death meta-analyzed across the five studies. Considering currently used prognostic factors, more variable telomere length in cancer cells appeared to be similarly associated with a higher risk of each outcome albeit only statistically significant for recurrence. Shorter telomeres in stromal cells were associated with a higher risk of prostate cancer death only (HR = 1.84, 95% CI 1.06–3.21, $p = 0.03$). Supplementary material, Table S6 shows study-specific HRs, and supplementary material, Table S7 shows the summary associations excluding the original HPFS study data.

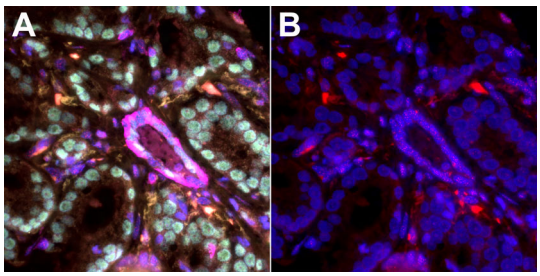

Figure 1. Combined telomere-specific FISH and multiplex immunofluorescence staining in prostate cancer. This newly developed assay was utilized to measure telomere lengths in individual cells of specific cell type. (A) In a prostate cancer that contains both benign and cancer regions, a basal-specific cytokeratin (magenta) delineates the benign prostate glands, two epithelial-cell specific nuclear markers (NKX3.1 and FOXA1; green) highlight prostatic epithelial cells, and two lymphocyte-specific markers (CD3 and CD20; magenta) identify lymphocytes in the surrounding tumor microenvironment. (B) In the same region, the telomeres are highlighted with a Cy3-labeled telomere-specific peptide nucleic acid probe (red). In both images, the DNA is stained with DAPI (blue). Original total magnification ×400.

The telomere biomarker and prostate cancer outcomes

Table 3 and Figure 2 show the summary associations between the telomere biomarker and recurrence, progression to metastasis, and prostate cancer death meta-analyzed across the five cohorts. Taking into account the pathologic prognostic markers, compared with men with less variable telomere length in their cancer cells and longer telomere length in their stromal cells, men with more variable telomere length in their cancer cells and shorter telomere length in their stromal cells had 3.76 times the risk of prostate cancer death (95% CI 1.37–10.3, $p = 0.01$) and had 2.23 times the risk of metastasis (95% CI 0.99–5.02, $p = 0.05$); men with the two other combinations had intermediate HRs. In contrast, only the telomere biomarker categories that include more variable telomere length were associated with an increased risk of recurrence. Supplementary material, Table S8 shows study-specific relative risks

Table 2. Meta-analytic summary RRs and 95% CIs of more variable telomere length among prostate cancer cells and shorter telomere length in prostate cancer-associated stromal cells with risk of recurrence, metastasis, and prostate cancer death after prostatectomy for clinically localized disease in five cohorts*

	Number of contributing cohorts	Summary RR†, 95% CI	
		More variable‡ telomere length among prostate cancer cells (versus less variable)	Shorter§ telomere length in cancer-associated stromal cells (versus longer)
Recurrence	3	1.40 1.13–1.74 $p = 0.002$	0.94 0.75–1.17 $p = 0.57$
Metastasis	4¶	1.36 0.90–2.04 $p = 0.14$	1.19 0.75–1.88 $p = 0.47$
Prostate cancer death	2	1.37 0.85–2.20 $p = 0.20$	1.84 1.06–3.21 $p = 0.03$

*Fifty prostate cancer deaths and 596 men were included in the original study in which we described the telomere biomarker.
†Each contributing RR and 95% CI was adjusted for currently used prognostic pathologic factors. RRs were summarized using inverse variance weights.
‡More variable was defined as the top tertile of variability in telomere length among prostate cancer cells. Less variable was defined as the bottom and middle tertiles.
§Shorter was defined as the shortest and middle tertiles of the median telomere length in cancer-associated stromal cells. Longer was defined as the longest tertile.
¶Includes all events ($N = 161$) from the Johns Hopkins Intermediate-High Risk – II. Including only the 35 metastatic events: more variable length among cancer cells (HR = 1.39, 95% CI 0.93–2.06, $p = 0.11$) and shorter length among stromal cells (HR = 1.12, 95% CI 0.71–1.75, $p = 0.64$).

