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Prostate stromal cell telomere shortening is associated with risk of prostate cancer in the placebo arm of the Prostate Cancer Prevention Trial*

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Abstract

Background—Telomeres are repetitive nucleoproteins that help maintain chromosomal stability by inhibiting exonucleolytic degradation, prohibiting inappropriate homologous recombination, and preventing chromosomal fusions by suppressing double-strand break signals. We recently observed that men treated for clinically localized prostate cancer with shorter telomeres in their cancer-associated stromal cells, in combination with greater variation in cancer cell telomere lengths, were significantly more likely to progress to distant metastases and die from their disease. Here, we hypothesized that shorter stromal cell telomere length would be associated with prostate cancer risk at time of biopsy.

Methods—Telomere-specific fluorescence *in situ* hybridization (FISH) analysis was performed in normal-appearing stromal, basal epithelial, and luminal epithelial cells in biopsies from men randomized to the placebo arm of the Prostate Cancer Prevention Trial. Prostate cancer cases (N=32) were either detected on a biopsy performed for cause or at the end of the study per trial protocol, and controls (N=50), defined as negative for cancer on an end-of-study biopsy performed

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per trial protocol (e.g. irrespective of indication), were sampled. Logistic regression was used to estimate the association between mean telomere length of the particular cell populations, cell-to-cell telomere length variability, and risk of prostate cancer.

Results—Men with short stromal cell telomere lengths (below median) had 2.66 (95% CI 1.04-3.06; p=0.04) times the odds of prostate cancer compared with men who had longer lengths (at or above median). Conversely, we did not observe statistically significant associations for short telomere lengths in normal-appearing basal (OR=2.15, 95% CI 0.86-5.39; p=0.10) or luminal (OR=1.15, 95% CI 0.47-2.80; p=0.77) cells.

Conclusions—These findings suggest that telomere shortening in normal stromal cells is associated with prostate cancer risk. It is essential to extend and validate these findings, while also identifying the cellular milieu that comprises the subset of cells with short telomeres within the prostate tumor microenvironment.

Keywords

Telomere; prostate cancer; stroma; tumor microenvironment

Introduction

New biomarkers are needed to improve risk stratification and prognosis of prostate cancer, a disease with a broad range of clinical outcomes. Tissue-based measurement of the lengths of telomeres, the tandem repetitive DNA elements located at the ends of human chromosomes, may be useful for this purpose (1). Telomeres are pivotal in maintaining chromosomal stability by masking telomere-induced double strand DNA break damage signals, inhibiting exonucleolytic degradation, and preventing chromosome fusions (2-4). Critical telomere shortening and subsequent chromosomal breakage-fusion-bridge cycles leads to genomic instability, thereby promoting malignant transformation and tumor progression (5,6).

We recently used a robust telomere-specific fluorescence *in situ* hybridization (FISH) assay that provides telomere length on a per cell basis in a prospective study cohort of men treated for clinically localized prostate cancer (7). We observed that men with shorter telomeres in their cancer-associated stromal cells had a significant increased risk of developing distant metastases and ultimately dying from their disease. In addition, when this cancer-associated stromal cell telomere length measurement was combined to include telomere length variation among the cancer cells, men with shorter stromal and more variable cancer telomeres had a 14-times higher risk of dying of their prostate cancer than men with longer and less variable telomeres. Importantly, these findings were independent of currently used prognostic indicators, and even performed well in men with intermediate risk disease (clinically-localized Gleason 7 prostate cancer).

In the current study, we test the hypothesis that men with shorter telomeres in normal-appearing cells in diagnostic biopsies will be at increased risk of harboring prostate cancer. We conducted a preliminary case-control study nested in the placebo arm of the Prostate Cancer Prevention Trial (PCPT) to evaluate in prostate biopsies the association between

median telomere length and cell-to-cell telomere length variability in normal-appearing prostate epithelial cells, stromal cells, and prostate cancer risk.