Table 3. Meta-analytic summary RRs and 95% CIs for the associations* between the telomere biomarker – the combination of variability in telomere length among prostate cancer cells and telomere length in prostate cancer-associated stromal cells – and risk of recurrence, metastasis, and prostate cancer death after prostatectomy in five cohorts†

	Number of contributing cohorts	Less variable/longer	More variable/longer	Less variable/shorter	More variable/shorter
Recurrence	3	1.00	1.50	1.13	1.68
		Reference	1.03–2.18	0.81–1.56	1.16–2.44
			$p = 0.04$	$p = 0.47$	$p = 0.007$
Metastasis	4‡	1.00	1.51	1.48	2.23
		Reference	0.69–3.30	0.73–3.01	0.99–5.02
			$p = 0.30$	$p = 0.28$	$p = 0.05$
Prostate cancer death	2	1.00	1.29	2.00	3.76
		Reference	0.41–4.07	0.75–5.32	1.37–10.3
			$p = 0.66$	$p = 0.17$	$p = 0.01$

*Each contributing RR was adjusted for prognostic pathologic factors. RRs were summarized using inverse variance weights.
†Fifty prostate cancer deaths and 596 men were included in the original study in which we described the telomere biomarker.
‡Includes all events ($N = 161$) from the Johns Hopkins Intermediate-High Risk – II. When including only the 35 metastatic events, the number was too small distributed across the four telomere biomarker categories to obtain a stable estimate to meta-analyze.

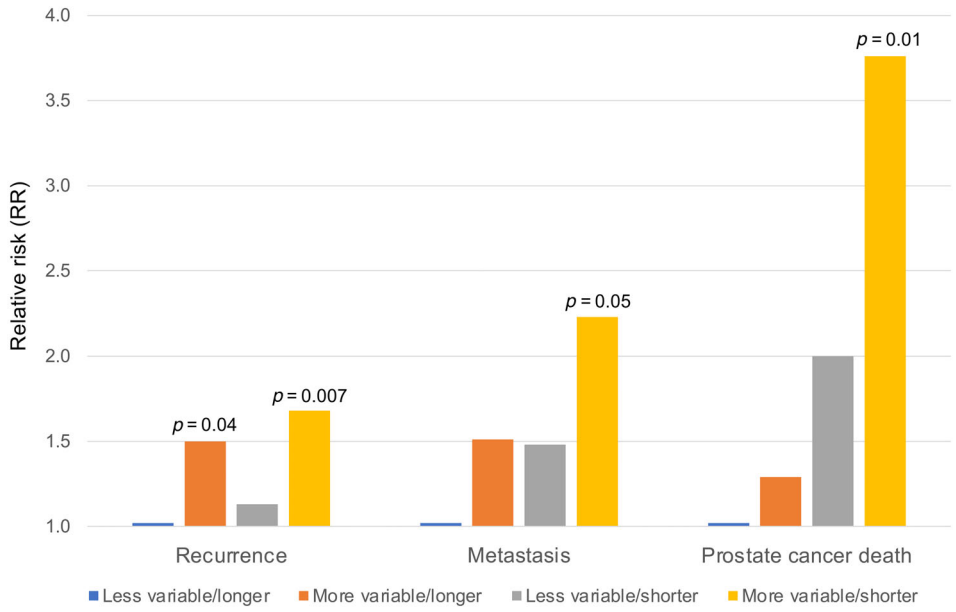

Figure 2. Meta-analytic summary associations between the telomere biomarker – combination of variability in telomere length among cancer cells and telomere length in cancer-associated stromal cells – and progression to recurrence, metastasis, and prostate cancer death after prostatectomy in five cohorts. Summary RRs from a meta-analysis of the HPFS, PHS, Johns Hopkins Recurrence, Johns Hopkins Intermediate-High Risk Study – I, and Johns Hopkins Intermediate-High Risk Study – II. Contributing RRs are adjusted for prognostic markers. *P* values are for the comparison of the specified category with the less variable/longer combination of the telomere biomarker.

(RRs), and supplementary material, Table S9 shows the summary HRs excluding the original HPFS data.

The telomere biomarker and prostate cancer outcomes among men with intermediate disease

Table 4 shows the summary associations between the telomere biomarker categories and prostate cancer outcomes when compared with the less variable/longer combination by Gleason sum (grade group). In men with Gleason 7 disease (grade groups 2/3), the more variable/shorter combination was statistically significantly associated with a higher risk of prostate cancer death (HR = 9.18, 95% CI 1.14–74.0, $p = 0.037$) when compared with the less variable/longer combination. All combinations of the telomere biomarker were statistically

Table 4. Meta-analytic summary RRs and 95% CIs for the associations* between the telomere biomarker (combination of variability in telomere length among prostate cancer cells and telomere length in prostate cancer-associated stromal cells) and risk of recurrence, metastasis, and prostate cancer death after prostatectomy by Gleason sum category and PTEN protein status in four cohorts†

	Gleason sum			PTEN protein status	
Telomere biomarker	<7 (grade group 1)	7 (grade groups 2, 3)	>7 (grade groups 4, 5)	Intact	Null
Recurrence					
Less variable/longer	1.00 Reference	1.00 Reference	1.00 Reference	1.00 Reference	1.00 Reference
More variable/longer	0.62 0.13–2.97	0.99 0.60–1.64	3.01 1.54–5.90 $p = 0.001$	1.27 0.76–2.13	2.71 1.25–5.88 $p = 0.012$
Less variable/shorter	0.16 0.04–0.73	0.86 0.55–1.32	2.00 1.05–3.82 $p = 0.035$	0.89 0.57–1.40	1.59 0.78–3.24
More variable/shorter	1.94 0.64–5.93	1.25 0.74–2.11	2.63 1.28–5.37 $p = 0.008$	1.40 0.83–2.39	3.05 1.37–6.78 $p = 0.006$
Metastasis					
Less variable/longer	1.00 Reference	1.00 Reference	1.00 Reference	1.00 Reference	1.00 Reference
More variable/longer	NE	2.24 0.43–11.7	1.17 0.40–3.44	2.37 0.62–9.07	0.95 0.16–5.87
Less variable/shorter	NE	3.98 0.91–17.7	0.65 0.23–1.81	2.74 0.78–9.59	0.49 0.11–2.28
More variable/shorter	NE	4.27 0.81–22.4	1.49 0.54–4.14	3.85 0.99–14.9 $p = 0.051$	2.72 0.25–30.1
Prostate cancer death					
Less variable/longer	1.00 Reference	1.00 Reference	1.00 Reference	1.00 Reference	1.00 Reference
More variable/longer	NE	0.23 0.01–4.06	1.31 0.36–4.76	0.87 0.14–5.56	3.99 0.28–55.8
Less variable/shorter	NE	4.16 0.54–31.9	0.88 0.27–2.84	1.70 0.36–8.01	2.89 0.27–36.8
More variable/shorter	NE	9.18 1.14–74.0 $p = 0.037$	1.95 0.62–6.14	6.74 1.46–37.6 $p = 0.015$	7.23 0.62–84.5

NE, not estimable (due to small sample size).
*Each contributing RR was adjusted for currently used prognostic pathologic factors. RRs were summarized using inverse variance weights.
†Of these, 50 prostate cancer deaths and 596 men were included in the original study in which we described the telomere biomarker. Of note, PTEN protein was not assessed in the Johns Hopkins Intermediate-High Risk Case-Cohort Study – II.

significantly associated with recurrence in men with Gleason sum >7 disease (grade groups 4/5) when compared with the less variable/longer combination.

The telomere biomarker, PTEN protein status, and prostate cancer outcomes

Table 4 shows the summary associations between the telomere biomarker categories and prostate cancer outcomes when compared with the less variable/longer combination by PTEN protein status. In men with PTEN protein intact cancers, the more variable/shorter combination was positively associated with prostate cancer death (HR = 6.74, 95% CI 1.46–37.6, $p = 0.015$) and progression to metastasis (HR = 3.85, 95% CI 0.99–14.9, $p = 0.051$). In men with PTEN protein null cancers, telomere biomarker categories that included the more variable telomere length in cancer cell component were associated with a higher risk of recurrence than those with intact PTEN protein.

Discussion

In this study of 2,255 men across five cohorts, we confirmed that the telomere biomarker is associated with progression to metastasis and prostate cancer death in men surgically treated for clinically localized prostate cancer.