Materials and Methods

Prostate cancer cases and controls

In the multisite PCPT, men were enrolled between 1993 and 1997 to test whether finasteride prevents prostate cancer (8). The eligibility criteria included men ≥ 55 years of age with a normal digital-rectal examination (DRE), a serum PSA ≤3 ng/mL, and an American Urological Association Symptom Index <20. All men were evaluated annually with PSA and DRE; if either result were abnormal, the men were recommended for prostate biopsy. Cancers detected on such biopsies were considered to be “for-cause” biopsy detected. All men not diagnosed with prostate cancer during the trial were recommended to undergo prostate biopsy after 7 years on the trial irrespective of their PSA concentration or DRE status. Cancers detected were considered to be “for-cause” biopsy-detected if serum PSA was >4 ng/mL or the DRE was abnormal; cancers detected when subjects completed the 7-year study period and underwent biopsy with a normal DRE and PSA ≤ 4 ng/mL were considered detected on “end-of-study” biopsy. For this telomere length study, we evaluated the first 32 prostate cancer cases and 50 controls of the 191 cases and 209 controls in the placebo arm that we sampled for tissue-based marker studies (9) from a case-control study nested in the PCPT (10). In the nested case-control study, the cases and controls were frequency matched on age, family history, and treatment arm, and all non-white controls were sampled. In the subset for tissue-based studies, we sampled the cases such that half were higher grade (Gleason sum ≥7). One biopsy core section (of the 6-10 taken in the PCPT) was selected for determination of telomere length. Most of the “cases” had only one biopsy core positive for cancer (8). In the “cases”, we did not preferentially select the core with cancer. The PCPT was approved by the Institutional Review Boards at the participating trial sites. The Institutional Review Board at the Johns Hopkins Bloomberg School of Public Health and the Colorado Multiple Institutional Review Board approved this study.

Measurement of telomere length using FISH

Telomere length was assessed by telomere-specific FISH staining as previously described (7,11). Briefly, 5μm biopsy slides were deparaffinized, hydrated, placed in target retrieval citrate buffer and steamed. Cy3-labelled telomere specific peptide nucleic acid (PNA) and FITC-labelled centromere specific PNA (used as a positive PNA hybridization control) were applied to the sample, denatured by incubation for 4 min at 83°C, and hybridized in the dark at room temperature for 2 hrs. Next, slides were rinsed in phosphate buffered saline with Tween 20 (PBST) followed by application of the prostate basal cell-specific anti-cytokeratin primary antibody (34BE12; Enzo Diagnostics, Farmingdale, NY) and incubated overnight at 4°C. Slides were then rinsed in PBST followed by application of fluorescent secondary antibody conjugated to Alexa Fluor 488 (diluted 1:100; Life Technologies, Grand Island, NY) and incubated at room temperature for 30 min. Slides were then counterstained with 4′, 6-diamidino-2-phenylindole (DAPI), and mounted with Prolong anti-fade mounting medium (Life Technologies, Grand Island, NY).

Microscopy

Each biopsy slide was imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, CA) using a 40×/0.95 NA PlanApo lens with correction collar. For each color channel, separate grayscale images were captured using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital cooled charged coupled device (CCD) camera, and saved as 12-bit uncompressed Tagged Image File Format (TIFF) files for use in downstream image analysis. Exposure times were set to avoid fluorescence signal saturation. Integration times typically ranged from 400-800 milliseconds for Cy3 (telomere) and FITC (centromere) signal capture, 50-100 milliseconds for the DAPI nuclear counterstain, and 100-400 milliseconds for the Alexa Fluor 488-conjugated antibody.

Image analysis

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. For individual cells, 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, and the immunofluorescence stain delineating benign prostatic basal epithelial cells. Telomeric signals identified by the binary segment mask, which were contained within the area inscribed by each circled nuclear DNA signal area, were then measured, and the data for each telomeric spot was tabulated and summed. For each case, we evaluated at least 30 individual benign-appearing cells from each cell population - luminal epithelial, basal epithelial, and stromal (fibroblasts and smooth muscle cells). Based on their unique morphologic features, other cell types (e.g. infiltrating lymphocytes) were excluded from the image analysis.

Statistical analysis

The median relative telomere length (measured as the total intensity of telomeric signals on a per cell basis) and the cell-to-cell telomere length variability (measured as the standard deviation of telomere lengths within a cell type) were calculated for all cell types for each individual. The cases were run across 10 different batches, and batch-specific median cut-points for relative telomere length and cell-to-cell telomere length variability were determined based on the distribution of the controls. Logistic regression was used to estimate the association between shorter (below the median) telomere length or higher (above the median) telomere length variability of the particular cell population and risk of prostate cancer adjusting for age. 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.

Results

Characteristics of prostate cancer cases and controls

Table 1 provides the characteristics of the 32 prostate cancer cases and 50 controls from the placebo arm of the PCPT. The cases and controls were similar on age at biopsy, family history, race, BMI, waist circumference, and cigarette smoking status. All of the cases were low stage, and about half of the cases were ≥7 Gleason sum (53%), and "for-cause" biopsy detected (53%).