With the use of PSA screening in the United States, most of the 248,530 prostate cancer cases [16] are detected when they are of small volume and apparently confined to the prostate, and thus should be curable by removal of the prostate. In the PSA era, despite having had their prostate removed, ~25% experienced PSA re-elevation months to years later [17], with 25% of these occurring five or more years later [18]. A third of men with PSA re-elevation developed overt metastases with a median time of 8 years after surgery [18] and 40% of these men died of their prostate cancer with a median time of 5 years after metastases are detected [18]. However, following the 2012 and 2018 changes to the US Preventive Services Task Force prostate cancer screening recommendations, the stage at diagnosis has shifted toward more advanced disease in the United States [19], which may result in a higher likelihood of recurrence among men treated by surgery. Thus, new molecular markers that improve prognostic accuracy are needed, particularly in men with intermediate risk disease.

In men with apparently organ-confined disease, the clinical tools currently used to predict disease behavior are inadequate and limit our ability to target men with optimal individualized treatment strategies. Furthermore, a thorough understanding of the molecular basis underlying aggressive cases is urgently needed for the continued development and refinement of prognostic indicators. We have now confirmed that the telomere biomarker is a promising molecular indicator of biological aggressiveness for use in prediction of prognosis. Telomere length variability and shortening are strongly associated with chromosomal instability, a hallmark of aggressive prostate cancer [20,21].

In particular, we demonstrated that the more variable/shorter combination of the telomere biomarker is associated with prostate cancer death in men with Gleason 7 disease (grade groups 2/3). These men have the most variable clinical course and are the group most in need of additional biomarkers for prognosis and treatment decision-making. We also showed that the more variable/shorter combination of the telomere biomarker is associated with both progression to metastasis and prostate cancer death in men with PTEN protein intact cancers. While PTEN protein null cancers have a worse prognosis, some men with PTEN protein intact cancers do progress. Thus, having an additional, independent biomarker may provide prognostic utility in this setting.

Only the more variable telomere length in cancer cells component of the telomere biomarker was associated with recurrence, as we previously observed in the HPFS [7]. The exception was in men with Gleason sum >7 disease (>grade group 2 or 3), in whom each category of the telomere biomarker was associated with a two to three times increased risk of recurrence compared to the less variable/longer combination.

We previously discovered the telomere biomarker in the HPFS [7]. In the current study, we followed the original 596 men for additional time and added 159 HPFS participants (represented across two TMAs) who were diagnosed and/or their tissue was arrayed subsequent to our initial study. This increased the number of prostate cancer deaths from 46 to 68 (48% increase) and the total follow-up time from 7,491 to 11,776 man-years (57% increase). While the patterns of association remained the same, the RRs were not as large as they were prior to these additions. For example, the RR of prostate cancer death for the more variable/shorter combination was 14.10 (95% CI 1.87–106) in our original study in the HPFS, 4.44 (95% CI 1.52–13.0) in the expanded study in the HPFS, and 3.76 (95% CI 1.37–10.3) when combining across all five of the cohorts, remaining well above the null and statistically significant. While we do not know the explanation for why the RRs are smaller, a key difference is that in the original study we visually selected 30–50 of each cell type for image analysis. For the additional two HPFS TMAs as well as in the four other cohorts, we used a multiplexed set of markers for the image analysis software to identify the relevant cell types for inclusion and exclusion, and investigated all relevant cancer and stromal (excluding lymphocytes) cells that were in the image's plane of focus. For the stromal cells, the new method identified hundreds to thousands of cells per cell type, likely capturing a different distribution of cells (compositionally and spatially) compared to the user-selected method. While the RR is smaller, with these additional HPFS and other cohort data provided greater precision (substantially narrower 95% CI).

Our study has a number of strengths. To confirm the telomere biomarker as prognostic for progression to metastatic disease and prostate cancer death, we used five studies of men who underwent prostatectomy developed with different criteria and different source populations (HPFS, PHS, Johns Hopkins). The cohorts we used had differing proportions of disease aggressiveness at diagnosis and likelihood of poor outcome (supplementary material, Tables S1–S5). For example, the Johns Hopkins Recurrence Nested Case–Control Study was enriched for biochemical recurrence. In contrast, the two Johns Hopkins Intermediate-High Risk Case–Cohort Studies were enriched for metastatic progression by design. Using these five studies allowed us to validate that the telomere biomarker is

specific for progression to metastatic disease and prostate cancer death, but not recurrence, a finding that does not always progress to lethal prostate cancer.

We used a validated, optimized, semi-automated method of telomere length measurement that we developed [7] and semi-automated and optimized [12]. The method allowed us to measure telomere length in fixed tissues, at single-cell resolution in specific cell types, with all in-focus cells assessed. These attributes reduced or eliminated the possibility of systematic error that could be introduced by the operator's bias in selecting individual cells for assessment, reduced or eliminated confounding by other cell types, and allowed for assessment of an important component of the biomarker – variability in telomere length among cancer cells (not the average length among these cells, as other methods would provide). Minimizing human error in measuring the components of the telomere biomarker is critical for routine clinical application of a molecular pathology-based prognostic tool.

Other aspects of the work warrant discussion. First, the method we developed does not determine actual telomere length (relative length is estimated and is linearly related to telomere length [15]) and does not determine chromosome-specific telomere length (chromosome-specific telomeric FISH probes for all chromosomes are not yet available). Second, the TMAs for each cohort were constructed using the largest cancer focus and/or with the highest Gleason pattern. We were not able to address whether the association between the telomere biomarker and poor outcome differs by which cancer foci was sampled. Nevertheless, we used the focus that is expected to impart the greatest risk. Third, we were not able to study recurrence, progression to metastasis, and prostate cancer death in each of the cohorts due to study designs and/or study populations used to construct the TMA sets we used. In addition, due to the small numbers of events, we were unable to separately analyze grade group 2 or 3; thus, we assessed the telomere biomarker in Gleason 7 disease as previously performed [7]. Fourth, given the five cohorts of men were surgically treated, we were not able to address whether the telomere biomarker is associated with poor outcome in men undergoing radiation therapy, hormonal therapy, or other single or combined prostate cancer treatment modalities. Fifth, our goal is to develop a tool for use at the time of prostatectomy to aid in decision-making about the need for additional treatment and more intensive surveillance. A complementary research question is whether the telomere biomarker has prognostic utility at the time of biopsy prior to any treatment. We have previously demonstrated the feasibility of assessing telomere lengths in biopsies from men randomized to the placebo arm of the Prostate Cancer Prevention Trial, and observed that telomere shortening in normal stromal cells was associated with prostate cancer risk [22]. Although, to determine whether men classified as low risk by the telomere biomarker might not require treatment at all, optimally, we would study men who are confirmed to have prostate cancer by biopsy and who are not treated and followed for outcome (i.e. men enrolled in active surveillance). However, men selected for active surveillance are, by definition, at a low risk of a poor outcome, and thus a very large study with long follow-up would be required to test the prognostic utility of the telomere biomarker.

In conclusion, we documented the robustness of the telomere biomarker as a prognostic tool for lethal prostate cancer. We focused on the length of the telomeres for this biomarker because abnormally shortened telomeres are intimately involved in promoting carcinogenesis, including by promoting the accumulation of chromosomal instability, a hallmark of prostate cancer [23]. We demonstrated that the telomere biomarker captures information in the prostatectomy specimen about tumor behavior beyond currently used indicators, thereby identifying men who are more or less likely to benefit from additional treatment. Thus, we expect that the telomere biomarker could be used to stratify men for individualized therapeutic strategies and has the potential of increasing the benefit to risk ratio for men and reducing healthcare costs associated with prostate cancer.

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Author contributions statement