Telomere-specific FISH staining provides single cell telomere length resolution

Representative examples of the intensities of telomere FISH signals for individual cells, reflecting the per cell telomere lengths, are shown in Figure 1. In prostate tissue biopsies from men in the placebo arm of the PCPT, some men displayed robust telomere FISH signals in their normal-appearing stromal cells (Figure 1A). In contrast, other men displayed less telomere FISH signals in their normal-appearing stromal cells, demonstrating a moderate degree of telomere shortening in this cell population (Figure 1B).

Stromal cell telomere lengths differ in prostate biopsies from men with and without prostate cancer

The association between cell-to-cell telomere length variability and median telomere length in the cell populations consisting of normal-appearing basal epithelial, luminal epithelial, and stromal cells in prostate biopsies from men with and without prostate cancer were examined.

We did not observe statistically significant associations for risk of prostate cancer and cell-to-cell telomere length variability in normal-appearing basal epithelial (OR=1.14, 95% CI 0.46-2.78; p=0.78), luminal epithelial (OR=0.49, 95% CI 0.19-1.25; p=0.14), and stromal (OR=0.46, 95% CI 0.18-1.16; p=0.10) cells. However, we could not rule out that more variable telomere length among the luminal epithelial or stromal cells was inversely associated with prostate cancer. As shown in Table 2, when comparing men with shorter telomere lengths (below median) to men who had longer telomere lengths (at or above median), we did not observe statistically significant associations for normal-appearing basal epithelial (OR=2.15, 95% CI 0.86-5.39; p=0.10) or luminal epithelial (OR=1.15, 95% CI 0.47-2.80; p=0.77) cells and presence of prostate cancer. Interestingly, we observed that men with shorter stromal cell telomere lengths had 2.66 (95% CI 1.04-3.06; p=0.04) times the odds of prostate cancer compared with men who had longer stromal cell telomere lengths. The association between shorter telomere length in stromal cells and risk of prostate cancer was positive for both higher- and lower-grade disease.

Discussion

In this study, we found that men with shorter telomere length in normal-appearing stromal cells on their biopsy specimen were associated with a higher odds of prostate cancer. While there was also a trend in the same direction for an association of shorter telomere length and cancer for normal appearing basal epithelial cells, this did not reach statistical significance. This association was not observed within normal-appearing luminal epithelial cells, suggesting the finding is not associated with constitutive telomere length, but rather with the stromal component of the tumor microenvironment.

Previous studies have shown that extensive telomere shortening in cancer cells compared with normal epithelial cells in the vast majority of prostate tumors (5,12,13), and even in high-grade prostatic intraepithelial neoplasia (14,15). Several studies have observed that decreased telomeric DNA content, a surrogate marker for bulk telomere length, in tumor tissue taken at time of prostatectomy (12,13) and even at biopsy (16) is associated with a higher risk of prostate cancer recurrence or death. Additionally, telomere shortening has been shown to occur in cancer-associated stromal cells (17,18), thereby reflecting a microenvironment that may further promote prostate carcinogenesis. Consistent with this notion, we recently found that shorter telomeres in cancer-associated stromal cells, in combination with increased cell-to-cell telomere length variation among cancer cells, was strongly associated with progression to metastasis and prostate cancer death in men treated for clinically localized disease (7). However, to our knowledge this is the first study attempting to link telomere length in benign stromal cells (not associated in space with cancer lesions) to the presence or grade of prostate cancer.

The present observations may have important clinical implications for men by improving risk stratification for subsequent prostate cancer development. If this preliminary result is confirmed in a larger population, men with negative prostate biopsies and who have short prostate stromal cell telomere lengths may be appropriate candidates for further investigation (e.g., prostatic imaging with MRI and targeted biopsy) and/or for prevention strategies. Moving forward, it will be important to determine if tumors with stromal telomere attrition are also associated with other emerging molecular biomarkers, such as ERG over expression, loss of PTEN, or presence of mutations in *TP53* or *SPOP* (19,20). Interestingly, a recent report found significant associations between ERG over expression and alterations in the cancer-associated stroma (e.g. increased vascular density, increased expression of hyaluronan and PDGFRβ, and decreased Caveolin-1 expression); thus, suggesting that alterations in the cancer and the stromal microenvironment may cooperate in promoting prostate cancer progression (21).