CMH, CEJ, EG, LAM, MJS, MH, AMDM, TLL, EAP and AKM conceived and designed the study. CMH, RZ and AKM developed methodology. CD and RZ acquired data. JRB, JL, CEJ and EAP provided statistical analyses. CMH, CEJ, EG, LAM, MJS, MH, AKM, TLL, EAP and AKM interpreted data. CMH, CEJ, EAP and AKM wrote the initial draft of the paper, and all authors contributed to the review, revision and approval of the final paper.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. Basourakos SP, Tzeng M, Lewicki PJ, *et al*. Tissue-based biomarkers for the risk stratification of men with clinically localized prostate cancer. *Front Oncol* 2021; **11**: 676716.
2. Jafri MA, Ansari SA, Alqahtani MH, *et al*. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. *Genome Med* 2016; **8**: 69.
3. de Lange T. Shelterin-mediated telomere protection. *Annu Rev Genet* 2018; **52**: 223–247.
4. Bhargava R, Fischer M, O'Sullivan RJ. Genome rearrangements associated with aberrant telomere maintenance. *Curr Opin Genet Dev* 2020; **60**: 31–40.
5. Meeker AK, Hicks JL, Platz EA, *et al*. Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. *Cancer Res* 2002; **62**: 6405–6409.
6. Artandi SE, Chang S, Lee SL, *et al*. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* 2000; **406**: 641–645.
7. Heaphy CM, Yoon GS, Peskoe SB, *et al*. Prostate cancer cell telomere length variability and stromal cell telomere length as prognostic markers for metastasis and death. *Cancer Discov* 2013; **3**: 1130–1141.
8. Epstein JI, Zelefsky MJ, Sjoberg DD, *et al*. A contemporary prostate cancer grading system: a validated alternative to the Gleason score. *Eur Urol* 2016; **69**: 428–435.
9. Jamaspishvili T, Patel PG, Niu Y, *et al*. Risk stratification of prostate cancer through quantitative assessment of PTEN loss (qPTEN). *J Natl Cancer Inst* 2020; **112**: 1098–1104.
10. Haney NM, Faisal FA, Lu J, *et al*. PTEN loss with ERG negative status is associated with lethal disease after radical prostatectomy. *J Urol* 2020; **203**: 344–350.
11. Jamaspishvili T, Berman DM, Ross AE, *et al*. Clinical implications of PTEN loss in prostate cancer. *Nat Rev Urol* 2018; **15**: 222–234.
12. Heaphy CM, Joshu CE, Barber JR, *et al*. Racial difference in prostate cancer cell telomere lengths in men with higher-grade prostate cancer: a clue to the racial disparity in prostate cancer outcomes. *Cancer Epidemiol Biomarkers Prev* 2020; **29**: 676–680.
13. Gunkel M, Chung I, Wörz S, *et al*. Quantification of telomere features in tumor tissue sections by an automated 3D imaging-based workflow. *Methods* 2017; **114**: 60–73.
14. Asaka S, Davis C, Lin SF, *et al*. Analysis of telomere lengths in p53 signatures and incidental serous tubal intraepithelial carcinomas without concurrent ovarian cancer. *Am J Surg Pathol* 2019; **43**: 1083–1091.
15. Meeker AK, Gage WR, Hicks JL, *et al*. Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining. *Am J Pathol* 2002; **160**: 1259–1268.
16. Siegel RL, Miller KD, Fuchs HE, *et al*. Cancer statistics, 2021. *CA Cancer J Clin* 2021; **71**: 7–33.
17. Carroll P. Rising PSA after a radical treatment. *Eur Urol* 2001; **40**: 9–16.
18. Pound CR, Partin AW, Eisenberger MA, *et al*. Natural history of progression after PSA elevation following radical prostatectomy. *JAMA* 1999; **281**: 1591–1597.
19. Sheng IY, Wei W, Chen YW, *et al*. Implications of the United States Preventive Services Task Force recommendations on prostate cancer stage migration. *Clin Genitourin Cancer* 2021; **19**: e12–e16.
20. Malihi PD, Graf RP, Rodriguez A, *et al*. Single-cell circulating tumor cell analysis reveals genomic instability as a distinctive feature of aggressive prostate cancer. *Clin Cancer Res* 2020; **26**: 4143–4153.
21. Wang S, Li H, Song M, *et al*. Copy number signature analysis tool and its application in prostate cancer reveals distinct mutational processes and clinical outcomes. *PLoS Genet* 2021; **17**: e1009557.
22. Heaphy CM, Gaonkar G, Peskoe SB, *et al*. Prostate stromal cell telomere shortening is associated with risk of prostate cancer in the placebo arm of the Prostate Cancer Prevention Trial. *Prostate* 2015; **75**: 1160–1166.
23. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
24. Dhillon PK, Barry M, Stampfer MJ, *et al*. Aberrant cytoplasmic expression of p63 and prostate cancer mortality. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 595–600.
25. Stark JR, Perner S, Stampfer MJ, *et al*. Gleason score and lethal prostate cancer: does $3 + 4 = 4 + 3$? *J Clin Oncol* 2009; **27**: 3459–3464.
26. Ahearn TU, Pettersson A, Ebot EM, *et al*. A prospective investigation of PTEN loss and ERG expression in lethal prostate cancer. *J Natl Cancer Inst* 2016; **108**: djv346.

27. Steering Committee of the Physicians' Health Study Research Group. Final report on the aspirin component of the ongoing Physicians' Health Study. *N Engl J Med* 1989; **321**: 129–135.
28. Fiorentino M, Judson G, Penney K, *et al*. Immunohistochemical expression of BRCA1 and lethal prostate cancer. *Cancer Res* 2009; **70**: 3136–3139.
29. Barry M, Dhillon PK, Stampfer MJ, *et al*. Alpha-methylacyl-CoA racemase expression and lethal prostate cancer in the Physicians' Health Study and Health Professionals Follow-up Study. *Prostate* 2011; **72**: 301–306.
30. Chaux A, Peskoe SB, Gonzalez-Roibon N, *et al*. Loss of PTEN expression is associated with increased risk of recurrence after prostatectomy for clinically localized prostate cancer. *Mod Pathol* 2012; **25**: 1543–1549.
31. Hempel HA, Cuka NS, Kulac I, *et al*. Low intratumoral mast cells are associated with a higher risk of prostate cancer recurrence. *Prostate* 2017; **77**: 412–424.
32. Ross AE, Johnson MH, Yousefi K, *et al*. Tissue-based genomics augments post-prostatectomy risk stratification in a natural history cohort of intermediate- and high-risk men. *Eur Urol* 2016; **69**: 157–165.
33. Johnson MH, Ross AE, Alshalalfa M, *et al*. SPINK1 defines a molecular subtype of prostate cancer in men with more rapid progression in an at risk, natural history radical prostatectomy cohort. *J Urol* 2016; **196**: 1436–1444.
34. Guedes LB, Almutairi F, Haffner MC, *et al*. Analytic, preanalytic, and clinical validation of p53 IHC for detection of TP53 missense mutation in prostate cancer. *Clin Cancer Res* 2017; **23**: 4693–4703.
35. Cooperberg MR, Hilton JF, Carroll PR. The CAPRA-S score: a straightforward tool for improved prediction of outcomes after radical prostatectomy. *Cancer* 2011; **117**: 5039–5046.
36. Lotan TL, Gurel B, Sutcliffe S, *et al*. PTEN protein loss by immunostaining: analytic validation and prognostic indicator for a high risk surgical cohort of prostate cancer patients. *Clin Cancer Res* 2011; **17**: 6563–6573.
37. D'Amico AV, Whittington R, Malkowicz SB, *et al*. Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *JAMA* 1998; **280**: 969–974.

References 24–37 are cited only in the supplementary material.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Table S1. Characteristics of the Health Professionals Follow-up Study

Table S2. Characteristics of the Physicians' Health Study

Table S3. Characteristics of 376 prostate cancer biochemical recurrence cases and 376 matched controls in the Johns Hopkins Recurrence Nested Case–Control Study

Table S4. Characteristics of men in the Johns Hopkins Intermediate-High Risk Case–Cohort Study – I

Table S5. Characteristics of men in the Johns Hopkins Intermediate-High Risk Case–Cohort Study – II

Table S6. Associations of more variable telomere length among prostate cancer cells and shorter telomere length in prostate cancer-associated stromal cells with risk of recurrence, metastasis, and prostate cancer death after prostatectomy in five cohorts

Table S7. Meta-analytic summary RRs and 95% CIs of more variable telomere length among prostate cancer cells and shorter telomere length in prostate cancer-associated stromal cells with risk of recurrence, metastasis, and prostate cancer death after prostatectomy in five cohorts excluding 50 prostate cancer deaths and 596 men from the original study in which we described the telomere biomarker

Table S8. Associations between the telomere biomarker – the combination of variability in telomere length among cancer cells and telomere length in cancer-associated stromal cells – and risk of recurrence, metastasis, and prostate cancer death after prostatectomy in five cohorts

Table S9. Meta-analytic summary RRs and 95% CIs for the associations[1] between the telomere biomarker (combination of variability in telomere length among prostate cancer cells and telomere length in prostate cancer-associated stromal cells) and risk of recurrence, metastasis, and prostate cancer death after prostatectomy in the five cohorts excluding 50 prostate cancer deaths and 596 men from the original study in which we described the telomere biomarker