Equally important to helping develop appropriate prevention strategies for men with negative prostate biopsies, is to determine the underlying mechanistic relationship between the presence of short telomeres in the stromal cells and an increased prostate cancer risk. For example, short telomeres in the stromal cells can activate a senescence-associated secretory pathway, including pro-inflammatory cytokines and matrix-degrading proteases, in the surrounding microenvironment (22,23). In turn, this altered microenvironment helps to facilitate tumor promotion and eventually tumor progression. Recent data from a study

conducted in the PCPT demonstrated that inflammation is common in benign prostate tissue, and the presence of any inflammation, mostly chronic, was positively associated with prostate cancer, particularly high grade disease (9). Future studies will focus on elucidating the potential causal link between chronic inflammation and the presence of stromal telomere shortening, as well as identifying the precise stromal cell type (s) which experience telomere loss and the exact mechanisms acting to cause this loss.

There are a number of strengths of this study. The PCPT was a large clinical trial with standardized pathology to confirm diagnoses and determine Gleason sum. The PCPT is unique in that all cancer-free study participants were recommended to undergo an end-of-study biopsy, to confirm the presence or absence of prostate cancer. Additionally, all telomere length measurements were determined in a blinded fashion to reduce potential bias. However, despite these strengths, there are also limitations to our study. Although the trial was prospective, the analysis was not, because we determined telomere length in the biopsies used to rule in or out the diagnosis of prostate cancer. In addition, although we confirmed the feasibility of assessing telomere lengths in biopsies, the relatively small sample size of this set of cases and controls meant that we could not address the association between telomere lengths and aggressive prostate cancer or potential differences that may exist among racial groups.

Conclusions

These findings suggest that telomere shortening in stromal cells not directly adjacent to a cancer lesion are associated with an increased concurrent prostate cancer risk. Future studies are necessary to validate and extend these findings. In addition, a better understanding of the cellular milieu that comprises the subset of cells with short telomeres within the prostate tumor microenvironment will be essential to appreciate the role telomere biology plays in the development of prostate cancer.

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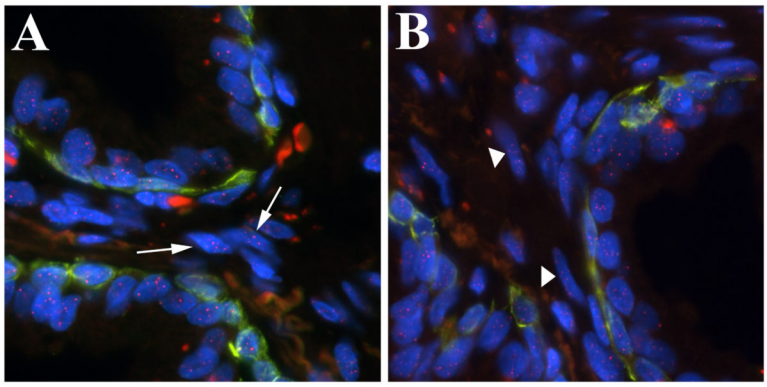

Figure 1. Telomere-specific FISH staining highlights shorter stromal cell telomere lengths

In normal-appearing stromal cells analyzed on prostate tissue biopsies from men in the placebo arm of the PCPT, (A) some men displayed robust telomere FISH signals (arrows) compared to (B) other men who displayed less telomere FISH signals (arrowheads). In both images, the DNA is stained with DAPI (blue), telomeric DNA is stained with a Cy3-labeled telomere-specific PNA probe (red), and basal cells are demarcated with a basal cell-specific cytokeratin antibody (green). Original magnification × 400.

Table 1
Characteristics of prostate cancer cases and controls, PCPT

Characteristic	Cases	Controls	P
N	32	50	
Mean age at biopsy in years (SD)	69.9 (5.1)	70.8 (6.0)	0.49
Mean BMI in kg/m^2 (SD)	27.6 (3.0)	26.8 (3.0)	0.21
Mean waist circumference in cm (SD)	104.2 (7.6)	100.4 (9.8)	0.08
Family history (%)	12.5	14.0	0.85
Smoke group (%)			
Current	6.3	4.0	0.89
Former	59.4	62.0	
Never	34.4	34.0	
Race (%)			
Caucasian	87.5	96.0	0.15
Minority (other)	12.5	4.0	
Stage (%)			
High	0	---	
Low*	100	---	
Reason for biopsy (%)			
For cause	53.1	---	
End of study	46.9	---	
Gleason sum (%)			
High (≥7)	53.1	---	
Low (<7)	46.9	---	

*Low stage is <T3 and not N+ and not M+

Table 2

Association between telomere length in normal-appearing prostate cell population and risk of prostate cancer, PCPT

Normal-appearing prostate cell populations	Low median telomere length*		p-trend
	OR	95% CI	
Basal epithelial	2.15	0.86, 5.39	0.10
Luminal epithelial	1.15	0.47, 2.80	0.77
Stromal	2.66	1.05, 6.69	0.04

*Age-adjusted values.